Prolonged glucocorticoid exposure dephosphorylates histone H1 and inactivates the MMTV promoter

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Glucocorticoids rapidly induce transcription from the mouse mammary tumour virus (MMTV) promoter via a glucocorticoid receptor (GR)-mediated chromatin disruption event. This remodelling of chromatin is transient such that upon prolonged exposure to hormone the promoter becomes refractory to glucocorticoids. We demonstrate that this refractory state requires the continual presence of hormone and can be reversed by its removal. Our experiments show that the promoter is inactivated via a mechanism whereby histone H1 is dephosphorylated in response to glucocorticoids. Removal of glucocorticoids results in the rephosphorylation of histone H1 and the reacquisition of transcriptional competence by the promoter. This response is specific for the MMTV promoter assembled as chromatin and is not observed for another inducible gene or transiently transfected MMTV DNA. Finally, we demonstrate that H1 on the MMTV promoter is dephosphorylated when the promoter is unresponsive to glucocorticoids. These studies indicate that phosphorylated H1 is intimately linked with the GR-mediated disruption of MMTV chromatin *in vivo*.

Keywords: chromatin remodelling/dephosphorylation/
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Introduction

An expanding body of knowledge provides clear mechanistic links between the architecture of DNA as chromatin and the regulation of transcription (Felsenfeld, 1992; Wolffe *et al.*, 1993; Kingston *et al.*, 1996). These investigations include elegant genetic experiments, precise structural characterization of the nucleosome and detailed *in vitro* biochemical analysis of the transcriptional process (Grunstein, 1990; Arents *et al.*, 1991; Adams and Workman, 1993; Almouzni and Wolffe, 1993). The majority of these studies suggest that the packaging of DNA into nucleosomes acts as a barrier to the initiation of transcription by preventing the access of transcription factors to their recognition sites (Hayes and Wolffe, 1992; Workman and Buchman, 1993). Genetic experiments in *Saccharomyces cerevisiae* and *Tetrahymena thermophila* have demonstrated specific roles for the core and linker histones in these processes (Grunstein, 1990; Shen and

Gorovsky, 1996). Studies in yeast have defined the contribution of individual histones to the repression of transcription such that the deletion of one or more of the core histones leads to the deregulation of a variety of inducible genes. Specifically, mutation of histone H4 activates transcription (Han and Grunstein, 1988) whereas decreased levels of H2A–H2B dimers de-repress transcription at a number of yeast genes (Norris and Osley, 1987). In *Tetrahymena* the deletion of H1 leads to specific gene activation without a pronounced effect on global transcription by Pol II or Pol III (Shen and Gorovsky, 1996). This is consistent with the specific repression of the 5S rRNA gene observed with the over-expression of histone H1 in *Xenopus laevis* (Bouvet *et al.*, 1994). In addition, genetic experiments examining mating type switching in yeast have also identified a number of protein complexes, exemplified by the SWI–SNF complex, which have profound effects on chromatin remodelling and transcription both *in vivo* and *in vitro* (Peterson and Herskowitz, 1992; Yoshinaga *et al.*, 1992; Côté *et al.*, 1994; Imbalzano *et al.*, 1994). Beyond these general features attributed to assembling DNA into chromatin, post-translational modifications of core histones have been shown to exert dramatic effects. Perhaps the most well-characterized example of this is the acetylation of lysines in the N-terminal tails of histones H3 and H4 (Roth *et al.*, 1992; Fisher-Adams and Grunstein, 1995). Both biochemical studies using agents that promote hyper-acetylation of the tails and genetic experiments whereby the tails are prevented from being acetylated reveal that hyper-acetylated histones are associated with activated chromatin (Durrin *et al.*, 1991; Lee *et al.*, 1993; Van Lint *et al.*, 1996). In agreement with these studies, analysis of heterochromatic silencing in *S.cerevisiae* reveals a correlation between hypo-acetylation of histones and a reduction in transcriptional activity (Braunstein *et al.*, 1993).

A clear repressive role for chromatin structure has been established by detailed biochemical studies (Hayes and Wolffe, 1992; Adams and Workman, 1993). They reveal that the activities of RNA Pol II and III are significantly inhibited by assembly of target sequences into nucleosomes (Knezetic *et al.*, 1988; Laybourn and Kadonaga, 1991; Hansen and Wolffe, 1994). This nucleosomal structure is also refractory to binding by many, but not all, transcription factors and members of the general transcription machinery (Archer *et al.*, 1991; Workman and Kingston, 1992). Consistent with the observations derived from genetic studies, *in vitro* nucleosomal reconstitution experiments indicate that the removal of H2A and H2B from nucleosomal arrays results in decreased chromatin compaction and enhanced gene activity (Hansen and Wolffe, 1994).

In addition to core histones, the linker histones, by

virtue of their participation in chromatin condensation, have been implicated as repressors of transcription (Croston *et al.*, 1991; Shen *et al.*, 1995). In most cases, histone H1 is thought to bind DNA in the nucleosome across the dyad axis and at the linker DNA as it enters and leaves the nucleosome (Ali and Singh, 1987). However, at least for the 5S rRNA gene, recent evidence suggests that a specific and asymmetric binding of the linker histone may be possible (Hayes and Wolffe, 1993; Hayes, 1996; Pruss *et al.*, 1996). The binding of histone H1 *in vitro* has been shown to stabilize the nucleosome and is proposed to facilitate the folding of nucleosomal arrays into 30 nm chromatin fibres *in vivo* (Felsenfeld and McGhee, 1986; Kamakaka and Thomas, 1990). Histone H1 can exert a repressive effect on transcription even when transcription factors are in excess (Croston *et al.*, 1991). *In vitro* studies indicate that the association of histone H1 with nucleosomal cores impairs the binding of transcription factor Gal4-AH and upstream stimulatory factor (USF) to DNA (Juan *et al.*, 1994). This observation is consistent with protein-DNA interaction experiments demonstrating that cross-linking of histone H1 to actively transcribed genes is significantly lower than to transcriptionally silent genes (Dimitrov *et al.*, 1990; Kamakaka and Thomas, 1990; Bresnick *et al.*, 1991; Dedon *et al.*, 1991).

The mouse mammary tumour virus (MMTV) represents a well established mammalian system where chromatin structure and transcriptional regulation have been intimately linked (Hager *et al.*, 1993; Truss *et al.*, 1993; Archer *et al.*, 1995). This promoter reproducibly acquires a phased array of six positioned nucleosomes when stably introduced into mammalian cells (Richard-Foy and Hager, 1987). Detailed analysis of the glucocorticoid induced transcription from this promoter demonstrates that the glucocorticoid receptor (GR) initiates a chromatin remodelling event which results in the loading of a pre-initiation complex (PIC) and active transcription from this promoter (Cordingley *et al.*, 1987; Archer *et al.*, 1992). We have confirmed a primary role for chromatin structure in regulating this promoter by the analysis of identical DNA sequences in transient transfection experiments (Archer *et al.*, 1992; Lee and Archer, 1994). Characterization of these transiently transfected molecules reveals that while they are probably associated with nucleosomes they do not display the phased nucleosomal array characteristic of the MMTV promoter in mouse chromatin (Cereghini and Yaniv, 1984; Reeves *et al.*, 1985; Archer *et al.*, 1992; Jeong and Stein, 1993). Consequently these templates are hypersensitive to endonucleases in both the absence and presence of hormone (Archer *et al.*, 1992). As would be predicted by this hypersensitivity, *in vivo* footprinting studies reveal that transcription factors normally excluded from the endogenous promoter are constitutively bound to the transient template (Archer *et al.*, 1992; Lee and Archer, 1994). However, these transient MMTV templates remain hormone inducible via a mechanism that involves the hormone-dependent recruitment of the TATA binding protein to the MMTV promoter (Lee and Archer, 1994).

Previously, we demonstrated that the MMTV promoter was maximally induced 1 h after hormone treatment and became refractory to GR activation after 24 h of continuous hormone exposure (Archer *et al.*, 1994a; Lee and Archer,

1994). This 'silencing' occurred despite the presence of relevant transcription factors in the nucleus during the refractory phase as evident by the fact that they were able to interact with the transiently introduced MMTV DNA (Lee and Archer, 1994). Consequently, we have suggested that these factors were prevented from binding to their recognition sequences by the reformation of nucleosome B (nuc-B) thus resulting in the inhibition of transcription (Lee and Archer, 1994). This decline in activation following hormone stimulation is characteristic and unique for templates assembled as chromatin as it is not observed when the same sequence is transiently transfected into these cells. This observation suggests that while chromatin structure is vital to the understanding of transcriptional activation, it also plays an important role in the deactivation or cessation of transcription after hormone stimulation. To investigate the mechanisms involved in this loss of activity or transcriptional competence from the promoter we have carried out a series of experiments in which we demonstrate that the maintenance of the refractory state requires the continuous presence of hormone. Prolonged exposure of the cells to hormone induces a refractory or silent state on the promoter that is accompanied by the global dephosphorylation of histone H1. Subsequent experiments demonstrated that the phosphorylation state of histone H1 parallels the activation status of the promoter, and that if H1 phosphorylation is blocked, the MMTV promoter remains refractory to glucocorticoid activation. In contrast, activation of the metallothionein (MT) promoter or the MMTV promoter transiently transfected into the same cells is not influenced by the phosphorylation status of histone H1. A direct role for phosphorylated H1 is suggested by the demonstration that when H1 on the MMTV promoter assembled as chromatin is dephosphorylated, the promoter no longer responds to glucocorticoid. Thus, the phosphorylation of histone H1 plays a pivotal role in transcriptional activation and modulates the ability of the GR to disrupt MMTV chromatin.

Results

Hormone withdrawal restores transcriptional competency to the MMTV promoter

The MMTV promoter has been the focus of numerous studies on the role of chromatin structure in the events leading to the activation of transcription by nuclear hormone receptors (Archer and Mymryk, 1995). The GR induces a rapid transcriptional response from the MMTV promoter by modifying chromatin structure to facilitate the assembly of the transcription pre-initiation complex (Zaret and Yamamoto, 1984; Cordingley *et al.*, 1987; Richard-Foy and Hager, 1987). Prolonged exposure to hormone results in a cessation of transcription such that the promoter returns to near basal levels of activity after 24 h of hormone treatment. This lack of transcription occurs despite the continued presence of hormone and is accompanied by the disruption of transcription complexes and the loss of hypersensitivity as a consequence of chromatin reformation (Lee and Archer, 1994). To explore the mechanism(s) underlying the GR's inability to activate transcription we have ascertained if the continued presence of the hormone is required to maintain the refractory phase. The approach we have taken is to examine if

the 'refractory' promoter could be re-stimulated after hormone withdrawal, growth in the absence of hormone and subsequent hormone re-administration prior to harvesting. A schematic of the experimental approach is presented in Figure 1A. Specifically, cells were either maintained initially in the absence (Figure 1B, lane 1) or presence of hormone for 4 h (Figure 1B, lane 2) and 24 h (Figure 1B, lanes 3–6). Cells grown for 24 h in the presence of hormone were then examined for the effects of continued hormone treatment (Figure 1B, lane 3), hormone removal for 20 h or 1 h prior to the re-addition of hormone (Figure 1B, lanes 5 and 6) and hormone removal without subsequent re-stimulation (Figure 1B, lane 4). Primer extension analysis of total RNA indicated that while 4 h of hormone administration prior to harvesting led to an increase in mRNA accumulation (Figure 1B, compare lanes 1 and 2), 48 h of hormone treatment resulted in a significant reduction in mRNA levels compared with 4 h of treatment (Figure 1B, compare lanes 1, 2 and 3). However, 20 h of dexamethasone withdrawal, prior to 4 h of hormone re-administration, resulted in the reactivation of the promoter and an increase in mRNA accumulation (Figure 1B, compare lanes 3 and 5). In contrast, if hormone was removed for only 1 h during the refractory phase then no increase in mRNA levels was detected if cells were re-treated with 4 h of dexamethasone (Figure 1B, lane 6). These hormone dependent changes were specific for MMTV as no change was observed for actin mRNA examined from the same cells (Figure 1B, lanes 1–6). This is somewhat surprising, given that glucocorticoids, which activate the promoter though the GR, also appear to be responsible for silencing transcription in cells that received prolonged hormone exposure.

We have established that silencing of the MMTV promoter in response to prolonged exposure to glucocorticoid is observed with stable chromatin templates but not transiently transfected templates (Lee and Archer, 1994). In the next series of experiments, we directly examined the effect of hormone withdrawal and re-addition on the activation of transcription from the transiently transfected MMTV plasmid. In these experiments the transient template displayed continued transactivation upon prolonged exposure to hormone (Figure 1C, compare lanes 1, 2 and 3). This is consistent with our previous studies indicating that transient templates fail to show an inhibition of activity at 24 or 48 h of hormone treatment (Lee and Archer, 1994). Further, in contrast with the chromatin templates, removal of hormone for 1 h is sufficient to allow the re-activation of the promoter upon hormone re-addition (Figure 1B and C, compare lanes 5 and 6).

As the establishment of the refractory phase is characterized by a 'closed' chromatin structure over nuc-B, we next determined if the changes in mRNA levels seen above correlate with alterations in chromatin structure. As shown in Figure 2, we conducted an *in vivo* restriction enzyme hypersensitivity assay with cells maintained under a regimen analogous to that described above except that hormone re-addition was for 1 h (Figure 1A). [We have previously established that while mRNA accumulation peaks at 4 h, changes in chromatin structure and transcription factor loading are maximal at 1 h of

Fig. 1. RNA accumulation in response to hormone removal and readdition during the refractory phase. (A) Experimental schematic indicating times of hormone exposure, removal and re-exposure prior to harvesting. (B) 1471.1 cells cultured under the conditions in (A); the levels of MMTV and actin mRNAs were analyzed by primer extension from total RNA (20 µg) using gene-specific primers as described in Materials and methods. Cells were cultured without hormone (lane 1), treated with dexamethasone (10^{-7} M) for 4 h (lane 2) and 48 h (lane 3), or treated with dexamethasone for 24 h (lanes 4 and 5), then hormone was removed for 24 h (lane 4) or 20 h (lane 5) and re-treated with dexamethasone (10^{-7} M) for 4 h prior to harvest (lane 5). Alternatively, cells were treated with dexamethasone for a period of 43 h, hormone was removed for 1 h and dexamethasone (10^{-7} M) was added for 4 h prior to harvest (lanes 6). (C) Cells transfected with 10 µg of pLTR LUC, a plasmid which contains the MMTV LTR attached to the firefly *Luciferase* gene, were cultured under the conditions described in (A). Total RNA (5 µg) was reverse transcribed to cDNA using a primer specific for the *Luciferase* gene and Superscript reverse transcriptase (Gibco-BRL). The cDNA was analyzed by *Taq* polymerase amplification with 32 P-labeled primers specific for the MMTV LTR. The levels of 18S mRNA were analyzed by primer extension of total RNA (3 µg) as described in Materials and methods. The purified extended products were analyzed on 7% polyacrylamide denaturing gels and exposed to Dupont reflection film.

Fig. 2. Restoration of restriction enzyme hypersensitivity and the loading of NF1 onto the stable template following hormone withdrawal during the refractory phase. (A) Mouse mammary cells (1471.1) containing the MMTV promoter, mobilized on a BPV-based multicopy plasmid as a stable replicating unit, were treated with dexamethasone (10^{-7} M) for 24 h followed by hormone withdrawal for 23 h (lane 5) or 24 h (lane 4) and were either not given any hormone (lane 4) or were treated with dexamethasone (10^{-7} M) for 1 h prior to harvest (lane 5). Alternatively, cells were treated with dexamethasone for a period of 46 h, hormone was removed for 1 h and dexamethasone (10^{-7} M) was added for 1 h prior to harvest (lane 6). Cells that did not receive any hormone or were treated with dexamethasone for 1 or 48 h are shown in lanes 1, 2 and 3, respectively. The nuclei were isolated and digested with *DpnII* *in vivo* as previously described. Following purification, all the samples were digested to completion with *HaeIII* *in vitro* as an internal standard for reiterative primer extension analysis using a 32 P-labeled primer. The purified extended products were separated on a 5% polyacrylamide denaturing gel before autoradiography at -80°C . The arrows indicate *HaeIII* and *DpnII* cleavage products. (B) 1471.1 cells were treated as described in Figure 2A and nuclei were isolated and digested with *HaeIII* and Exonuclease III. The purified DNA were recut with *HaeIII* *in vitro*, treated with Mung bean nuclease and then analyzed by primer extension with *Taq* polymerase using primers that were specific for the MMTV promoter.

hormone treatment (Archer *et al.*, 1994a; Lee and Archer, 1994).] Congruent with the RNA analysis, cleavage within nuc-B was elevated 8-fold when cells were re-stimulated with dexamethasone after 23 h of hormone removal (Figure 2A, compare lanes 1, 2 and 5). This is in contrast to cells that were treated with dexamethasone for 48 h, (Figure 2A, lane 3) or had undergone hormone withdrawal without subsequent re-stimulation (Figure 2A, lane 4). In these cells cleavage within nuc-B was similar to that seen in unstimulated cells, 1.3- to 1.6-fold elevation,

indicative of a 'closed' nucleosomal structure. This suggests that 23 h after the removal of hormone the refractory phase is abrogated and chromatin can once again be remodelled by the GR. Interestingly, cleavage was minimal if hormone was removed from cells for only 1 h prior to hormone re-stimulation, 1.9-fold versus 8-fold (Figure 2A, compare lanes 1, 6 and 5), indicating that short term hormone removal is insufficient to allow reactivation. Thus the recovery of transcriptional competence by the MMTV promoter that has become refractory to the GR requires hormone withdrawal for a period of 20 h.

Coincident with the GR-induced remodelling of MMTV chromatin is the hormone dependent formation of a PIC at the promoter. As shown in Figure 2B, this complex can be monitored by examining the hormone dependent loading of transcription factor NF1 using *in vivo* footprinting analysis (Figure 2B, lanes 1 and 2). Consequently, it was important to examine the assembly of the PIC when the promoter was re-activated during the refractory phase upon hormone withdrawal and re-addition. NF1 binding was observed in cells that were treated with hormone for 24 h and subsequently had hormone removed for 23 h prior to re-stimulation, but not in cells that were maintained in media containing hormone for 48 h (Figure 2B, compare lanes 3 and 5). Consistent with the restriction enzyme hypersensitivity assay, NF1 was not detected when cells were re-stimulated with dexamethasone after only 1 h of hormone removal (Figure 2B, lane 6). These experiments confirm that chronic exposure to glucocorticoid results in a non-inducible promoter. Further, they demonstrate that hormone removal for a protracted period restores the promoter to a 'conformation' that can be re-activated upon a second exposure to hormone.

Phosphorylation status of histone H1 and transcriptional competency of the MMTV promoter

Our experiments suggest that it is the distinct arrangement of chromatin during the refractory phase that prevents the GR-mediated activation of MMTV transcription (Figures 1 and 2). This new arrangement of chromatin that results from prolonged exposure to hormone is itself reversible upon removal of the hormone (Figure 2). Consequently, we turned our attention to reversible aspects of chromatin structure, such as post-translational modification of histones, which might contribute to the loss and recovery of transcriptional competence at this promoter. In addition to the disruption of core histones, the GR-mediated activation of MMTV transcription has been correlated with the displacement of histone H1 (Bresnick *et al.*, 1991). This linker histone is known to undergo a number of post-translational modifications including phosphorylation (Bradbury, 1992; Roth and Allis, 1992). The phosphorylation of histone H1 influences its interaction with DNA and has been proposed to result in an 'opening' of chromatin to increase transcription factor access to DNA (Roth and Allis, 1992). To pursue this possibility, we have examined the level of phosphorylated H1 in cells exposed to dexamethasone under a similar time course that results in the loss of transcriptional competence at the promoter (Figure 3). Western blot analysis indicated that phosphorylated H1 was abundant

Fig. 3. Histone H1 is progressively dephosphorylated in the continued presence of dexamethasone. Mouse 1471.1 cells were either not given any hormone (lane 1) or were treated with dexamethasone (10^{-7} M) for a period of 1, 7 or 24 h (lanes 2–4 respectively). Total histones were prepared from nuclei by acid extraction as described in Materials and methods. Histones (40 μ g) were separated on a 16% acrylamide acid-urea gel, transferred to a nitrocellulose membrane and Western blot analysis was performed with a polyclonal antibody against phosphorylated H1. As a loading control the blot was then stained with amido black to reveal total histone H1 present.

in untreated cells (Figure 3, lane 1) and in cells that were treated with dexamethasone for 1 h (Figure 3, lane 2). However, histone H1 was progressively dephosphorylated in the continued presence of dexamethasone. After 7 h of hormone treatment the level of phosphorylated H1 present was decreased (Figure 3, lane 3) and became barely detectable after 24 h of dexamethasone treatment (Figure 3, lane 4). Under these conditions no changes in total H1 levels were evident (Figure 3, lanes 1–4). Hence, the 'opening' and 'closing' of nuc-B, the ability of transcription factors to bind chromatin and the level of transcription following hormone treatment correlate tightly with the phosphorylation and the dephosphorylation of histone H1.

If the 'closing' of nuc-B after 24 h of dexamethasone treatment results from the dephosphorylation of histone H1 this would imply that histone H1 is rephosphorylated under conditions that reactivate transcription (Figure 1B). Indeed, in sharp contrast to cells that were grown in media containing dexamethasone for a period of 48 h (Figure 4, lane 4), histone H1 was highly phosphorylated after hormone was removed from cells for 23 or 24 h (Figure 4, lanes 5 and 6). Furthermore, histone H1 remained dephosphorylated when dexamethasone was removed for only 1 h from cells exposed to hormone for 46 h (Figure 4, lane 7). This is consistent with the restriction enzyme hypersensitivity and transcription factor loading assay, indicating that 1 h of hormone removal was insufficient to obtain activation during the refractory phase (Figures 1 and 2). Finally, while the phosphorylation status of H1 was altered by hormone treatment, the levels of total histone H1 were unchanged by hormone administration (Figure 3 and data not shown). These observations strengthen the correlation between H1 phosphorylation and induction of transcription from the MMTV promoter, linking changes in histone H1 phosphorylation with precise alterations in the hormone responsiveness of the MMTV promoter assembled as chromatin.

These results imply that the phosphorylation of histone H1 plays an important role in the recovery from the refractory phase, including the ability of the GR to modify

Fig. 4. Histone H1 is rephosphorylated after prolonged hormone withdrawal. Cells were treated with dexamethasone (10^{-7} M) for 24 h, hormone was removed for 23 h (lane 6) or 24 h (lane 5) and cells were either not given any hormone (lane 5) or were treated with dexamethasone (10^{-7} M) for 1 h prior to harvest (lane 6). Alternatively, cells were treated with dexamethasone for a period of 46 h, hormone was removed for 1 h and dexamethasone (10^{-7} M) was added for 1 h prior to harvest (lane 7). Cells that did not receive any hormone or were treated with dexamethasone for 1 and 48 h are shown in lanes 2, 3 and 4 respectively. Purified histone H1 (10 μ g) (lane 1) and total histones (40 μ g) (lanes 2–7) were separated on a 16% acrylamide acid-urea gel, transferred to a nitrocellulose membrane and Western blot analysis was performed using a polyclonal antibody against phosphorylated H1.

MMTV chromatin and activate transcription. Thus we would predict that blocking H1 rephosphorylation when hormone is removed would prevent the reactivation of the promoter. For these experiments, we used the kinase inhibitor staurosporine to prevent the rephosphorylation of H1 following hormone removal. Western blot analysis of the phosphorylation state of histone H1 indicated that staurosporine inhibited histone H1 rephosphorylation after hormone removal (Figure 5A, lanes 6 and 7) to a level that was not detectable by the chemiluminescent detection procedure. In fact, the level of phosphorylated H1 was lower in cells treated with staurosporine (Figure 5A, lanes 5, 6 and 7) than in cells treated with dexamethasone for 48 h (Figure 5A, lane 2). In contrast, cells not treated with staurosporine displayed high levels of phosphorylated H1 following dexamethasone removal (Figure 5A, lanes 3 and 4).

Having established that staurosporine blocked the rephosphorylation of H1 after hormone removal, we next examined the ability of the GR to trans-activate under these conditions (Figure 5B). Consistent with our prediction, the promoter remained refractory despite 23 h of hormone removal and re-addition when H1 was maintained in a dephosphorylated state. Primer extension analysis demonstrated that no MMTV mRNA was detected in cells maintained with staurosporine after hormone removal and re-addition (Figure 5B, lane 4). This is in contrast to cells that were not treated with staurosporine where a substantial increase in MMTV mRNA after 20 h of hormone withdrawal and 4 h of hormone administration (Figure 5B, lane 2) was observed. These data suggest that H1 phosphorylation is associated with the GR-mediated chromatin remodelling and activation of transcription from the MMTV promoter. Further, they strengthen the hypothesis that histone H1 dephosphorylation resulting from prolonged hormone treatment is a key component of the

mechanism by which this promoter becomes refractory to further stimulation.

As shown above, transiently transfected MMTV reporter plasmids failed to become refractory to hormone stimulation upon prolonged exposure to dexamethasone (Figure 1C). Given that H1 would be dephosphorylated under

these conditions, this observation suggests that the phosphorylation status of H1 should not influence transcription from the transient templates. In the next series of experiments, we examined the impact of inhibiting histone H1 phosphorylation with staurosporine on transcription from transiently introduced promoters. We would predict that the transient promoter is active when H1 was dephosphorylated, since inhibiting phosphorylation did not influence transactivation from this transiently introduced reporter (Figure 1C). In agreement with this prediction, the results demonstrated that a transiently introduced reporter was induced by dexamethasone (Figure 5C, compare lanes 1 and 2) within cells where the rephosphorylation of histone H1 was prevented by staurosporine. These experiments establish that while staurosporine is a general kinase inhibitor and may influence other kinases in these cells, it has no significant impact on transcriptional activation, by the GR, from the transiently transfected MMTV promoter.

Histone H1 phosphorylation does not influence hormone activation of the metallothionein gene

As our experiments establish that there is a bulk dephosphorylation of histone H1 in response to the prolonged exposure to hormone, it would be anticipated that all chromatin in the cells would be complexed with dephosphorylated H1. To assess the 'global' consequences of dephosphorylating H1, we have investigated the transcriptional activity from two endogenous chromosomal genes, the glucocorticoid inducible MT gene and the glucocorticoid independent glyceraldehyde-3P dehydro-

Fig. 5. Effects of the kinase inhibitor staurosporine on MMTV activation. (A) Staurosporine inhibits the rephosphorylation of H1. Dexamethasone (10^{-7} M) was added to cells for 24 h and hormone was removed for 23 h (lanes 4 and 7) or 24 h (lanes 3 and 6), either in the absence (lanes 2–4) or the presence (lanes 5–7) of staurosporine (100 ng/ml). After hormone removal, cells were either not given any hormone (lanes 3 and 6) or were treated with dexamethasone (10^{-7} M) for 1 h prior to harvest (lanes 4 and 7). Cells that were treated with dexamethasone (10^{-7} M) for 48 h are shown in lanes 2 and 5, respectively. Purified histone H1 (10 µg) (lane 1) and total histones (35 µg) (lanes 2–7) were separated on a 16% acrylamide acid-urea gel, transferred to a nitrocellulose membrane, and Western blot analysis was performed using a polyclonal antibody against phosphorylated H1. (B) Staurosporine inhibits MMTV transcription from chromatin. Levels of MMTV and 18S rRNA transcripts in 1471.1 cells were analyzed by primer extension from total RNA (20 µg) using primers specific for either the MMTV or 18S rRNA. Dexamethasone (10^{-7} M) was added to cells for 24 h, hormone was removed for 20 h (lanes 2 and 4) or 24 h (lanes 1 and 3) either in the absence (lanes 1 and 2) or the presence (lanes 3 and 4) of 100 ng/ml of staurosporine, and cells were either not given any hormone (lanes 1 and 3) or were treated with dexamethasone (10^{-7} M) for 4 h prior to harvest (lanes 2 and 4). (C) Staurosporine does not inhibit MMTV transcription from the transiently introduced DNA. Cells were pretreated with dexamethasone (10^{-7} M) for 24 h and transfected with 10 µg of plasmid pLTR LUC. After a period of 1 h, hormone was removed and 100 ng/ml of staurosporine was added to tissue culture media. Cells were either not given any hormone (lane 1) or were restimulated with dexamethasone (10^{-7} M) for 4 h (lane 2) prior to RNA isolation. Total RNA (5 µg) was reverse-transcribed to cDNA using an oligo dT primer and Superscript reverse transcriptase (Gibco-BRL). The cDNA was analyzed by *Taq* polymerase amplification with 32 P-labelled primers specific for the *Luciferase* gene. The levels of 18S mRNA were analyzed by primer extension of total RNA (3 µg) as described in Materials and methods. The purified products were analyzed on 7% polyacrylamide denaturing gels and exposed to Dupont reflection film.

Fig. 6. Transcription of the metallothionein and glyceraldehyde-3P dehydrogenase genes in response to glucocorticoids and in the presence of staurosporine. (A) Prolonged hormone treatment does not affect transcription from MT and GAPDH promoters. 1471.1 cells were either not given any hormone (lane 1) or were treated with dexamethasone (10^{-7} M) for 4 h (lane 2), 24 h (lane 3) or 48 h (lane 4) prior to RNA isolation. Total RNA (5 μ g) was reverse transcribed to cDNA using an oligo dT primer and Superscript reverse transcriptase. The cDNA was analyzed by *Taq* polymerase amplification with 32 P-labelled primers specific for the MT or GAPDH. The purified extended products were analyzed on 7% polyacrylamide denaturing gels and exposed to Dupont reflection film. (B) Staurosporine does not affect MT or GAPDH transcription. Dexamethasone (10^{-7} M) was added to cells for 24 h, hormone was removed for 20 h (lanes 2 and 4) or 24 h (lanes 1 and 3), either in the absence (lanes 1 and 2) or the presence (lanes 3 and 4) of 100 ng/ml of staurosporine, and cells were either not given any hormone (lanes 1 and 3) or were treated (lanes 2 and 4) with dexamethasone (10^{-7} M) for 4 h prior to RNA isolation. Total RNA (5 μ g) was reverse transcribed to cDNA using an oligo dT primer and superscript reverse transcriptase. The cDNA was analyzed by *Taq* polymerase amplification with 32 P-labelled primers specific for the MT or GAPDH mRNA. The purified extended products were analyzed on 7% polyacrylamide denaturing gels and exposed to Dupont reflection film.

genase gene (GAPDH) (Karin *et al.*, 1984; Piechaczyk *et al.*, 1984; Koropatnick and Duerksen, 1987). In contrast with what is observed with the MMTV promoter in these cells, the MT mRNA levels were elevated at 24 and 48 h of hormone treatment (Figure 6A, compare lanes 1–4). Thus, the MT promoter did not display the characteristic refractory period observed on the MMTV promoter in the same cells. Similarly, the GAPDH promoter, which is constitutive and unresponsive to glucocorticoids, did not show any significant changes over the same time period

(Figure 6A, compare lanes 1–4). These results suggest that the activation profile of the MMTV promoter does not result from pleiotropic effects of prolonged exposure to glucocorticoid. They also confirm that the consequences of H1 dephosphorylation are intimately tied to the activation and cessation of transcription from this promoter.

To establish that H1 phosphorylation was not required for MT activation by the GR, we examined whether the MT promoter could be induced by glucocorticoid in cells cultured with staurosporine to maintain H1 in a dephosphorylated state. The results demonstrated that the MT promoter, in contrast to the MMTV promoter, is fully activated by glucocorticoid when H1 phosphorylation is blocked by staurosporine (compare Figures 5B and 6B). Thus, while staurosporine will inactivate a number of kinases, its effects are not so broad as to inhibit the entire GR signalling pathway in the cell. Indeed, these experiments suggest a direct and specific role for histone H1 phosphorylation in the regulation of transcription from the MMTV promoter assembled as chromatin.

Phosphorylation status of histone H1 on the MMTV promoter

The preceding experiments examined changes in bulk histone H1 phosphorylation with alterations in MMTV chromatin remodelling and as such they are not informative with respect to the phosphorylation status of H1 on the MMTV promoter. Indeed, they leave open the possibility that a small sub-population of histone H1 remains phosphorylated and associated with the MMTV promoter. To remove this ambiguity we used a UV cross-linking strategy to examine the fate of histone H1 on inducible and refractory MMTV promoters. For these experiments nuclei from control cells or cells maintained in hormone for 24 h were isolated and subjected to DNA protein cross-linking by UV irradiation (Bresnick *et al.*, 1991). Subsequently, nuclei were digested and promoter fragments were immunoprecipitated with an antibody that recognized phosphorylated histone H1. MMTV proximal promoter sequence was detected by PCR amplification using primers specific for MMTV. The results from these experiments demonstrated that, prior to hormone treatment, a significant portion of histone H1 on the MMTV promoter was phosphorylated (Figure 7A, lane 2). However, within 24 h of hormone treatment the majority of H1 associated with the MMTV promoter was dephosphorylated, as indicated by the reduced yield of MMTV promoter DNA in the PCR assay (Figure 7A, lane 3).

Previous studies established that the initial GR activation of MMTV transcription is associated with the loss of H1 protein from the promoter (Bresnick *et al.*, 1991). Thus, it was possible that the loss of phosphorylated H1 seen in the above experiment could result from the loss of H1 protein from the promoter rather than reflecting a change in phosphorylation. To address this possibility we made use of an antibody specific for the unphosphorylated H1 (Lu *et al.*, 1995). A prediction from these experiments is that we would find a reciprocal relationship between the levels of dephosphorylated H1 and the transcriptionally competent promoter with an antibody that was specific for H1 in the dephosphorylated state. The results shown in Figure 7B confirm this prediction. Immunoprecipitation

Fig. 7. Phosphorylation status of histone H1 on the MMTV promoter *in vivo*. **(A)** Loss of phosphorylated histone H1 at the MMTV promoter. 1471.1 cells were either not given any hormone (lanes 1 and 2) or were treated with dexamethasone (10^{-7} M) for 24 h (lane 3). Nuclei were isolated, exposed to UV light, then digested with *Hae*III prior to immunoprecipitation with (lanes 2 and 3) or without (lane 1) an antibody specific for phosphorylated histone H1. MMTV proximal promoter sequence, +80 to -225, was detected by PCR amplification. The purified extended products were analyzed on 7% polyacrylamide denaturing gels and exposed to Dupont reflection film. **(B)** Histone H1 on the MMTV promoter is dephosphorylated after long term hormone treatment. UV cross-linked DNA from naïve cells (lanes 1 and 3) or cells treated with hormone for 24 h (lanes 2 and 4) was immunoprecipitated with an antibody specific for either phosphorylated histone H1 (lanes 1 and 2) or dephosphorylated histone H1 (lanes 3 and 4), and analyzed as above. **(C)** Hormone withdrawal results in the rephosphorylation of histone H1 on the MMTV promoter. Cells were either untreated (lanes 1 and 4), treated with hormone for 48 h (lanes 2 and 5) or treated with hormone for 24 h and then hormone removed for 24 h (lanes 3 and 6). UV cross-linked chromatin was then immunoprecipitated with an antibody to phosphorylated histone H1 (lanes 4–6). Samples of the input DNA (lanes 1–3) and the immunoprecipitated DNA (lanes 4–6) were then analyzed as above. **(D)** Hormone withdrawal results in the rephosphorylation of histone H1 on 5' distal (nuc-F) portion of the MMTV promoter. Cells were either untreated (lane 1), treated with hormone for 48 h (lane 2) or treated with hormone for 24 h and then hormone removed for 24 h (lane 3). UV cross-linked chromatin was then immunoprecipitated with an antibody to phosphorylated histone H1. MMTV distal promoter sequence, -782 to -1044, was detected by PCR amplification. The purified extended products were analyzed on 7% polyacrylamide denaturing gels and exposed to Dupont reflection film.

of MMTV promoter sequences with the antibody to dephosphorylated H1 demonstrated a greater amount of dephosphorylated H1 prior to hormone addition than at 24 h (Figure 7B, lanes 1 and 2). These experiments clearly demonstrate that the consequences of bulk dephosphorylation of histone H1, after 24 h of hormone treatment, is the reformation of 'refractory' MMTV chromatin with dephosphorylated H1.

As seen in our previous experiments, the GR-mediated dephosphorylation of H1 is reversed with hormone removal and the promoter is reactivated (Figure 4). In the next set of experiments we used a UV cross-linking assay to confirm that these changes in H1 phosphorylation occur on the MMTV proximal promoter. Under conditions that lead to the rephosphorylation of H1 there is a re-association of the phosphorylated H1 with MMTV proximal promoter sequences (Figure 7C, lanes 4–6). Thus the ability of the promoter to be reactivated following prolonged hormone removal is reflected in the interaction of phosphorylated

H1 with the promoter. A prediction from these studies is that as prolonged exposure to glucocorticoid leads to a complete dephosphorylation of H1, the entire MMTV promoter, and indeed all chromatin, would be organized with dephosphorylated H1. We confirmed this prediction by analyzing sequences in the 5' portion of the LTR that are >1 kb away from the nuc-B region probed previously (Figure 7A, B and C) (Richard-Foy and Hager, 1987). Consistent with the global dephosphorylation of H1 there is a decrease in phosphorylated H1 associated with this distal region of the promoter with prolonged exposure (Figure 7D, lanes 1 and 2). Further, as seen with the proximal promoter (Figure 7C) removal of hormone results in the return of phosphorylated H1 at the promoter in the 5' portion of the LTR (Figure 7D, lanes 2 and 3). These data establish that prolonged exposure to glucocorticoids leads to a global dephosphorylation of H1 which results in an MMTV promoter organized into a chromatin structure that fails to respond to the activated GR.

Discussion

Glucocorticoids induce a rapid transcriptional activation of the MMTV promoter via the disruption of chromatin, the loss of histone H1 and the binding of NF1 (Hager *et al.*, 1993; Archer *et al.*, 1995). Prolonged hormone treatment results in the disassembly of an active transcription complex from the MMTV promoter, the cessation of transcription and the establishment of a repressive chromatin structure that is refractory to further stimulation by the GR (Archer *et al.*, 1994a; Lee and Archer, 1994). This assembly and disassembly of the transcription complex is specific for the MMTV promoter assembled as a phased array of nucleosomes and is not observed when the promoter is introduced into cells by transient transfection. We have proposed that these observations reveal a dominant role for chromatin structure in the MMTV response to steroid hormones in mammalian cells (Archer *et al.*, 1994b).

In this work, we examined the mechanism(s) by which this 'refractory' state is maintained and the role of the ligand-activated GR in both the activation and inactivation of the MMTV promoter. Our experiments demonstrate a requirement for the continuous presence of hormone to establish the refractory state (Figure 1B). This is not the case for transcription from a transiently transfected MMTV reporter which does not become refractory to induction upon prolonged exposure to glucocorticoid (Figure 1C). However, the refractory state may be reversed by hormone removal for a similar time period such that the promoter became fully responsive to the GR again (Figure 1B). Similarly, the protracted period of hormone withdrawal required to reactivate the chromatin template was dispensable for the transiently transfected MMTV DNA.

We show that histone H1 was highly phosphorylated in naïve cells where the GR, upon hormone stimulation, induced a rapid transcriptional response (Figures 1 and 7). In contrast, histone H1 was dephosphorylated in cells that were treated with hormone for a period of 24 h (Figure 7). Further, the GR and NF1, the major transcription factors in MMTV activation, were present in the nucleus during this period of time but unable to initiate transcription in the presence of hormone (H.-L.L. and T.K.A., unpublished). However, after 20 h of hormone withdrawal, histone H1 was rephosphorylated and the MMTV promoter regained its transcriptional competency (Figures 1 and 4). Further support for this view comes from experiments that used staurosporine to prevent the rephosphorylation of histone H1, in which transcription was not reactivated following hormone removal (Figure 5). Although staurosporine is a general kinase inhibitor, its effects on transcription appear to be specific for the MMTV promoter assembled as chromatin. Indeed, in the presence of staurosporine, the transiently transfected MMTV promoter remains hormone inducible while the stable MMTV promoter is not. Thus, while the inhibitor may affect the phosphorylation state of numerous proteins in the cell, it does not appear to affect the ability of the GR to activate transcription *per se*, but rather to activate transcription from MMTV chromatin templates.

The impact of H1 phosphorylation appeared to be specific for MMTV chromatin as a second glucocorticoid responsive promoter, MT displayed a different response

to prolonged exposure to glucocorticoids (Figure 6A). The MT promoter did not become refractory upon long term hormone treatment, nor was it sensitive to the staurosporine inhibition of the rephosphorylation of H1 (Figure 6B). Consequently the GR was able to activate MT transcription. DNA-protein cross-linking experiments reveal that H1 on the MMTV promoter, adjacent to the HREs, was dephosphorylated when the promoter was inactivated (Figure 7A). The prolonged removal of hormone resulted in the rephosphorylation of H1 bound to the proximal promoter (Figure 7C) and a reciprocal relationship between phosphorylated and dephosphorylated H1 at the promoter (Figure 7B). Finally, additional UV cross-linking experiments demonstrated that H1 at a distal 5' region of the MMTV LTR was dephosphorylated in response to hormone exposure and rephosphorylated when hormone was removed (Figure 7D). These experiments suggest a model for MMTV activation whereby the GR interacts with MMTV chromatin associated with phosphorylated histone H1, resulting in the release and dephosphorylation of histone H1. Subsequently, MMTV chromatin is reformed into a refractory structure with dephosphorylated H1 and is unable to be induced by the activated GR (Figure 8).

The global dephosphorylation of histone H1 suggests that dephosphorylated H1 would be present throughout the entire genome and not be restricted to the MMTV promoter. However, as seen with our functional analysis of the actin, 18S, GAPDH and MT genes, expression does not appear to be modulated by the phosphorylation status of H1 (Figures 1, 5 and 6). The observation that the global dephosphorylation of histone H1 in mammary cells is associated with a specific activation process, namely glucocorticoid activation of the MMTV promoter, is analogous to recent findings in *Xenopus* and *Tetrahymena*. Experiments that either over- or under-express histone H1 protein in *Xenopus* embryos revealed that the differential expression of oocyte and somatic 5S rRNA genes was mediated by the levels of histone H1 during *Xenopus* development (Bouvet *et al.*, 1994; Wolffe, 1994). The recent deletion of both histone H1s in *Tetrahymena* also failed to elicit a global effect on transcription, rather specific and divergent effects on individual genes were recorded (Shen and Gorovsky, 1996). In this case, activated expression of one gene (*ngoA*) in starved cells was independent of H1 but its repression in growing cells required the presence of H1. Conversely, a second gene (*CyP*) displayed a phenotype that required H1 for activated expression in starved cells but not for repression of expression in growing cells (Shen and Gorovsky, 1996). Our data are congruent with this study in that our examination of four genes, MMTV, actin, GAPDH and MT, reveals a specific role of histone H1 phosphorylation for GR-induced activation of the MMTV promoter but not for the GR-mediated induction of the MT promoter.

The case for post-translational modification of histones regulating gene transcription has been strengthened by the recent description of the targeted deacetylation mediated by the nuclear receptors and the Mad-Max heterodimers (Alland *et al.*, 1997; Hassig *et al.*, 1997; Heinzel *et al.*, 1997; Laherty *et al.*, 1997; Nagy *et al.*, 1997). Histone deacetylase activity physically associates with the transcription factor(s) and is delivered to a specific site on

Histone H1 phosphorylation modulates transcriptional activation from the MMTV promoter

Fig. 8. The phosphorylation status of histone H1 regulates MMTV chromatin remodeling by the GR. In naïve cells, the MMTV promoter is associated with phosphorylated H1 and the promoter is transcriptionally competent. The addition of dexamethasone leads to GR-mediated transcriptional activation which results in the displacement of histone H1, the remodeling of nuc-B and the binding of transcription factors including NF1. Prolonged hormone treatment results in the global dephosphorylation of H1, disruption of the bound transcription factors and reassembly of nuc-B with dephosphorylated H1. This results in a promoter that is refractory to hormone stimulation. Hormone withdrawal leads to the rephosphorylation of H1 and restores transcriptional competence to the promoter.

the promoter where it presumably leads to the deacetylation of histone H3 and the silencing of the promoter. Several pieces of evidence do not support a direct targeting mechanism for GR-mediated histone H1 dephosphorylation. In the first case, the loss of phosphorylated H1 is gradual and does not result from short term hormone exposure (Figure 3). Thus it is possible to 'cycle' the promoter between active and inactive states by successive hormone addition and removal for 1 h periods (H.-L.L. and T.K.A., unpublished). During this time period there is no appreciable change in H1 phosphorylation. In the second case, as indicated above, GR activation of the promoter results in the displacement of H1 and its subsequent re-association leads to the inactivation of the promoter. This suggests that the dephosphorylation and rephosphorylation of H1 may not take place on the DNA. Finally, and most importantly, while we demonstrate that H1 adjacent to the HRE on the MMTV promoter is dephosphorylated upon prolonged hormone treatment, the loss of histone H1 phosphorylation is global and would be expected to occur on the entire genome (Figure 3).

As indicated above, our data suggest a model whereby the GR-mediated dephosphorylation of histone H1 is important in regulating the glucocorticoid induction of MMTV transcription. We show that this result is restricted to the MMTV promoter assembled as a phased array of nucleosomes and not seen upon transient transfection of the MMTV DNA (Figure 5C and H.-L.L. and T.K.A., unpublished). Previous studies have suggested that the GR and NF1 may compete for binding to purified MMTV

DNA (Brüggemeier *et al.*, 1990). A possible consequence of the assembly of the proximal promoter as a phased array of nucleosomes would be to allow the GR to enhance NF1 binding *in vivo*. Thus, phosphorylated H1 on the promoter would provide a compatible target for the GR interaction with chromatin. However, when H1 was dephosphorylated the GR would be prevented from activating the promoter. Alternatively, the position of the NF1 binding site at or near the 3' boundary of nuc-B may result in a competition between H1 and NF1 that is sensitive to the phosphorylation of H1 (Archer *et al.*, 1991; Bresnick *et al.*, 1991; Fragoso *et al.*, 1995). In support of this idea is the observation that histone H1 can display specific binding to a consensus NF1 binding site on the mouse $\alpha_2(1)$ collagen promoter (Ristiniemi and Oikarinen, 1989).

A signature of the GR-induced activation of the MMTV promoter is the loss of H1 from the promoter that accompanies the loading of the pre-initiation complex onto a disrupted nuc-B *in vivo* (Archer *et al.*, 1991; Bresnick *et al.*, 1991). Our experiments demonstrate that the phosphorylation status of histone H1 is tightly linked to the transcriptional competency of the MMTV promoter (Figure 8). These findings suggest at least three mechanisms by which H1 phosphorylation status is relevant to activation by the GR. One could envision that when H1 is dephosphorylated: (i) it is unable to interact directly with the GR and hence not be displaced; (ii) it may package chromatin in a condensed conformation that precludes the interaction of the GR with its cognate

binding sites or (iii) it promotes a strong association with the nucleosome which then prevents the H1 displacement and thus the disruption of the nucleosome core. This second mechanism is reminiscent of that proposed by Allis and colleagues, whereby a dephosphorylated H1 would occlude a transcription factor binding site to repress transcription (Roth and Allis, 1992). In this case, hyperphosphorylation of H1 would lead to repulsion of H1 from the negatively charged DNA and thus provide a window for a *trans*-acting factor to find its site which is otherwise occluded by the dephosphorylated H1. The recent demonstration that H1 binds asymmetrically and inside the gyres of DNA at the 5S gene would also be consistent with the differential effects of H1 phosphorylation on gene expression (Hayes, 1996; Pruss *et al.*, 1996). The phosphorylation of histone H1 may lead to a specific stereochemical organization of MMTV chromatin that is critical to the activation process. As transiently transfected MMTV does not adopt the same chromatin architecture as the endogenous MMTV promoter, the effect of H1 phosphorylation would not necessarily be expected to be the same (Archer *et al.*, 1992; Lee and Archer, 1994). In support of this idea neither prolonged exposure of glucocorticoids to dephosphorylate H1 nor exposure to staurosporine to prevent H1 rephosphorylation blocks GR activation of transient templates. Thus, in combination with recent *in vivo* and *in vitro* experiments that describe specific functional and structural roles for histone H1 in transcription, our data suggest an additional and novel mechanism by which chromatin structure may regulate transcription (Hayes, 1996; Pruss *et al.*, 1996; Shen and Gorovsky, 1996).

Materials and methods

Cell culture

Cell line 1471.1 is a mouse C127 transformant which stably maintains ~300 copies of a bovine papillomavirus (BPV) vector with the MMTV LTR linked to the chloramphenicol acetyl transferase (CAT) gene (Archer *et al.*, 1991). Cells were grown as monolayers on 150 mm plates at 37°C with 5% CO₂ in Dulbecco's modified Eagle medium containing 10% fetal bovine serum as previously described (Lee and Archer, 1994).

In vivo analysis of restriction enzyme hypersensitivity

Cells were treated with dexamethasone (10⁻⁷ M) for the times and conditions indicated (see Figure legends) and nuclei were isolated and then digested with restriction endonucleases as previously described (Lee and Archer, 1994). Following purification, samples were digested to completion with *Hae*III *in vitro* as an internal standard relative to the *in vivo* cleavage which verified that equivalent amounts of DNA (10–20 µg) were used for reiterative primer extension analysis using ³²P-labeled primers. The primer, 5'-TCTGGAAAGTGAAGGATAA-GTGACG-3', corresponds to +60 to +84 portion of the MMTV LTR and is specific for the MMTV promoter. The purified extended products were separated on 5 or 7% polyacrylamide denaturing gels before autoradiography at -80°C.

In vivo analysis of transcription factor loading

Cells were transfected with 10 µg of plasmid PM18, a plasmid which contains the MMTV LTR linked to the *ras* oncogene (Ostrowski *et al.*, 1983) and were treated with hormone as described (see Figure legends). Isolated nuclei were digested with *Hae*III (1000 U/ml) and Exonuclease III (625 U/ml) to detect Exonuclease stops corresponding to the 5' boundaries of transcription factors on the MMTV LTR (Lee and Archer, 1994). DNA was purified and the removal of single stranded overhangs was accomplished using Mung bean nuclease. All the samples were digested to completion with *Hae*III prior to analysis by reiterative primer extension using *Taq* polymerase and ³²P-labeled primers that were specific for either the stable (MMTV-CAT) or the transient (MMTV-

RAS) template. For the stable chromatin, the primer used, 5'-TTAGCTT-CCTTAGCTCCTGAAAAT-3', corresponds to the +120 to +145 portion of the stable template that is absent in the transient plasmid. The primer, 5'-GCTTCGGTACCAAAGTGAACC-3', was used to analyze the transiently transfected MMTV template and it corresponds to the +160 to +139 portion of the transient plasmid that is absent in the stable episome and is therefore specific for the transient DNA. Purified extended products were analyzed on 5 and 7% polyacrylamide denaturing gels before autoradiography at -80°C.

Isolation of histones, gel electrophoresis and analysis of phosphorylated H1

Nuclei were isolated from cells grown as described above and acid soluble proteins were prepared by resuspending nuclei in 100 µl of 0.4 N H₂SO₄ at 4°C for 1 h. The suspension was centrifuged for 5 min at 16 000 g and basic proteins were precipitated from the supernatant overnight at -20°C by addition of 1 ml of acetone. Proteins were collected by centrifugation for 10 min at 16 000 g, air-dried and dissolved in 50 µl of 0.9 M acetic acid and 25 µl of 75% sucrose. Samples (40 µg) were separated on a 16% acrylamide acid-urea gel as previously described (Panyim and Chalkley, 1969) and electroblotted to Hybond-ECL nitrocellulose (Amersham) for 24 h at 50 V and 4°C. Membranes were probed with an antibody specific against the phosphorylated histone H1 or an antibody specific against the unphosphorylated histone H1 (Lu *et al.*, 1995) at a dilution of 1:5000 using the enhanced chemiluminescent procedure (Kirkgaard and Perry Laboratories) and visualized with the Dupont Reflection film.

RNA isolation and primer extension

Total RNA was prepared using Trizol reagents (Gibco-BRL) as described by the manufacturer. Nucleotide sequences of the oligonucleotide primers for MMTV, β-actin and 18S were 5'-TCTGGAAAGTGAAGGATAA-GTGACG-3', 5'-ACCAGCGCAGCGATATCGTCATCCAT-3' and 5'-ACCAAAGGAACCATAACTG-3', respectively. Primer extension was carried out with oligonucleotides that were labelled with [³²P]ATP using T4 polynucleotide kinase. Total RNA (20 µg) and labelled oligonucleotide were dissolved in 15 µl hybridization buffer (0.15 M KCl, 10 mM Tris pH 8.3 and 1 mM EDTA). The primer and RNA were heated to 65°C for 90 min and allowed to cool slowly to 42°C. Primer extension was carried out at 42°C for 1 h after the addition of 30 µl of reverse transcriptase buffer (30 mM Tris-Cl pH 8.3, 15 mM MgCl₂, 8.3 mM DTT, 75 µg/ml actinomycin D and 0.22 mM dNTP mix) and 20 U superscript reverse transcriptase (Gibco-BRL). 105 µl RNase reaction mix (100 µg/ml salmon sperm DNA, 20 µg/ml RNase A, 100 mM NaCl, 10 mM Tris-Cl pH 7.5, 1 mM EDTA) was added to each primer extension reaction tube and RNase digestion was carried out at 37°C for 15 min. The reaction was terminated by the addition of 15 µl of 3 M sodium acetate. DNA was purified by extraction with 150 µl phenol/chloroform/isoamyl alcohol and precipitated by the addition of 300 µl of 100% ethanol. The cDNA pellet was washed with 100 µl of 70% ethanol, air dried and resuspended in 5 µl loading buffer. Products were analyzed on a 7% polyacrylamide denaturing gel.

Reverse transcriptase-PCR

Total cellular RNA (5 µg) and 750 ng of oligo dT₁₂₋₁₈ primer (Pharmacia) or Luc 651, an oligonucleotide specific for the *Luciferase* gene (30 pmol), were used for cDNA synthesis. The reaction was carried out in a solution containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 500 µM dNTP and 20 U superscript reverse transcriptase (Gibco-BRL). After 1 h at 37 °C and 10 min at 75°C, PCR was carried out with cDNA derived from 500 ng of RNA, 5 U of *Taq* DNA polymerase in a final volume of 50 µl. The reaction solution contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 100 µM dNTP and 5 pmol of each primer. Primers MMTV 619, 5'-CCTCTTGTGTTTGTGTCT-3' (+18 to +42) and MMTV 22 5'-TCTGGAAAGTGAAGGATAAGTGACG-3' (+60 to +84) were used to PCR amplify cDNA synthesized from Luc 651, 5'-CCTTTT-CITTAATGTTTGGCGTCTTC-3'. Alternatively, cDNA was synthesized using oligo dT₁₂₋₁₈, and primers MMTV 619 and Luc 651 were used for PCR amplification. For the MT gene, primers 5'-CGGATCCCGGAATGGACCCCACTGCT-3' and 5'-CGGATCCCA-GACTCAAACAGGCTTTTAT-3' were used. For GAPDH, 5'-TAT-TGGGCGCCTGGTCACCA-3' and 5'-CCACCTTCTTGATGTCATCA-3' were used for PCR amplification. Transiently introduced MMTV DNA was eliminated prior to RT-PCR amplification by incubating 5 µg RNA with 2 U of DNase I (Gibco-BRL) in a buffer containing 20 mM Tris-HCl pH 8.4, 2 mM MgCl₂ and 50 mM KCl at room temperature

for 15 min. DNase I was inactivated by the addition of 1 µl of 25 mM EDTA solution. Samples were heated at 65°C for 10 min and the RNA samples were used in the cDNA synthesis reaction. Alternatively, after cDNA synthesis, transient MMTV DNA was eliminated by digesting double stranded plasmid with 40 U restriction enzyme *Sau3A*, which cuts between the two primers, in a buffer containing 100 mM NaCl, 10 mM Bis Tris Propane-HCl, 10 mM MgCl₂ and 1 mM DTT at 37°C. After restriction enzyme digestion, cDNA was used in the PCR amplification.

Analysis of histone H1 cross-linked to the MMTV promoter DNA *in vivo*

Cross-linking of histone H1 to the MMTV DNA and subsequent immunoprecipitation analysis were described previously (Bresnick *et al.*, 1991). The protocol was modified to use a PCR approach with forward and reverse primers, 5'-TTAGCTTCCTTAGCTCCTGAAAAT-3' and 5'-TTAAGTAAGTTTTGGTTACAAACT-3', which amplify a 325 bp fragment that encompassed nucleosome B, or primers 5'-CAAACCTGGCAT-AGCCTCTGC-3' and 5'-CCTATTGGATTGGTCTTATTGG-3', which amplify a 262 bp fragment that encompassed nucleosome F within the *HaeIII*-digested MMTV promoter.

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