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Inhibition of MMTV transcription by HDAC inhibitors occurs independent of changes in chromatin remodeling and increased histone acetylation

Niveen M Mulholland[1,2,3], Edlyn Soeth[2,4] and Catharine L Smith*[,2]

[1]*Department of Genetics, George Washington University, Washington, DC 20052, USA;* [2]*Signal Transduction Group, Laboratory of Receptor Biology and Gene Expression, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA*

Increased histone acetylation has been associated with activated gene transcription and decreased acetylation with repression. However, there is a growing number of genes known, which are downregulated by histone deacetylase (HDAC) inhibitors through unknown mechanisms. This study examines the mechanism by which the mouse mammary tumor virus (MMTV) promoter is repressed by the HDAC inhibitor, trichostatin A (TSA). We find that this repression is transcriptional in nature and that it occurs in the presence and absence of glucocorticoids. TSA decreases MMTV transcription at a rapid rate, reaching maximum in 30–60 min. In contrast with previous reports, the repression does not correlate with an inhibition of glucocorticoid-induced nuclease hypersensitivity or NF1-binding at the MMTV promoter. Surprisingly, TSA does not induce sizable increases in histone acetylation at the MMTV promoter nor does it inhibit histone deacetylation, which accompanies deactivation of the glucocorticoid-activated MMTV promoter. Repression of MMTV transcription by TSA does not depend on the chromatin organization of the promoter because a transiently transfected MMTV promoter construct with a disorganized nucleoprotein structure was also repressed by TSA treatment. Mutational analysis of the MMTV promoter indicates that repression by TSA is mediated through the TATA box region. These results suggest a novel mechanism that involves acetylation of nonhistone proteins necessary for basal transcription.
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Introduction

Acetylation of histones has long been correlated with transcriptional activity (Allfrey *et al*., 1964). This relationship was solidified when several transcriptional coactivators were identified as histone acetyltransferases (HATs) (Brownell *et al*., 1996; Mizzen *et al*., 1996; Ogryzko *et al*., 1996) and a number of corepressor proteins were found in association with histone deacetylases (HDACs) (Alland *et al*., 1997; Heinzel *et al*., 1997; Kadosh and Struhl 1997; Laherty *et al*., 1997; Nagy *et al*., 1997). These results have led to the general idea that increases in histone acetylation are generally conducive to transcription. Acetylation of histones is thought to loosen the structure of chromatin to allow the transcriptional machinery to function (Hong *et al*., 1993) or, along with other types of histone modification, serve to attract and facilitate binding of various proteins involved in transcriptional regulation (Strahl and Allis, 2000).

A number of studies provide evidence of the fact that the relationship between histone acetylation and transcription is more complicated. In yeast, acetylation of particular lysines in the H4 N-terminal tail region appears to be required for silencing (Braunstein *et al*., 1996). In addition, knockout of the HDAC-encoding Rpd3 gene leads to increased genomic silencing in yeast and Drosophila (De Rubertis 1996; Rundlett *et al*., 1996) and yeast strains carrying deletions of Rpd3 fail to show proper activation of particular genes (Vidal *et al*., 1991; Vidal and Gaber 1991; Rundlett *et al*., 1996). Histones at some yeast gene promoters have been shown to become deacetylated upon activation of the promoter (Deckert and Struhl, 2001), and in mammalian cells, some genes are repressed by HDAC inhibitors. Examples include c-*myc* (Van Lint *et al*., 1996; Koyama *et al*., 2000) and cyclin D1 (Lallemand 1996; Siavoshian *et al*., 2000).

In addition to their ability to modulate gene expression, HDAC inhibitors have been found to have effects on cultured tumor cells including growth arrest, the induction of differentiation, and apoptosis (Marks *et al*., 2000). These attributes have generated efforts to use these drugs as antitumor agents *in vivo* and several are currently in clinical trials. However, questions remain about how these agents actually work to modify gene

*Correspondence: CL Smith, Laboratory of Receptor Biology and Gene Expression, National Institutes of Health, Bldg. 41, Room B608, 41 Library Dr. MSC 5055, Bethesda, MD 20892-5055, USA;
E-mail: smithcat@exchange.nih.gov
[3]Current address: Massachusetts General Hospital, Wellman 10, 50 Blossom St., Boston, MA 02114, USA
[4]Current address: Molecular Oncology, Department of General Surgery and Thoracic Surgery, Christian-Albrechts-University, 24105 Kiel, Germany
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expression. First, these inhibitors are selective in that they affect the expression of only a small fraction of genes (Van Lint *et al*., 1996). The basis of this selectivity is unknown. Second, they can both activate and repress the expression of genes. Since both histones and nonhistone proteins can be acetylated, it is not clear whether changes in gene expression are mediated primarily through increased acetylation of histones or nonhistone proteins.

The mouse mammary tumor virus long terminal repeat (LTR) contains a transcription regulatory region that is relatively well-characterized in terms of the transcription factors that bind to it, the structure it adopts in organized chromatin, and the mechanism by which it is activated by steroid hormones. This promoter, when assembled into organized chromatin, can be repressed by the HDAC inhibitors trichostatin A (TSA) or sodium butyrate at concentrations which cause the accumulation of acetylated histones (Bresnick *et al*., 1990; Bartsch *et al*., 1996; Lambert and Nordeen 1998; Myers *et al*., 1998; Wilson *et al*., 2002). We have investigated the mechanism by which TSA represses the MMTV promoter in organized chromatin. Our results provide new insights into how HDAC inhibitors work and challenge assumptions often made in studies utilizing these drugs.

Results

Effects of TSA on MMTV transcription

Previous studies examining regulation of the MMTV promoter by HDAC inhibitors found that, depending on a variety of conditions, butyrate or TSA could activate or repress the MMTV promoter in organized chromatin (Bresnick *et al*., 1990; Bartsch *et al*., 1996; Lambert and Nordeen 1998; Myers *et al*., 1998; Wilson *et al*., 2002). Repression was observed in seven distinct cell lines with two exceptions. In one report, inhibition of MMTV by these drugs was dose-dependent; at doses which resulted in high levels of histone acetylation, inhibition was observed, while lower doses gave rise to stimulation (Bartsch *et al*., 1996). In another report, the response of the MMTV promoter appeared to be influenced by position effects from chromatin surrounding the integrated MMTV promoter (Lambert and Nordeen, 1998). However, since HDAC inhibitors are most often associated with promoter activation, we were interested in the mechanism by which these drugs mediate the repression of this well-characterized promoter.

Exposure of cells to HDAC inhibitors in previous studies of MMTV promoter activity had been carried out for relatively long periods of time, on the order of 6–32 h. This raises the possibility that the effects of the drugs on MMTV regulation are not direct, but may be mediated through effects on other genes. We assayed the rates of MMTV-CAT and β-actin transcription by nuclear run-on at 0, 3.5, and 18 h of TSA treatment in the presence or absence of Dex. A representative analysis is shown in Figure 1a with a graphic summary of four independent experiments. It is clear that repression of MMTV transcription is complete by 3.5 h of TSA treatment. In addition to the inhibition of Dex-induced transcription as previously reported

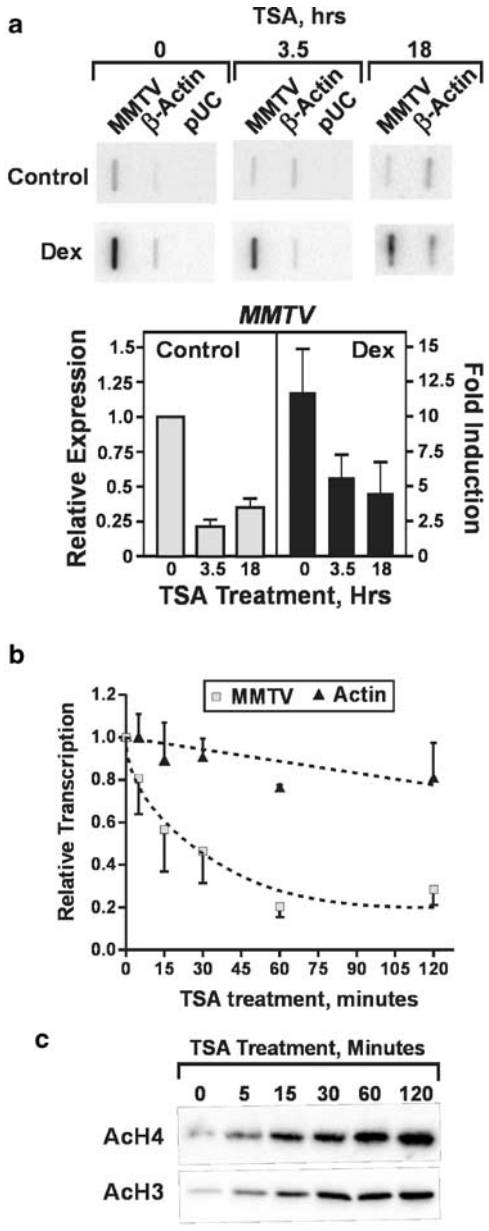

Figure 1 Transcriptional regulation of MMTV by TSA and glucocorticoids. (**a**) Nuclear run-on analysis was carried out on nuclei from cells treated with TSA (50 ng/ml) for 0, 3.5 or 18 h. Dex (100 nM) was added to half the cultures for 1 h coincident with the last hour of TSA treatment. Newly synthesized RNA was hybridized with slot blots to which DNA containing MMTV, β-actin or pUC18 sequences had been applied. The latter was used as a control for background hybridization. The top panel shows a representative analysis while the bottom panel is a graphic summary of four–eight independent experiments. The error bars represent SEM. (**b**) Nuclear run-on analysis was carried out on cells treated for 0, 5, 15, 30 and 60 min with TSA in the absence of Dex. The data represent the results of three independent experiments. (**c**) Histones were extracted from cells treated for the times indicated with TSA and subjected to Western blotting with antibodies against acetylated H3 and H4

(Bresnick *et al*., 1990; Bartsch *et al*., 1996; Lambert and Nordeen, 1998), there is also a strong inhibition of basal (control) transcription, an observation also reported by Bartsch *et al*. (1996). Transcription of the β-actin gene was not significantly changed by these TSA treatments (Figure 1a and data not shown). These results suggest that TSA targets the MMTV promoter in a steroid-independent fashion.

To further characterize the kinetics of TSA repression, we carried out a shorter time course analysis in the absence of Dex (Figure 1b). TSA-induced repression of basal MMTV transcription is extremely rapid, being detectable after only 5 min of treatment and complete within 1 h. There is a slight inhibitory effect on β-actin transcription but, unlike MMTV repression, it is temporary, since longer TSA treatments did not show any effect (data not shown). The rapid rate at which MMTV transcription is repressed strongly indicates that TSA targets the MMTV promoter directly. To ensure that inhibition of HDAC activity occurs in this relatively short-time frame, we measured the level of bulk histone H3 and H4 acetylation in the nuclei used for run-on analysis. Western blotting for acetylated H3 (AcH3) and H4 (AcH4) as seen in Figure 1c clearly shows that significant increases in histone acetylation are evident between 5 and 60 min of treatment. Thus, TSA inhibition of HDACs occurs very rapidly *in vivo* and the repression of MMTV transcription is correlated with immediate changes in HDAC activity.

TSA effects on chromatin remodeling at the MMTV promoter

Glucocorticoids induce a change in chromatin structure at the MMTV promoter which is measured by nuclease hypersensitivity and the binding of ubiquitous transcription factors (Cordingley *et al*., 1987; Archer *et al*., 1991). Previous studies of MMTV regulation by HDAC inhibitors had shown that the inhibition of MMTV promoter activity correlated with a loss of glucocorticoid-induced nuclease hypersensitivity (Bresnick *et al*., 1990; Bartsch *et al*., 1996), indicating that TSA may be mediating its inhibitory effects on the MMTV promoter through changes in chromatin structure. However, TSA treatment times in these studies were in the 12–24 h range.

To examine the kinetic relation between TSA-induced repression of MMTV transcription and chromatin remodeling more closely, we carried out a time course of TSA treatment and measured the ability of the restriction enzyme *Sac*I to cleave at a recognition site in the B nucleosome region of the MMTV promoter. Increased cleavage indicates increased accessibility of

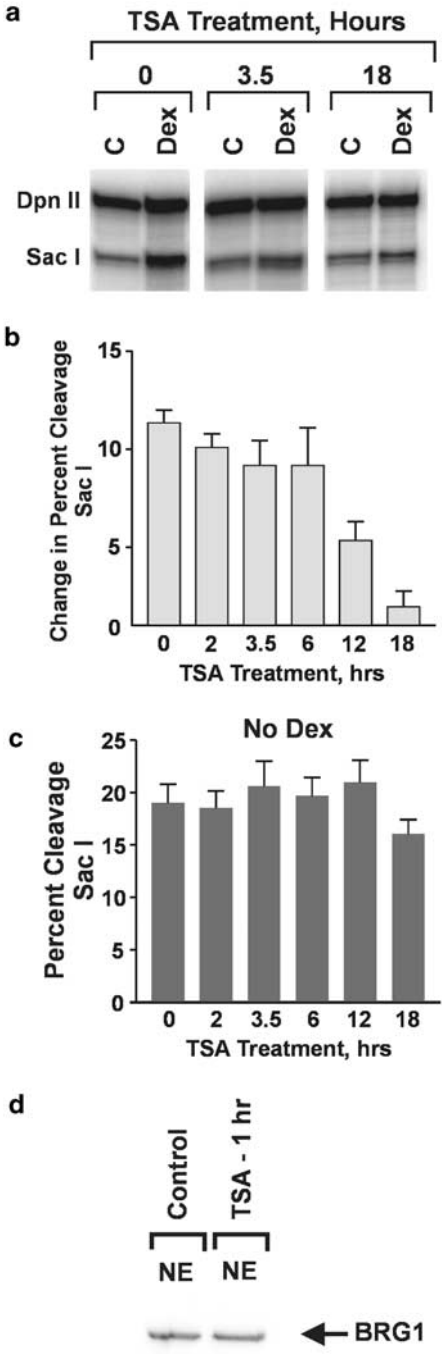

Figure 2 TSA effects on chromatin remodeling at the MMTV promoter. The cells were treated with TSA for 0, 2, 3.5, 6, 12, and 18 h with TSA (50 ng/ml). In the last hour of TSA treatment, half the cultures were treated with Dex (100 nM). Nuclei were isolated and treated as described in Materials and methods. Digestion products were detected through linear amplification with a radiolabeled, MMTV-specific primer. (**a**) shows a representative experiment with selected time points. Samples from cells that were not treated with Dex are indicated with a C (control). (**b**) shows statistical analysis of the Dex-induced change in *Sac*I cleavage for all time points from three independent experiments. The fractional cleavage of *Sac*I was calculated by dividing the intensity of the *Sac*I digestion product by the sum of the intensities of the *Sac*I and *Dpn*II digestion products for an individual sample. The change in cleavage induced by glucocorticoids is the difference, expressed as a percentage, between the fractional cleavage of *Sac*I in the presence and absence of Dex for each TSA treatment length. (**c**) shows statistical analysis of *Sac*I cleavage at different lengths of TSA treatment in the absence of Dex. Fractional *Sac*I cleavage [calculated as described in (**b**)] for each sample was converted to a percentage. The results from three independent experiments were included in the analysis. (**d**) Effects of TSA treatment on levels of BRG1. Nuclear extracts from untreated cells and cells treated with TSA for 1 h were subjected to electrophoresis on 8% SDS–PAGE. After Western transfer, membranes were blotted with antibody specific for BRG1

the enzyme to its cleavage site, or a loosening of chromatin structure. The results, seen in Figure 2a and b, show the typical glucocorticoid induction of *Sac*I cleavage in the absence of TSA (0 h). Consistent with the other studies, TSA almost completely inhibits this induction, but the time course shows that this effect is very gradual. Within the first 2 h of TSA treatment, the time frame in which MMTV transcription declines precipitously (Figure 1b), there is no significant effect on the ability of the GR to induce this change in chromatin structure. TSA-induced inhibition of GR-dependent chromatin remodeling at the MMTV promoter is not evident until after 6 h of treatment, long after transcription from the promoter is fully repressed. It is possible, however, that TSA causes MMTV chromatin to become more inaccessible to cleavage by nucleases independent of glucocorticoids. This would be indicated by a decline in *Sac*I cleavage at the promoter. Therefore, we measured the cleavage of *Sac*I in the absence of glucocorticoids at various times of TSA treatment. The results, shown in Figure 2c, clearly indicate that this is not the case, since the cleavage of *Sac*I stays constant over a 12 h time period.

A recent study showed that TSA treatment blocks the ability of the progesterone receptor to induce chromatin remodeling at an integrated MMTV promoter (Wilson *et al*., 2002). The loss in remodeling correlated with a downregulation of proteins contained within the BRG-1 chromatin remodeling complex, which is thought to be involved in GR-dependent chromatin remodeling at the MMTV promoter (Fryer and Archer 1998; Wallberg *et al*., 2000; Fletcher *et al*., 2002). We made nuclear extracts from untreated cells as well as those treated for 1 h with TSA, a time frame that results in maximal MMTV transcriptional repression (Figure 1b), and assayed them by Western blotting for BRG-1 levels (Figure 2d). We found that BRG-1 levels remain constant over 1 h of TSA treatment, consistent with the lack of change in the extent of GR-dependent chromatin remodeling that we observed with 2 h of TSA treatment (Figure 2b).

The glucocorticoid-induced binding of NF1 is another hallmark of MMTV promoter activation and is very tightly correlated with the induction of nuclease hypersensitivity. To measure NF1 binding to the MMTV promoter *in vivo*, we used an exonuclease block assay as shown in Figure 3. The Dex-induced binding of NF1 in the absence of TSA treatment is shown in lanes 1 and 2 as well as lanes 5 and 6. At 18 h of TSA treatment, the binding of NF1 in the presence of Dex was severely inhibited (lanes 3 and 4) while at 1 h of treatment, NF1 binding was quite efficient (lanes 7 and 8). This result, along with the *Sac*I cleavage data, shows that TSA-induced repression of MMTV transcription is not mediated through changes in chromatin remodeling. The inhibition of chromatin remodeling likely represents a secondary outcome of drug treatment and may have to do with a loss of remodeling factors within several hours of TSA treatment, as reported recently in a different cell line (Wilson *et al*., 2002).

Effects of TSA on histone acetylation at the MMTV promoter

Although TSA treatment modulates the activity of a variety of mammalian promoters, it is often not clear whether there are accompanying changes in histone acetylation at target promoters. Using chromatin immunoprecipitation (ChIP) assays carried out on mononucleosomal material, we measured the effects of TSA treatment on acetylation of histones at nucleosomes B and F in the MMTV promoter region in the presence and absence of Dex (Figure 4a). The B nucleosome contains most of the GR-binding sites,

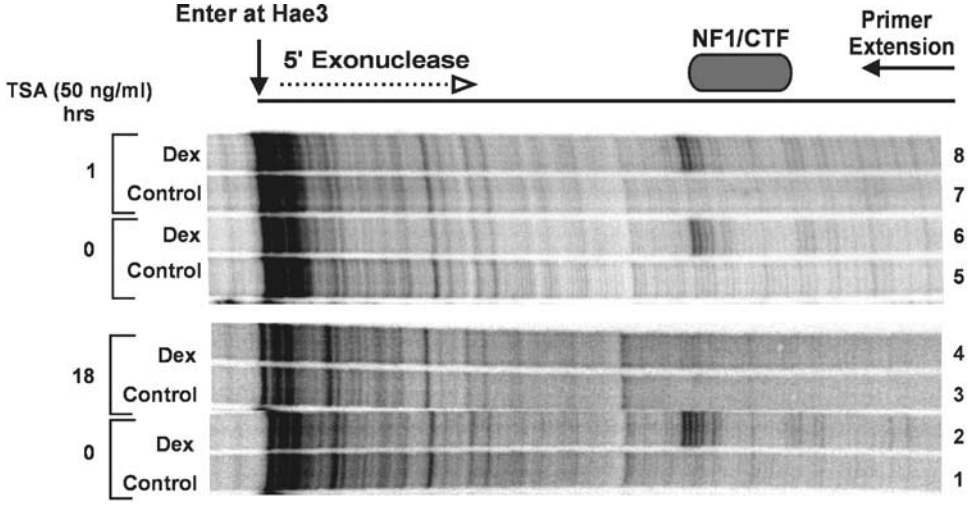

Figure 3 Effects of TSA treatment on GR-induced NF1 binding to the MMTV promoter. The cells were treated for 0, 1, and 18 h with TSA. Dex was added to half the cultures in the last hour of TSA treatment. Nuclei were isolated and digested with *Hae*III and λ exonuclease. DNA was purified and subjected to linear amplification with a radiolabeled MMTV primer. Digestion products were detected after electrophoresis on 8% denaturing gels followed by autoradiography. The experiment shown is representative of several others

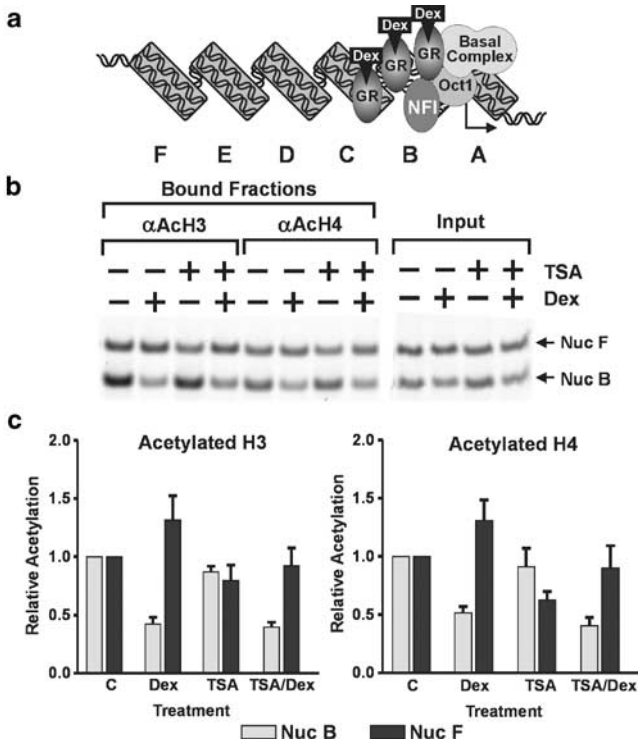

Figure 4 Effects of TSA on acetylation of H3 and H4 at MMTV promoter nucleosomes in the presence and absence of glucocorticoids. The cells were treated with or without TSA for 2 h. Dex was added to half these cultures after 1 h of TSA treatment. After formaldehyde crosslinking, nuclei were isolated and subjected to micrococcal nuclease digestion. ChIP assays were performed as described in Materials and methods. (**a**) A schematic representation of the MMTV promoter showing the approximate positions of the nucleosomes as well as the binding sites of factors in the proximal promoter region. (**b**) shows a representative analysis of a ChIP assay. Samples from cells receiving no treatment are indicated with C. (**c**) shows a summary of the results of three–four independent experiments carried out with antibodies against acetylated H3 (left panel) or acetylated H4 (right panel). In each experiment, band intensities from the bound fractions were normalized with the corresponding inputs. The normalized intensities from the untreated (control) samples were set to 1 and the normalized intensities observed in each treatment condition are expressed as a fraction of control

while the F nucleosome lies just 3′ of the mammary selective enhancer (Mellentin-Michelotti *et al*., 1994). A representative analysis is shown in Figure 4b and a graphic summary of 3–4 independent experiments is shown in Figure 4c. As we recently reported (Sheldon *et al*., 2001), glucocorticoid treatment resulted in a loss of acetylation of histones H3 and H4 in the nucleosome B region of the MMTV promoter, which correlates with a decline in activated transcription after 30 min of exposure to glucocorticoids. Unexpectedly, we observed that TSA treatment does not reverse this GR-induced histone deacetylation at nucleosome B. Even more striking is the fact that TSA does not increase histone acetylation under any treatment condition, even though bulk levels of histone acetylation are greatly increased with 2 h of TSA treatment (Figure 1c). In fact, in the absence of Dex, TSA treatment of 2 h appears to result in an average decline in H4 acetylation at nucleosome F. These results are consistent with a recent report showing that acetylation of histone H4 at the MMTV promoter is moderately decreased after 25 h of TSA treatment in a different cell line (Wilson *et al*., 2002).

To monitor histone acetylation in the time period in which MMTV transcription declines, we carried out ChIP assays at various times after TSA treatment. Bound and input fractions were assayed for the presence of MMTV DNA from four nucleosomes, A,B,C, and F (see Figure 4a). A statistical summary of the results of at least three independent experiments is shown in Figure 5. The asterisks denote values that are statistically different ($P<0.05$) from basal (time 0). At the A nucleosome, which borders the TATA box and contains the start site of transcription, acetylation of histone H4 increased by about 20% in 5 min of treatment. By 1 h of treatment, the levels of H3 and H4 acetylation declined 20% relative to basal levels. At nucleosome B acetylation of histone H3 decreased by about 25% between 15 and 60 min. In contrast, acetylation of histone H4 increased by about 20% initially and then fell back to near basal levels.

Nucleosome C contains two upstream binding sites for the GR and falls in part within the nuclease hypersensitive region generated by glucocorticoid treatment (Fragoso *et al*., 1998). Acetylation of histones at nucleosome C decreased significantly over 1 h of treatment, falling 55% relative to basal levels. At nucleosome F, acetylation of H4 declined over time 45% relative to basal. In general, histone acetylation increased slightly, but significantly, at nucleosomes A and B at early treatment times, but fell thereafter to levels about 20% below control. At nucleosomes C and F, histone acetylation decreased significantly between 5 and 60 min of treatment and the transient rise in acetylation at early time points was not observed. This is very surprising since bulk levels of histone acetylation are rising and should not be expected to fall given the continued presence of the drug.

Effects of TSA treatment on a transiently transfected MMTV template

Our observations that TSA-induced repression of the MMTV promoter occurs independent of GR activation and without sizable increases in histone acetylation suggest that the drug may exert its effects at the promoter primarily on a nonhistone protein involved in basal transcription. A transiently transfected MMTV promoter does not acquire the organized nucleoprotein structure observed at the stably replicating MMTV template in cellular chromatin (Archer *et al*., 1992; Lee and Archer 1994). GR activates it by a mechanism which does not involve chromatin remodeling and derepression, but rather, GR recruitment or stabilization of the basal transcription machinery. If TSA-induced repression is mediated through a factor necessary for basal transcription at the MMTV promoter, this template may also respond to the drug.

Bresnick *et al*. reported that glucocorticoid activation of a transiently transfected MMTV reporter construct

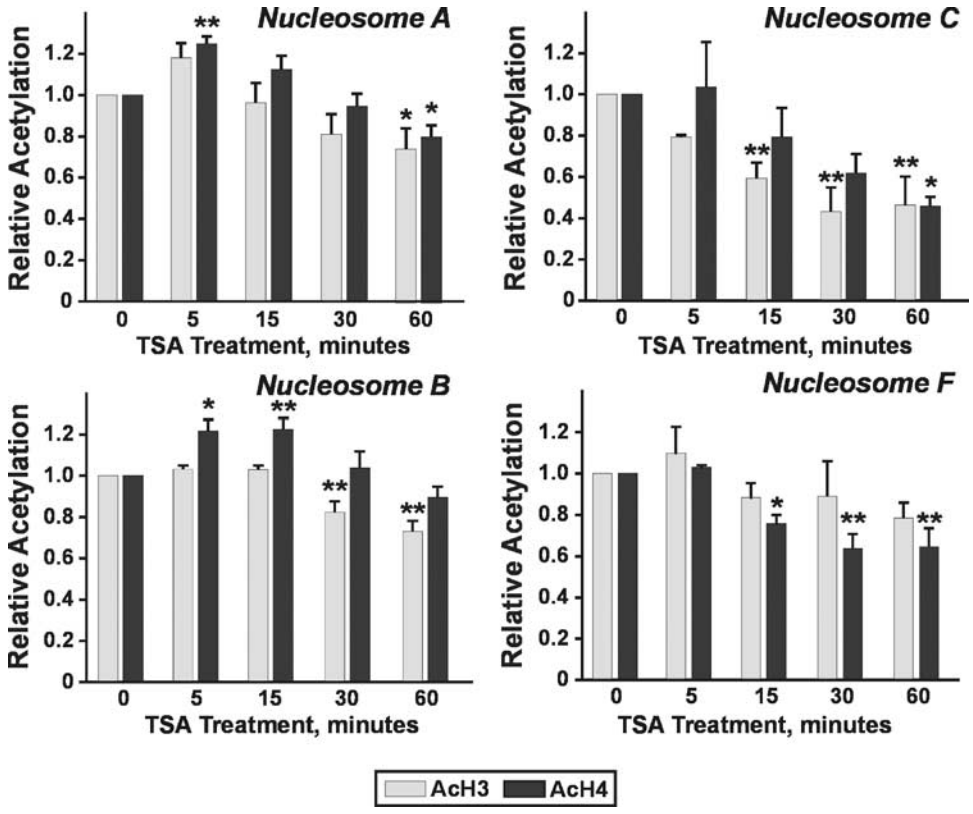

Figure 5 Time course of TSA effects on histone acetylation at nucleosomes in the MMTV promoter region. The cells were treated with TSA for the times indicated. After formaldehyde crosslinking, the cells were processed as described in the legend to Figure 4. The results of three–four independent experiments were subjected to statistical analysis and are shown for nucleosomes A, B, C, and F. The lightly shaded bars represent the levels of acetylated H3, while the black bars represent acetylated H4. The data were subjected to one-way ANOVA (GraphPad Prism) to determine statistically significant differences between the various time points and time zero. Single asterisks denote a *P*-value less than 0.05 and double asterisks denote a *P*-value less than 0.01

was not repressed by sodium butyrate treatment (Bresnick *et al*., 1990). We reexamined this issue in our cell line with TSA treatment. Figure 6a shows that TSA induced significant repression of MMTV promoter activity both in the presence and absence of glucocorticoids, resulting in a pattern very similar to that observed for the stably replicating template (Figure 1a). In the same time frame, TSA treatment resulted in increased activity of the Rous sarcoma virus (RSV) promoter on a cotransfected construct containing a β-galactosidase (β-gal) reporter, indicating that TSA does not have a general inhibitory effect on all transcription in our cell line. A time course of TSA treatment (Figure 6b) shows that these trends are consistent. The transiently transfected MMTV promoter remains repressed at longer treatment times, while RSV promoter activity continues to increase. The longer treatment times are necessary to observe changes in protein levels (i.e. luciferase and β-gal) as opposed to the measurement of RNA levels used above to analyse TSA effects on the MMTV template in organized chromatin.

To determine which part of the MMTV promoter is necessary for TSA-induced repression, we examined various 5′ deletions of the LTR region, as shown in Figure 7a. The reporter pLTRluc contains the full-length LTR plus approximately 100 bp of transcribed region upstream of the luciferase gene. The p220luc construct is deleted to −220 bp relative to the transcription start site and contains just the proximal promoter with four GR-binding sites, an NF1 site, two Oct1-binding sites, a TATA box, and a putative initiator element at the start site of transcription (Pierce *et al*., 1993). Although the extent of activation by Dex treatment is lower for p220luc compared to pLTRluc, TSA treatment causes potent repression of both basal and activated transcription (left panel, Figure 7a). The pMluc construct is deleted to −102 bp, which removes all the GREs except for two half-sites. These sites are not active however, because there is no significant activation by Dex treatment (right panel, Figure 7a). However, TSA treatment also causes repression of pMluc, indicating that the TSA response element is in the proximal MMTV promoter region.

To determine which transcription factor binding site may be mediating the repression, we made constructs containing point mutations in the NF1, Oct1, or putative initiator binding sites. The NF1 and Oct1 mutations were modeled on those made by Brüggemeier and colleagues (Bruggemeier *et al*., 1991), who showed that these mutations resulted in a loss of factor binding.

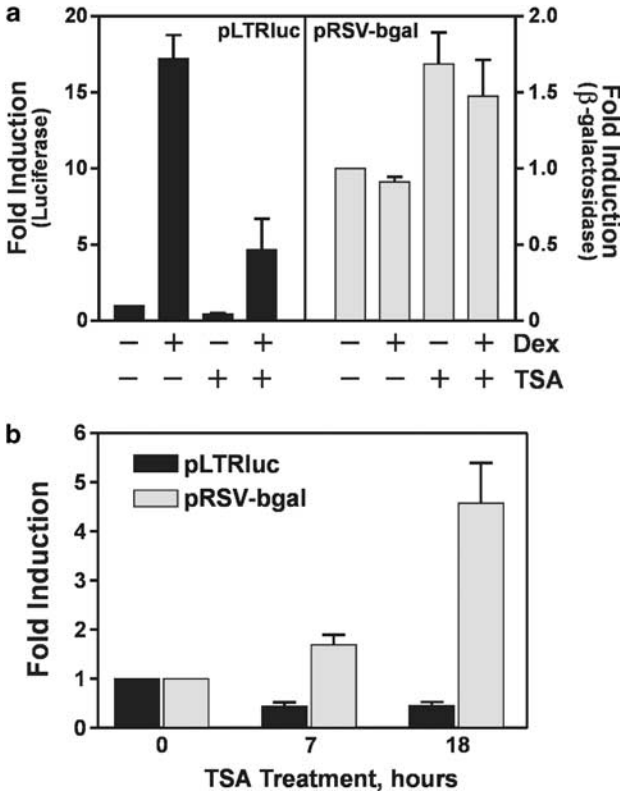

Figure 6 Effects of TSA on a transiently transfected MMTV promoter template. The cells were transfected with pLTRluc, pRSV-bgal, and pUC18. After treatment with TSA, the cells were harvested and extracts were prepared. Luciferase and β-gal activity were measured by chemiluminescent assays. (**a**) TSA effects on promoter activity in the presence and absence of Dex. TSA treatments were for 7 h and Dex treatments were for 6 h. (**b**) Time course of TSA effects on promoter activity. Transfected cells were treated with TSA in the absence of Dex for 0, 7, or 18 h prior to harvest

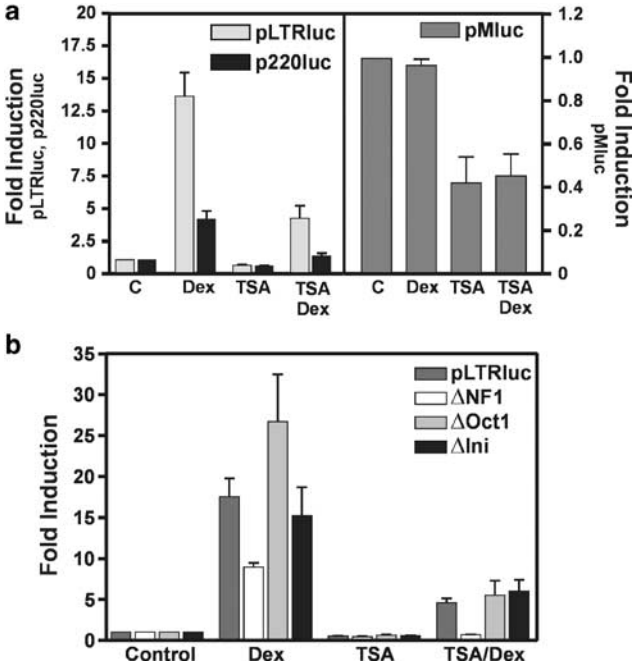

Figure 7 Effects of TSA on MMTV Promoter Mutants. The cells were transfected with the plasmids indicated. The treatments were carried out as described for Figure 6a. Cell extracts were subjected to luciferase assays. Luciferase activities were normalized to protein concentration for each sample and expressed graphically as fold inductions over the untreated controls. The results shown were derived from at least three independent experiments. (**a**) Activities of the the full-length MMTV promoter (pLTRluc) and the promoter deleted to either -220 bp (p220luc) or -102 bp (pMluc). (**b**) Activities of the full-length MMTV promoter containing point mutations in the binding sites for various factors shown to contribute to promoter activity

The mutations made in the initiator were identical to those made by Pierce and colleagues, (Pierce *et al*., 1993). Figure 7b shows the results of transfection analysis. Mutation of the binding sites for NF1 (ΔNF1), Oct1 (both distal and proximal sites - ΔOct1), or the initiator element (ΔIni), did not result in a loss of TSA-induced repression. The initiator element did not appear to be active in our cell lines because the activity profile of the ΔIni construct was identical to that for pLTRluc. Mutation of the NF1 site appeared to make the promoter more vulnerable to TSA-induced repression, since any activation of the ΔNF1 construct caused by Dex treatment was completely inhibited in the presence of TSA.

The remaining factor-binding site in the proximal MMTV promoter region is the TATA box. Since its mutation would knock out all specific transcription, we tested to see if the MMTV TATA box alone could mediate the TSA repression. We also tested other constructs in which promoter activity would be driven mostly through the TATA box region. Descriptions of these constructs can be found in Materials and Methods. Surprisingly, Figure 8 shows that each of the four different TATA elements tested was repressed by TSA treatment. TATA elements from the CMV and ovalbumin promoters were repressed to a lesser extent than those from either the thymidine kinase or MMTV promoters. However, this did not correlate with overall transcriptional activity, as the ovalbumin TATA-containing construct generated much less luciferase activity than that containing the CMV TATA element (data not shown). The two constructs containing the thymidine kinase promoter contain the same sequences from the promoter (-81 bp to $+52$ bp relative to the transcription start site) but different sequence contexts, thus the observed repression by TSA is owing to its effect on the promoter rather than fortuitous effects on surrounding sequences. Given that TSA often has an activating effect on transcription, it is noteworthy that none of the constructs was activated by TSA treatment.

It is unlikely that all TATA-containing promoters in our cells are repressed by TSA treatment. In fact, the TATA-containing RSV promoter was activated by TSA as shown in Figure 6. It is possible that other factors present at a promoter are able to negate any negative effects of TSA on the transcription complex at the TATA box. In Figure 8b, we provide two examples of this. First, we tested two versions of the thymidine

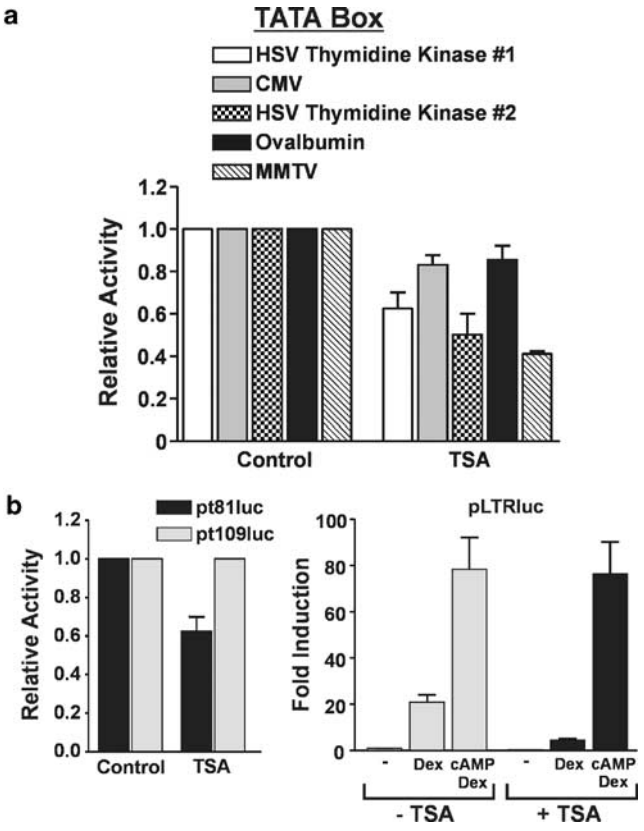

Figure 8 (**a**) Effects of TSA on TATA-driven promoter activity. Reporters containing various TATA elements are described in Materials and methods. The cells were transfected with the various reporters and were either left untreated (Control) or treated with TSA for 7 h. Cell extracts and the resulting data were processed as described in the legend to Figure 7. The reporters were as follows: HSV thymidine kinase #1 – pt81luc, CMV – pUHC13–3, HSV thymidine kinase #2 – pTetTKluc, ovalbumin – pPRE-TATA-luc, MMTV – pMTV-TATA-luc. (**b**) Modulation of TSA effects on the HSV tk and MMTV promoters. The cells were transfected, treated, and processed as described above and in the legend to Figures 6 and 7

kinase promoter, one being very minimal with a GC box and the TATA box (pt81luc—designated as HSV thymidine kinase #1 in Figure 8a), and the other (pt109luc) containing additional regulatory elements that are important in cell cycle regulation of the promoter (McKnight, 1983). In Figure 8b (left panel), we show that, while the minimal promoter is repressed by TSA, the longer version of the promoter is resistant to TSA repression. Second, the MMTV promoter can be synergistically activated by both glucocorticoid and cAMP signaling (Rangarajan *et al*., 1992; Moyer *et al*., 1993). This is shown in the absence of TSA in the right panel of Figure 8b. As we have shown, TSA treatment represses both basal and Dex-activated promoter activity. However, in the presence of both cAMP and Dex, the synergistic activation is unaffected by TSA treatment. These results indicate that the combination of other factors present at particular TATA-containing promoters may protect the promoter from any negative effects of TSA on the basal transcription machinery. In the absence of these factors however, the promoter may be vulnerable to TSA repression through its TATA element.

Discussion

HDAC inhibitors represent an exciting new class of anticancer drugs, but the precise mechanisms by which they target and change gene expression to induce differentiation or apoptosis are largely unknown (Weidle and Grossmann, 2000; Kramer *et al*., 2001). In this study, we have investigated the mechanism by which an HDAC inhibitor represses the MMTV promoter. We have established that the MMTV promoter can be a primary target for these drugs and have determined that the mechanism of transcriptional repression does not involve changes in chromatin remodeling, as previously suggested (Bresnick *et al*., 1990; Bartsch *et al*., 1996). Unexpectedly, this repression is not associated with significant increases in histone acetylation at various nucleosomes within the MMTV promoter. Instead, TSA induces a general deacetylation of promoter nucleosomes. The response of the MMTV promoter to TSA is also not influenced by its nucleoprotein structure and appears to be mediated through the TATA box region. The activity of several other TATA elements was also repressed by TSA. Together, these results suggest that the primary target of HDAC inhibition at the MMTV promoter is a nonhistone protein that is involved in an essential step of basal transcription. Our work defines a previously unrecognized mechanism by which HDAC inhibitors modulate transcription and provides important insights into how target gene specificity might be achieved.

The kinetic detail of our study provides significant mechanistic insights into the mode of repression mediated by HDAC inhibitors. First, the rapid drop in transcription within a time frame of significant increases in bulk histone acetylation indicates that MMTV repression is the direct result of TSA inhibition of HDAC activity. Thus the promoter is likely to be a primary target for the drug. Second, we show that the repression occurs independent of GR action. Transcription is repressed whether the promoter is in the basal or GR-activated state.

Third, in agreement with other reports, we find that TSA inhibits glucocorticoid-induced nuclease hypersensitivity and chromatin remodeling at the MMTV promoter. However, it does so long after MMTV transcription is repressed. Our extensive kinetic analysis of both transcription rates and chromatin remodeling shows that the two effects are unlinked and the changes in chromatin remodeling are not involved directly in transcriptional repression. Only one previous study of MMTV regulation by HDAC inhibitors measured TSA effects on both transcription and chromatin remodeling in a time course, but the shortest treatment was 6 h (Bartsch *et al*., 1996). However, in concordance with our results, they observed a repression of transcription in the absence of glucocorticoids. Another study examined

time-dependent TSA effects on chromatin remodeling induced by progesterone receptor at the MMTV promoter in a different cell type and observed an inhibition of remodeling that occurred between 1 and 4 h of treatment (Wilson *et al*., 2002). However, a corresponding analysis of transcriptional kinetics was not carried out, so it is unclear if the TSA-dependent inhibition of progesterone-mediated MMTV activation they observed was due solely to effects on remodeling or an independent mechanism. They did not observe any effect of TSA on MMTV promoter activity in the absence of glucocorticoids, so it is possible that the mechanism that we observed may have some cell type specificity.

Further mechanistic information is provided by ChIP analysis. Surprisingly, TSA represses the MMTV promoter without inducing sizable increases in histone acetylation at promoter nucleosomes, suggesting that, at this promoter, nonhistone proteins are the ultimate targets of the drug and its inhibition of HDACs. The functional significance of the small increase in histone acetylation at the nucleosomes in the proximal promoter region is unknown; the size of the increase (20%) is much smaller than the increases observed at other gene promoters exposed to TSA (Thomson *et al*., 2001; Ghoshal *et al*., 2002; Kim *et al*., 2003). However, histone acetylation at all MMTV nucleosomes that we tested was generally decreased by TSA within 1 h of treatment. A loss of histone acetylation has also been observed in the continued presence of TSA at the c-jun promoter, suggesting the presence of TSA-insensitive HDACs in mammalian cells (Thomson *et al*., 2001). Histone deacetylation has been associated with transcriptional repression in a number of experimental systems; however, it is unclear whether it plays a causative role in repression at the MMTV promoter since the loss of histone acetylation at most of the nucleosomes is small to moderate and the rate of that loss (Figure 5) lags behind that of the drop in transcription (Figure 1b).

Consistent with the idea that the target of HDAC inhibition at the MMTV promoter is not histones, the repression of the MMTV promoter is not strictly dependent on its overall chromatin configuration because TSA inhibits structurally distinct forms of the promoter. Mutational analysis of the MMTV promoter indicates that the only *cis*-acting element required for TSA repression is the TATA box. In fact, TATA-dependent promoter activity was repressed by TSA from constructs containing several different TATA elements. Together, these observations strongly suggest the acetylation-sensitive regulation of a protein necessary for a basic step in the initiation of MMTV transcription. In support of this contention, we observe that TSA represses the promoter in the presence or absence of glucocorticoids. GR is thought to activate the MMTV promoter in organized chromatin first by derepressing it through chromatin remodeling and second, by recruiting and/or stabilizing the basal transcription machinery (Archer *et al*., 1992). Since GR-dependent chromatin remodeling is unaffected by TSA, the block to transcription occurs downstream of this event. If the assembly or function of the basal transcription complex at the MMTV promoter is inhibited by TSA, repression would be observed independent of GR action.

One way in which inhibition of HDAC activity could be affecting the TATA element is through binding of TBP/TFIID. One study, designed to address a link between protein acetylation and RNA polymerase II transcription in a histone-free system, found that addition of acetyl-CoA stimulated the DNA-binding activity of TFIID. However, they determined that it did so not through direct acetylation of TFIID or TFIIA, but through a different mechanism (Galasinski *et al*., 2000). Other studies have addressed the question of histone acetylation and TBP binding. In one case, TBP binding to chromatin-assembled SV40 minichromosomes was facilitated by histone acetylation at an underlying nucleosome (Sewack *et al*., 2001). However, in yeast gene systems, binding of TBP to promoters does not appear to strictly correlate with the acetylation status of promoter histones (Sekinger and Gross 2001; Katan-Khaykovich and Struhl 2002; Kristjuhan *et al*., 2002).

Inhibition of HDAC activity could also be leading to increased acetylation of a member of the transcription initiation complex, including TBP-associated factors (TAFs), general transcription factors, mediator complex components, or coactivators. Two of the general transcription factors (TFIIE and TFIIF) (Imhof *et al*., 1997) and several coactivators known to interact with the basal machinery, such as p300, PCAF, and SRC1, are known to be acetylated (Chen *et al*., 1999; Sterner and Berger, 2000). In fact, we observe increased acetylation of p300 in response to TSA treatment (SK Snyder and CL Smith, unpublished observations). However, the effects of acetylation on the function of these proteins is largely unknown. Acetylation has been shown to have both positive and negative effects on activities of other factors (Sterner and Berger, 2000). Acetylation of the Drosophila transcription factor TCF weakened its interaction with its key coactivator (Waltzer and Bienz, 1998). In addition, acetylation of the coactivator ACTR by p300 inhibited its ability to interact with nuclear receptors (Chen *et al*., 1999), and HMG I/Y can destabilize the enhanceosome at the interferon-β promoter if acetylated by CBP at lysine 65 (Munshi *et al*., 1998).

Changes in the acetylation of nonhistone proteins mediated by inhibition of HDAC activity provides an attractive mechanism for the target gene specificity of these drugs, since transcriptional regulatory factors are often recruited in a promoter-specific manner. Although our analysis of TATA-driven transcription indicates that it is negatively affected by increased cellular acetylation levels, not all TATA-containing genes are repressed by TSA because other factors binding to promoters, either constitutively or as a result of cell cycle or signaling, may counteract negative effects of protein acetylation on the basal machinery. This is evidenced by the fact that the TK promoter is unaffected by TSA if upstream elements are included. In addition, the MMTV promoter can become refractory to TSA

effects when both cAMP and glucocorticoid signaling pathways are activated. This type of mechanism would further increase the specificity of these drugs and imply that they could have tissue-specific or conditional effects on various gene targets.

Our study has raised questions about the long-standing assumption that the effects of HDAC inhibitors on target gene promoters are mediated largely through changes in histone acetylation. It is possible that increased acetylation of nonhistone proteins may be of equal or greater importance in mediating the primary and specific effects of the drugs. Identification of more primary target promoters should result in a better understanding of how these drugs modify the genetic program of cells. As more HDAC inhibitors and specific reagents for detecting acetylation of nonhistone factors become available, we look toward defining both the HDACs and transcriptionally relevant factors that are at work in the response of the MMTV promoter to changes in acetylation. This well-characterized promoter will provide a very useful model for further analysis of HDAC-mediated effects on transcription as well as understanding mechanisms of mediating specificity by HDAC inhibitors.

Materials and methods

Cell lines and reagents

Cell line 1470.2 was derived from C127i mouse mammary adenocarcinoma cells through transformation with episomes containing the transforming (69%) fragment of the bovine papilloma virus (BPV) genome (Pennie *et al*., 1995). It contains multiple copies of MMTV-CAT (chloramphenicol acetyltransferase) transcription units in the context of BPV sequences. Cells were maintained in DMEM containing 10% fetal bovine serum. Final concentrations of Dex (Sigma) and TSA (Wako) used in cell treatment were 100 nM and 50 ng/ml, respectively. Oligonucleotide primers used to detect the B and F nucleosome regions have been described (Sheldon *et al*., 2001). Primers for the A nucleosome region are: upper strand – 5′ TGCAACAGTCCTAACATTCACCTC-3′ and lower strand – 5′-GTGAAGGATAAGTGACGAGCGG-3′. Primers for the C nucleosome region are: upper strand – 5′-TCTCAAGAAGAAAAAGACGACATG-3′ and lower strand – 5′ CTGTCCCCTCCTTGGTATGG-3′.

Plasmids

Plasmids used include pM25, containing MMTV-CAT sequences (kindly provided by Dr Gordon Hager, NIH), pβ-actin (kindly provided by Dr Bruce Patterson, NIH), containing β-actin cDNA cloned into pUC18, and pLTRluc, which contains the full-length MMTV LTR driving transcription of the luciferase gene (Lefebvre *et al*., 1991). Plasmids p220luc (originally named pHHluc), pMluc, pt81luc, and pt109luc have been described (Nordeen, 1988) and were a generous gift from Dr Steve Nordeen (University of Colorado Health Science Center) (Nordeen, 1988). Plasmid pUHC13-3 (Gossen and Bujard, 1992) contains the minimal CMV promoter (TATA element) downstream from several copies of the bacterial tetracycline operator and was purchased from Life Technologies. Plasmid pTetTKluc was generated by replacing the CMV TATA element in pUHC13-3 with a PCR fragment containing sequences from the herpes simplex virus (HSV) thymidine kinase promoter (-81 bp to $+52$ bp relative to the transcription start site). Plasmid pPRE-TATA-luc contains two copies of a progesterone/glucocorticoid response element upstream of the ovalbumin TATA element and the luciferase reporter gene (kindly provided by S Stoney Simons, NIDDK). The constructs indicated as ΔNF1, ΔOct1 and ΔIni were synthesized from pLTRluc using site-directed mutagenesis (Quikchange, Stratagene). The distal and proximal regions of the NF1-binding site were replaced with cleavage sites for *Xho*I and *Bgl*II, respectively, to generate ΔNF1. The distal and proximal Oct1-binding sites were replaced with cleavage sites for *Nhe*I and *Kpn*I, respectively, to generate ΔOct1. The putative initiator element was replaced with a *Bgl*II cleavage site to generate ΔIni. This mutation is identical to that published by Pierce *et al*. (1993). The pMTV-TATA-luc construct was generated by exchanging a *Kpn*I/*Pac*I fragment from pMluc, containing the promoter region and part of the luciferase gene, with a *Kpn*I/*Pac*I fragment from the ΔOct1 construct, which extends from the *Kpn*I site at the mutated proximal Oct1-binding site into the luciferase gene. It contains the MMTV promoter from just upstream of the TATA box through part of the transcribed region of the LTR.

RNA isolation and S1 nuclease analysis

RNA was isolated from 1470.2 cells as previously described (Pennie *et al*., 1995). Radiolabeled probes for S1 analysis were prepared by linear amplification of both MMTV and β-actin sequences using specific oligonucleotide primers and linearized template plasmids in the presence of α-[^{32}P]dATP. S1 nuclease analysis was carried out as described previously (Pennie *et al*., 1995).

Nuclear run-on transcription

Nuclei were isolated as described previously (Pennie *et al*., 1995) and frozen in liquid nitrogen. Run-on analysis was carried out as described (Pennie *et al*., 1995). Approximately 3×10^6 c.p.m. of each RNA sample was diluted in 1 ml hybridization buffer [3 × SSC, 20 mM sodium phosphate pH 7.3, 0.02% polyvinyl pyrrolidone, 0.02% Ficoll, 0.1% SDS, 100 μg/ml yeast tRNA (Life Technologies)] and exposed to membranes to which DNA had been affixed. Both prehybridization and hybridization were carried out overnight at 60°C in 3 × SSC, 20 mM sodium phosphate pH 7.3, 0.02% polyvinyl pyrolidone, 0.02% Ficoll 400, 0.1% SDS and 100 μg/ml yeast tRNA. After washing, membranes were dried and exposed to phosphorimaging screens.

Histone extraction and analysis

Aliquots of nuclei isolated for run-on analysis were pelleted and resuspended in 0.2 M H_2SO_4 at an approximate DNA concentration of 2 mg/ml. After overnight incubation at 4°C, acid-soluble proteins were clarified by microcentrifugation and precipitated with TCA. The precipitates were washed with acidified acetone and acetone prior to drying and resuspension in 10 mM β-mercaptoethanol. In total, 5 μg of acid-soluble protein was separated on 15% SDS–PAGE gels and analysed for the presence of acetylated H3 and acetylated H4 by Western blotting with antibodies specific for these modifications. Antiacetyl(K9/K14)H3 and antiacetylH4 (Upstate Biotechnology, Inc.) were used at a dilution of 1 : 2000.

Sac*I access and* NF*1 binding assays*

Nuclei were isolated and digested with either *Sac*I (10 U/μg DNA) or a combination of *Hae*III (5 U/μg DNA) and λ exonuclease (0.1 U/μg DNA) as described previously (Pennie *et al*., 1995). DNA was purified from digested nuclei by phenol/chloroform extraction and ethanol precipitation. DNA from nuclei cleaved with *Sac*I was digested to completion with *Dpn*II. The digestion products were linearly amplified by *Taq* polymerase using a radiolabeled oligonucleotide primer corresponding to sequences around the start site of MMTV transcription. After separation on 8% denaturing polyacrylamide gels, digestion products were visualized using a phosphorimager.

ChIP assays

Cells were treated with TSA prior to fixation with 0.5% formaldehyde for 10 min at 37°C. The cells were washed immediately with ice-cold PBS (w/o Ca and Mg) and harvested. Nuclei were isolated as described above and digested with micrococcal nuclease (0.375 U/μg DNA) for 10 min at 37°C. Soluble material containing predominantly mononucleosomes was isolated by centrifugation and diluted with buffer containing 0.01% SDS, 1% Triton X-100, 1.5 mM EDTA, 15 mM Tris pH 8.0, 15 mM NaCl, 5 mM sodium butyrate, 10 mM NaF, and 1 × Protease inhibitor cocktail (Calbiochem). Acetylated histones were immunoprecipitated overnight with 10 μg of either antiacetylH3 or antiacetylH4 (Upstate Biotechnology). A control with no antibody added was included for nonspecific binding. Samples were incubated with protein A-agarose (Pierce) for 3 h. Agarose-conjugated immune complexes were washed sequentially (5 min/wash) according to the ChIP protocol from Upstate Biotechnology Inc. Bound nucleosomal material was eluted by sequential washes in Elution buffer (50 mM NaCl, 10 mM Tris pH 7.5, 5 mM EDTA, 5 mM sodium butyrate, 10 mM NaF) containing 1.5 and 0.5% SDS. After crosslink reversal, DNA was purified by extraction with phenol/chloroform and ethanol precipitation. DNA concentrations were obtained by fluorimetry with Hoechst dye. Equal amounts of DNA from each sample were subjected to PCR (20 cycles) with individual oligonucleotide primer sets specific for MMTV nucleosome regions A, B, C, and F (see Figure 4a). PCR products were separated on 8% polyacrylamide gels (TBE) and visualized after incubation with Sybrgreen (Molecular Probes) using a fluorimager (Molecular Dynamics).

Cell transfections

Transfections were carried out by electroporation using a BTX Squareporator (Genetronics). Aliquots of 5×10^6 cells (1470.2) were transfected with 5–10 μg pLTRluc, 2 μg pRSV-bgal (containing RSV enhancer/promoter driving expression of the β-gal gene), and 5 μg pUC18 as a carrier. The cells were treated, harvested, and assayed for luciferase activity as described previously (Pennie *et al*., 1995). β-gal activity was measured by chemiluminesence (Tropix). All assays were carried out in a Berthold Microlumat 96P luminometer (Wallac).

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