Dissection of progesterone receptor-mediated chromatin remodeling and transcriptional activation in vivo

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We have investigated whether constitutive binding by the progesterone receptor (PR) to a promoter is required for the maintenance of an open chromatin structure in vivo. For these experiments, we used human T47D breast cancer cells in which the mouse mammary tumor virus (MMTV) promoter, stably assembled as chromatin, is constitutively hypersensitive to endonucleolytic cleavage. In vivo footprinting revealed that transcription factors nuclear factor 1 and the PR were constitutively bound to the MMTV promoter in these cells. Treatment of these cells for 1 hr with the steroid antagonist ZK98299 prevented PR binding to chromatin in vivo and reversed hypersensitivity, leading to the loss of transcription factor binding. The reduction in hypersensitivity induced by ZK98299 was readily reversed by treatment with the progestin R5020. The chromatin organization of the promoter could be cycled between the open and closed states by consecutive treatments with agonist or antagonist. The antagonist RU486 also blocked activation of transcription and the assembly of a transcription preinitiation complex, but in contrast to ZK98299, maintained the hypersensitive chromatin state. Taken together, these results suggest that PR binding to chromatin is sufficient to induce hypersensitivity to endonucleolytic cleavage. Furthermore, they indicate that the PR binding to DNA and the resulting chromatin hypersensitivity is functionally separate from transcriptional activation in vivo.

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In eukaryotic cells, the packaging of DNA and histones as chromatin allows DNA sequences to be compacted efficiently and economically (van Holde 1988). This chromatin organization also provides a mechanism to restrict the access of *trans*-acting proteins and thus controls or modulates the expression of the genes these factors regulate (Hager and Archer 1991; Felsenfeld 1992; Workman and Buchman 1993; Wolffe 1994). In *Saccharomyces cerevisiae*, genetic analyses have revealed that deletion of the genes for one or more of the core histones deregulates a variety of inducible genes (Grunstein 1990). In higher eukaryotes, many lines of evidence suggest that compact regions of DNA are transcriptionally silent with transcription originating from areas of decondensation within chromatin (Groudine and Weintraub 1981; McGhee et al. 1981; Elgin 1988; Gross and Garrard 1988). These regions of active gene expression are characterized by increased accessibility to various nucleases, a property indicative of an open chromatin conformation (Elgin 1990). In certain cases, there is a coincidence of

hypersensitivity to DNase I and binding by transcription factors, which may result from the loss of one or more nucleosomes from the promoter (Wu 1980; Zaret and Yamamoto 1984; Almer and Hörz 1986; Richard-Foy and Hager 1987).

The ability of *trans*-acting factors to modulate chromatin structure has been studied extensively in a number of systems, including the mouse mammary tumor virus (MMTV) promoter (Archer et al. 1989). A detailed analysis of glucocorticoid activation demonstrated that the glucocorticoid receptor (GR) binds to and disrupts one of the proximal nucleosomes (Nuc-B) in a phased array that is found when the promoter is stably incorporated into mouse cells (Richard-Foy and Hager 1987; Archer et al. 1989). This allows the subsequent assembly of a preinitiation complex (PIC) of transcription factors that includes nuclear factor 1 (NF1), octamer transcription factors (OTFs) and TATA-binding protein (TBP) (Lee and Archer 1994).

In contrast, significantly less is known of the mechanisms by which the closely related progesterone receptor (PR) activates transcription from this promoter (Hager and Archer 1991; Archer et al. 1994a). Intriguingly, when

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the PR is transiently introduced into GR + /pr – mouse cells, it is unable to activate transcription from an MMTV promoter stably maintained as chromatin. However, in the same cells it stimulates transcription efficiently from a transiently introduced nonchromatin template (Archer et al. 1994a). In addition, in human T47D cells expressing comparable levels of the PR and GR, transcription from both stably maintained and transiently introduced copies of the MMTV promoter is stimulated efficiently by the GR but not the PR (Nordeen et al. 1989; Archer et al. 1994b). These data suggest that the mechanism by which the PR activates transcription from chromatin templates differs from that of the GR.

More recently, we have made the surprising observation that when stably introduced into PR + /gr – human T47D breast cancer cells (designated T47D/2963.1 cells) the MMTV promoter adopts a novel chromatin structure that is constitutively hypersensitive over the Nuc-B region (Mymryk et al. 1995). In mouse cell lines, this region comprising nucleotides approximately –220 to –75 of the promoter, displays glucocorticoid-inducible hypersensitivity (Zaret and Yamamoto 1984; Archer et al. 1991). Furthermore, in vivo footprinting experiments reveal that in T47D/2963.1 cells the open chromatin structure is coincident with the constitutive binding of several transcription factors, including the PR and NF1 (Mymryk et al. 1995). One interpretation of these results is that the binding of the PR, alone or in conjunction with another factor, is sufficient to lead to a constitutively open chromatin structure. We have taken advantage of this cell line to ask whether the normal closed chromatin structure could be imposed by preventing the PR from binding to its cognate site. Utilizing steroid hormone antagonists that block PR-mediated transcription, we show that inhibition of PR binding results in the elimination of the constitutive hypersensitivity characteristic of the MMTV promoter in these cells. Ensuing the imposition of a closed chromatin state, there is loss of transcription factor binding to the promoter in vivo.

Results

PR binding maintains an open chromatin organization

In human T47D-derived T47D/2963.1 cells the region of the MMTV promoter encompassed by Nuc-B is constitutively hypersensitive to restriction enzyme cleavage (Mymryk et al. 1995). Unlike the normally closed organization of the MMTV promoter in mouse cells, the novel chromatin architecture in T47D/2963.1 cells does not exclude binding by NF1 and, furthermore, exhibits occupancy by the PR even in the absence of ligand (Mymryk et al. 1995). To examine whether constitutive binding by the PR is required to maintain the constitutively open chromatin organization of the MMTV promoter in T47D/2963.1 cells, we used the steroid hormone antagonists RU486 and ZK98299, both potent inhibitors of progesterone action (Neef et al. 1984). ZK98299 antagonizes progesterone action by preventing the progesterone receptor from binding to DNA (Klein-

Hitpass et al. 1991; Nath et al. 1992; Takimoto et al. 1992; Allan et al. 1992; Bocquel et al. 1993). In contrast, the proposed mechanism of action of RU486 differs from that of ZK98299, as it does not prevent the receptor from binding to DNA in vitro (Beck et al. 1993 and references therein). However, either antagonist ZK98299 (10^{-7} M) or RU486 (10^{-7} M) completely masked the approximately eightfold induction in MMTV-regulated expression of chloramphenicol acetyltransferase (CAT) activity obtained by treating T47D/2963.1 cells with the synthetic progesterin R5020 (10^{-9} M) for 24 hr (Fig. 1). To ascertain the mechanistic implications of preventing PR binding to DNA in vivo, we treated T47D/2963.1 cells with ZK98299 and monitored changes in chromatin hypersensitivity. For this study we made use of restriction enzymes that cleave at sites within or immediately adjacent to the region that would normally be occupied by Nuc-B (see Fig. 2A). The addition of the synthetic progesterin R5020 did not substantially elevate the cleavage by *Sst*I or *Mbo*I relative to the untreated control (Fig. 2A, cf. lanes 5 and 6 and 9 and 10). However, when cells were exposed to ZK98299 with or without added R5020 for 1 hr, the cleavage by *Mbo*I and *Sst*I was reduced (Fig. 2A, cf. lanes 7 and 8 and 11 and 12 with lanes 5 and 9, respectively). In some instances, the loss of hypersensitivity extended into the region normally organized as a linker between the B and C nucleosomes in mouse cells, as cleavage by *Hae*III was also slightly reduced upon antagonist treatment (Fig. 2A, cf. lanes 3 and 4 with lane 1; see also Fig. 5B, below). This effect may be related to the close proximity between the upstream boundary of Nuc-B and the *Hae*III cleavage site. In contrast to cells treated with ZK98299, the chromatin in T47D/2963.1 cells treated with the progesterone antagonist RU486 for 1 hr, at a concentration (10^{-7} M) sufficient to block transcription from the promoter (Fig. 1), remained constitutively hypersensitive (Fig. 2B, cf. lanes 5 and 6 with 7 and 8 and lanes 9 and 10 with 11 and 12).

Antagonist-mediated exclusion of transcription factors from the MMTV promoter

The ability of ZK98299 treatment to effectively reverse the constitutive hypersensitivity and impose a closed chromatin state suggests that binding of the PR to the promoter would also be reduced. In vivo exonuclease III footprinting experiments demonstrated that ZK98299 effectively prevented PR binding (Fig. 3). In T47D/2963.1 cells, the PR was bound in the absence of exogenous progesterin R5020 and the subsequent addition of R5020 for 1 hr led to a modest increase in binding (Fig. 3, cf. lanes 1 and 2). In contrast, the addition of ZK98299 alone or the coadministration of ZK98299 (10^{-7} M) with R5020 (10^{-9} M) for 1 hr significantly reduced the level of bound PR present (Fig. 3, cf. lanes 3 and 4 with lane 1). Thus, unliganded and transcriptionally inactive PR, bound to the MMTV promoter, was sufficient to maintain an open chromatin structure, an activity ascribed previously only to agonist-activated GR. When PR binding to chromatin

Figure 1. ZK98299 and RU486 inhibit progestin induction of transcription from a MMTV template stably maintained as chromatin in T47D (T47D/2963.1) cells. T47D/2963.1 cells were left untreated ($\blacktriangle$, control) or treated with the synthetic progestin R5020 (10^{-9} M; star) alone or in combination with the progesterone antagonists ZK98299 (10^{-9} M and 10^{-7} M, respectively; $\blacksquare$) or RU486 (10^{-9} M and 10^{-7} M, respectively; $\bullet$) for 24 hr. Lysates were prepared and analyzed for CAT activity using a kinetic assay (Neumann et al. 1987). The data shown was normalized for total protein and represents the average of two independent experiments. The fold induction represents the average of the inductions for each of the four time points.

was prevented by ZK98299, the constitutive hypersensitivity characteristic of these cells was lost (Fig. 2A).

A signature response of the MMTV promoter to glucocorticoids is the hormone-dependent loading of a transcription preinitiation complex that includes NF1. We have demonstrated that assembly of the promoter into a specific chromatin structure prevents NF1 binding, as NF1 binds to transiently introduced non-nucleosomal templates in the absence of hormone (Archer et al. 1992). As indicated above, treatment with ZK98299 for 1 hr blocked PR binding and led to a loss of hypersensitivity at the promoter by the reformation of a closed architecture within the region encompassed by Nuc-B (Figs. 2A and 3). In this case, it might be expected that this assembly of a repressive chromatin architecture would lead to reduced NF1 binding. In agreement with this prediction, NF1 levels on the promoter decreased significantly in cells treated with ZK98299 for 1 hr (Fig. 3, lanes 1 and 2 vs. 3 and 4). Hence, these experiments provide firm support for a model that proposes (1) it is the organization of a specific chromatin structure that prevents the binding of ubiquitous transcription factors such as NF1, and (2) it is the binding of the steroid receptor that is necessary and sufficient to open the chromatin. Thus, in T47D/2963.1 cells, the continued binding of the PR appears essential for the maintenance of the open chromatin state and this argues that the *trans*-activation potential of the PR is not required for NF1 binding.

To further dissect the role of PR binding, we examined the effect of the antagonist RU486-bound PR on transcription factor binding to the MMTV promoter (Fig. 4). In contrast to ZK98299, RU486 has been reported to stimulate PR binding *in vitro* (Beck et al. 1993 and references therein) but prevents activation *in vivo*. In T47D/2963.1 cells treated with RU486 (10^{-7} M) for 1 hr, *in vivo* exonuclease III footprinting experiments de-

tected a reduced level of PR binding and a more distinct reduction in NF1 binding, as compared with that observed in untreated cells (Fig. 4A, cf. lanes 1 and 3). A direct comparison of the effect of ZK98299 and RU486 on transcription factor loading to the MMTV promoter is shown in Figure 4B. Overall, the effect of RU486 treatment on transcription factor loading was not as pronounced as that observed for ZK98299 treatment (Fig. 4B, cf. lanes 1 and 2). Cotreatment of cells with RU486 (10^{-7} M) and R5020 (10^{-9} M) for 1 hr blocked the enhanced level of PR and NF1 binding observed upon R5020 treatment alone (Fig. 4A, cf. lanes 2 and 4). These results separate the ability of the PR to modify chromatin and to recruit transcription factors. Specifically, although the RU486-occupied receptor is fully capable of maintaining the open chromatin organization of the MMTV promoter in T47D/2963.1 cells (Fig. 2B), it differs from agonist-occupied receptor as it appears unable to enhance the binding of NF1 (Fig. 4).

Chromatin organization can be cycled between the open and closed states

ZK98299 antagonizes progestin action by competing for binding to the PR, suggesting that it might be possible to reversibly cycle the chromatin organization of the MMTV promoter in T47D/2963.1 cells between the open and closed states by manipulating the equilibrium between agonist and antagonist. As depicted in Figure 5A, T47D/2963.1 cells were treated with ZK98299 and R5020 for consecutive 1-hr periods and analyzed for restriction enzyme hypersensitivity (Fig. 5B). As compared with untreated cells, treatment with ZK98299 for 1 hr (Z) reduced cleavage by *Mbo*I and *Sst*I (Fig. 5B, lanes 6,10). In cells exposed initially to ZK98299 and then subsequently exposed to R5020 for 1 hr (ZR), enhanced

Figure 2. Hormone antagonist ZK98299, but not RU486, reduces the constitutively high access of in vivo restriction enzyme cleavage within MMTV chromatin. (A) Schematic of the proximal portion of the MMTV promoter indicating the chromatin structure, sites of restriction enzyme cleavage, and the oligonucleotide used for the reiterative primer extension analysis. T47D/2963.1 cells were left untreated (lanes 1,5,9), treated with R5020 (10^{-9} M; lanes 2,6,10), ZK98299 (10^{-7} M; lanes 3,7,11), or both R5020 and ZK98299 (10^{-9} M and 10^{-7} M, respectively; lanes 4,8,12) for 1 hr. Nuclei were isolated and digested in vivo with restriction endonucleases cutting within the MMTV promoter (*HaeIII*, *MboI*, and *SstI*). Purified DNA was redigested with *HaeIII* to provide an internal standard, as described in Materials and methods and analyzed by reiterative primer extension analysis with 32 P-labeled primer 22 and *Taq* polymerase as described [Archer et al. 1991; Lee and Archer 1994]. (Lanes 1–4) *HaeIII*; (lanes 5–8) *MboI*; (lanes 9–12) *SstI*. (Φ_x) Φ X174 replicative form (RF) DNA cut with *HaeIII*; (*) in vitro *HaeIII* cleavage products, (lanes 5–12). (B) T47D/2963.1 cells were left untreated (lanes 1,5,9), treated with R5020 (10^{-9} M; lanes 2,6,10), RU486 (10^{-7} M; lanes 3,7,11), or both R5020 and RU486 (10^{-9} M and 10^{-7} M, respectively; lanes 4,8,12) for 1 hr prior to analysis as above [Archer et al. 1991; Lee and Archer 1994]. (Lanes 1–4) *HaeIII*; (lanes 5–8) *MboI*; (lanes 9–12) *SstI*. (*) In vitro *HaeIII* cleavage products (lanes 5–12).

cleavage by *MboI* and *SstI* was restored (Fig. 5B, lanes 7,11). Treatment of these cells with a subsequent additional 1-hr cycle of ZK98299 (ZRZ) again reduced cleavage by *MboI* and *SstI* (Fig. 5B, lanes 8,12). Thus, the alterations in chromatin organization induced by PR binding are dynamic and rapidly reversible.

We also examined the effect of manipulating the equilibrium between agonist and antagonist on the binding of transcription factors to the MMTV promoter using exonuclease III footprinting analysis (Fig. 5C). In concert with the changes in chromatin structure, treatment with ZK98299 for 1 hr dramatically reduced the level of bound PR and NF1 as compared with that in untreated cells (Fig. 5C, cf. lanes 1 and 2). Subsequent treatment with R5020 for 1 hr restored PR and NF1 binding (Fig. 5C, lane 3), and a further 1-hr treatment with ZK98299 abolished this binding. These results provide a clear demonstration that chromatin hypersensitivity and transcription factor

binding are intimately linked to the occupancy of the promoter by the PR.

Prolonged exposure to ZK98299 establishes a repressive chromatin architecture

A prediction from the previous experiments would be that prolonged exposure to ZK98299 will lead to the appearance of the normal repressive chromatin structure observed in GR+/pr- mouse cells [Archer et al. 1989]. To test this hypothesis, T47D/2963.1 cells were grown for 2 weeks in ZK98299 (10^{-7} M), at which time the drug was removed and replaced with either fresh media containing ZK98299 (10^{-7} M) or fresh media containing R5020 (10^{-9} M). Cells were analyzed subsequently for restriction enzyme hypersensitivity, and a clear effect of the ZK98299 pretreatment was observed (Fig. 6). Under these conditions, treatment with R5020 for 1 hr led to a

Figure 3. In vivo exonuclease III detection of the effect of ZK98299 on DNA-protein interactions on the MMTV promoter. Schematic of the proximal portion of the MMTV promoter indicating the chromatin structure, 5' boundaries of the sites of protein interaction, and the oligonucleotide used for the PCR analysis. T47D/2963.1 cells were left untreated (lane 1), treated with R5020 (10^{-9} M; lane 2), ZK98299 (10^{-7} M; lane 3), or both R5020 and ZK98299 (10^{-9} M and 10^{-7} M, respectively; lane 4) for 1 hr. Isolated nuclei were digested with *Hae*III and exonuclease III to detect the 5' boundaries of factors bound to the MMTV promoter [Mymryk and Archer 1994]. Purified DNA was treated with mung bean nuclease to remove single-stranded overhangs and analyzed by reiterative primer extension analysis with 32 P-labeled primer 22 and *Taq* polymerase [Mymryk and Archer 1994]. The arrows indicate exonuclease stops corresponding to the PR- and NF1-binding sites. (Φ_x) ΦX174 RF DNA cut with *Hae*III; (C) C sequencing track.

substantial induction of restriction enzyme hypersensitivity relative to that obtained in cells not grown previously in ZK98299 (cf. lanes 3 and 4 and 5 and 6 in Fig. 6 with lanes 5 and 6 and 9 and 10 in Fig. 2A). This reduced hypersensitivity might be expected to lead to a significantly lower basal level of activity from the promoter. CAT assays performed on cells similarly maintained in ZK98299 exhibited a 30- to 40-fold induction upon removal of the antagonist and addition of R5020 (10^{-9} M) (Fig. 7). This is an induction approximately four- to five-

fold larger than observed for cells not pretreated with ZK98299, largely because of a dramatic decrease in expression in the absence of agonist (cf. Figs. 1 and 7). These experiments thus support the concept that inhibition of PR binding by ZK98299 leads to the establishment of a repressive chromatin state and, hence, lower basal activity.

Discussion

PR binding maintains an open chromatin organization

The constitutively open chromatin organization observed for the MMTV promoter in the T47D/2963.1 cell line differs dramatically from that characterized previously in mouse and human cells containing the GR [Archer et al. 1989, 1994b; Mymryk et al. 1995]. In those cell lines, the region encompassed by Nuc-B is assembled into a closed conformation that is reorganized into a more accessible state by activated GR [Archer et al. 1989, 1994b; Mymryk et al. 1995]. In T47D/2963.1 cells, this region of the promoter is organized into an open chromatin architecture that is constitutively accessible to nucleolytic agents and transcription factor binding (Fig. 8; Mymryk et al. 1995). As the positioning of this nucleosome coincides with the binding sites for the PR [von der Ahe et al. 1985; Truss et al. 1994] and PR binding was detected in vivo in the absence of progestin treatment, this suggested to us that PR binding could be required for maintenance of the open chromatin region. We tested this hypothesis by employing two widely used progestin antagonists, RU486 and ZK98299, which differ in their ability to block PR binding to DNA in vitro.

Treatment with the progestin antagonist RU486, which permits PR binding to DNA in vitro [Klein-Hitpass et al. 1991], was sufficient to block transcriptional activation (Fig. 1) but had no effect on the chromatin organization of the promoter (Figs. 2B and 8). In vivo footprinting analysis did not detect enhanced PR binding by RU486 alone (Fig. 4). Importantly, although RU486 treatment maintains the constitutive hypersensitivity of the MMTV promoter (Fig. 2B), it blocked the enhanced binding by the PR and NF1 induced by the agonist R5020 (Fig. 4A). This suggests that while the RU486-bound PR is able to interact with chromatin, as seen by the maintenance of hypersensitivity to restriction enzyme cleavage with the MMTV promoter, the interaction is less stable than the R5020-occupied PR as the interaction appears labile to exonuclease III. In this case, the RU486/PR complex is remarkably like the agonist-bound GR, which induces hypersensitivity but is not detected by exonuclease III [Cordingley et al. 1987; Lee and Archer 1994]. However, unlike the agonist-bound GR or PR, the RU486-bound receptor fails to recruit transcription factors required for the formation of a productive transcription initiation complex (Fig. 8).

In contrast to experiments with RU486, treatment with the antagonist ZK98299, which precludes PR binding to the promoter in vitro [Klein-Hitpass et al. 1991;

Figure 4. The effect of RU486 treatment on DNA-protein interactions on the MMTV promoter. (A) In vivo exonuclease III detection of the effect of RU486 on DNA-protein interactions on the MMTV promoter. T47D/2963.1 cells were left untreated (lane 1), treated with R5020 (10^{-9} M; lane 2), RU486 (10^{-7} M; lane 3), or both R5020 and RU486 (10^{-9} M and 10^{-7} M, respectively; lane 4) for 1 hr. Isolated nuclei were digested with *Hae*III and exonuclease III to detect the 5' boundaries of factors bound to the MMTV promoter (Mymryk and Archer 1994). Purified DNA was treated with mung bean nuclease to remove single-stranded overhangs and analyzed by reiterative primer extension analysis with 32 P-labeled primer 22 and *Taq* polymerase (Mymryk and Archer 1994). The arrows indicate exonuclease stops corresponding to the PR- and NF1-binding sites. (Φ_x) Φ X174 RF DNA cut with *Hae*III; (C) C sequencing track. (B) Comparison of the effect of ZK98299 and RU486 on DNA-protein interactions on the MMTV promoter. T47D/2963.1 cells were left untreated (lane 3), or treated with ZK98299 (10^{-7} M; lane 1) or RU486 (10^{-7} M; lane 2) for 1 hr. Analysis was as described in A. The arrows indicate exonuclease stops corresponding to the PR- and NF1-binding sites. (Φ_x) Φ X174 RF DNA cut with *Hae*III; (G) G sequencing track.

Allan et al. 1992; Nath et al. 1992; Takimoto et al. 1992; Bocquel et al. 1993) and in vivo (Fig. 3; Truss et al. 1994), prevented activation of transcription (Fig. 1) and caused a reorganization of the chromatin into a closed conformation (Figs. 2A and 8). Coincident with the appearance of a closed chromatin structure at the promoter is a loss of transcription factor binding. Thus, by exploiting the differences in mechanism of action of these two antagonists, we have demonstrated a direct requirement for PR binding in the maintenance of an open chromatin region.

Remodeling of chromatin structure by the PR is separable from activation of transcription

Although both RU486 and ZK98299 efficiently block transcription from the MMTV promoter in T47D/2963.1 cells (Fig. 1), only treatment with ZK98299 resulted in a closure of the chromatin in the MMTV promoter (Figs. 2 and 8). These experiments allow us to formally separate the remodeling of chromatin structure by the steroid receptor and its ability to activate transcription. Recipro-

Figure 5. Loss of restriction enzyme hypersensitivity induced by ZK98299 is reversible. (A) Schematic of experimental protocol. Prior to harvesting for analysis, T47D/2963.1 cells were left untreated (C); treated with ZK98299 for 1 hr (Z); treated with ZK98299 for 1 hr, washed and treated with R5020 for 1 hr (ZR); or treated with ZK98299 for 1 hr, washed and treated with R5020 for 1 hr, and washed and treated with ZK98299 for an additional hr (ZRZ). (B) T47D/2963.1 cells were left untreated (lanes 1,5,9) or treated with ZK98299 (10^{-7} M) and R5020 (10^{-9} M) for consecutive 1-hr periods as depicted in A. Nuclei were isolated and digested in vivo with restriction endonucleases cutting within the MMTV promoter (*Hae*III, *Mbo*I, and *Sst*I). Purified DNA was redigested with *Hae*III to provide an internal standard, as described in the Materials and methods, and analyzed by reiterative primer extension analysis with 32 P-labeled primer 22 and *Taq* polymerase as described (Archer et al. 1991; Lee and Archer 1994). (Lanes 1–4) *Hae*III; (lanes 5–8) *Mbo*I; (lanes 9–12) *Sst*I. (Φ_x) Φ X174 RF DNA cut with *Hae*III. (C) control; (Z) ZK98299; (ZR) ZK98299 followed by R5020; (ZRZ) ZK98299 followed by R5020 followed by ZK98299. (All treatments were for 1 hr.) (*) In vitro *Hae*III cleavage products. (C) T47D/2963.1 cells were left untreated, or treated with ZK98299 (10^{-7} M) and R5020 (10^{-9} M) for consecutive 1-hr periods as depicted in A. Isolated nuclei were digested with *Hae*III and exonuclease III to detect the 5' boundaries of factors bound to the MMTV promoter (Mymryk and Archer 1994). Purified DNA was treated with mung bean nuclease to remove single-stranded overhangs and analyzed by reiterative primer extension analysis with 32 P-labeled primer 22 and *Taq* polymerase (Mymryk and Archer 1994). The arrows indicate exonuclease stops corresponding to the PR- and NF1-binding sites. (Φ_x) Φ X174 RF DNA cut with *Hae*III. (C) Control; (Z) ZK98299; (ZR) ZK98299 followed by R5020; (ZRZ) ZK98299 followed by R5020 followed by ZK98299. (All treatments were for 1 hr.)

cal experiments with the GR mutant 556 (Godowski et al. 1987), which can activate transcription from a transient template but not from a stable chromatin template (Archer et al. 1994a), further support the concept that activation of transcription and remodeling of chromatin are separable functions within the steroid receptors.

Analogous results obtained from studies on nucleo-

some disruption in yeast by GAL4 also demonstrate a requirement for DNA binding but not activation of transcription (Morse 1993). This suggests that a variety of distinctly different *trans*-acting factors may share in common the ability to disrupt repressive nucleosomal structures to facilitate formation of transcription complexes. This ability is not generally associated with tran-

Figure 6. Prolonged exposure to ZK98299 restores hormone-inducible *in vivo* restriction enzyme cleavage within MMTV chromatin. T47D/2963.1 cells were maintained in ZK98299 (10^{-7} M) for 2 weeks. Medium was removed and replaced with fresh medium containing ZK98299 (10^{-7} M; lanes 1,3,5) or R5020 (10^{-9} M; lanes 2,4,6) for 60 min. Cells were harvested and analysis was performed as described in the legend to Fig. 2. (Lanes 1,2) *Hae*III; (lanes 3,4) *Mbo*I; (lanes 5,6) *Sst*I. (*) *In vitro* *Hae*III cleavage products (lanes 3–6).

scription factors that bind DNA specifically, as many such factors are unable to access their respective binding sites assembled as chromatin. As seen for the MMTV promoter in mouse cells, neither NF1 nor the OTFs bind

to the promoter as chromatin in the absence of hormone, yet both readily bind to a transiently introduced non-chromatin template in the absence of hormone (Lee and Archer 1994). Thus, activation of transcription from a chromatin locus likely incorporates two steps. The first step would be the derepression of the locus by disruption of an interfering nucleosomal structure. This step would necessarily require binding by a key transcription factor, such as the hormone receptors, that can recognize their binding sites in the context of chromatin (Archer et al. 1991) and, through an as yet undefined process, disrupt the nucleosome. The second step of this bimodal process would be the formation of a transcription initiation complex containing ubiquitously available transcription factors that were previously prevented from binding to the template by the presence of a nucleosome (Archer et al. 1992).

Differences in mechanism of activation between-steroid receptors

Our observations suggest essential differences between the modes of action of the PR and that reported previously for the GR (Archer et al. 1989; Lee and Archer 1994). Although these two receptors share a high degree of homology, particularly within their DNA-binding domains (Evans 1988), and recognize identical hormone response elements with nearly identical affinity (von der Ahe et al. 1985), we detect binding by the PR but not the GR to the MMTV promoter *in vivo* (Mymryk et al. 1995). The lack of detectable binding by the GR could suggest that the interaction of the GR with the promoter is transient (Archer et al. 1994a; Lee and Archer 1994) while binding by the PR is not. [Our results with RU486 would suggest that in this case the interaction of the RU486/PR complex with the promoter is also transient, as it is detected less efficiently by exonuclease III footprinting (Fig. 4)]. The results presented here show that constitu-

Figure 7. Prolonged exposure to ZK98299 reduces basal expression from the MMTV promoter. T47D/2963.1 cells were maintained in ZK98299 (10^{-7} M) for 2 weeks. Medium was removed and replaced with fresh medium containing ZK98299 (10^{-7} M; ■), or R5020 (10^{-9} M; ★) for 24 hr. Lysates were prepared and analyzed for CAT activity using a kinetic assay (Neumann et al. 1987). The data shown were normalized for total protein and represent the average of two independent experiments. The fold induction represents the average of inductions at each of the four time points.

Figure 8. Agonist- and antagonist-mediated regulation of MMTV transcription by the PR. In the absence of R5020, the Nuc-B region of the MMTV promoter is constitutively hypersensitive to cleavage by restriction enzymes (REs) and exhibits binding by the PR and NF1 transcription factors. The addition of the antagonist RU486 does not alter the chromatin architecture of the promoter but blocks transcription. Binding by the PR and NF1 appears reduced. Treatment with the antagonist ZK98299 results in a closing of Nuc-B, which excludes transcription factor binding and blocks transcription. The addition of the agonist R5020 reverses the effect of ZK98299, re-establishing an open chromatin organization for Nuc-B. This allows transcription factor access and the establishment of a productive transcription initiation complex.

tive binding by the PR maintains the MMTV promoter in an altered, more accessible chromatin structure. One obvious consequence of this novel organization is the failure of the nucleosome to block binding by ubiquitous *trans*-acting factors including NF1 (Figs. 3 and 8; Mymryk et al. 1995), leading to an enhanced level of basal transcription. The prolonged growth of T47D/2963.1 cells in ZK98299 re-established a closed chromatin structure (Figs. 6 and 8) by blocking PR binding and drastically reduced the level of uninduced transcription (Fig. 7). Thus, transcription from the MMTV promoter by progestins is regulated less tightly than that by glucocorticoids.

An additional consequence of the altered chromatin organization of the MMTV promoter induced by PR binding could be a change in the kinetics of transcriptional induction by steroid hormones. Induction of transcription from chromatin copies of the MMTV promoter by glucocorticoids is transient, returning to basal levels after 24 hr of hormone treatment [Archer et al. 1994a; Lee and Archer 1994]. The cessation of transcription correlates with a reformation of a repressive chromatin organization, suggesting that the disassembly of the transcription complex is mediated by a change in chromatin structure [Lee and Archer 1994]. In contrast, no changes in chromatin organization and transcription factor loading are detected in T47D/2963.1 cells over the same period of time, suggesting that transcription remains elevated over a correspondingly longer time frame [Mymryk et al. 1995]. In support of this proposal, we detect significantly elevated levels of MMTV mRNA at 24 hr of hormone treatment relative to that seen at 4 hr of treatment (J. Mymryk and T. Archer, unpubl.).

Mechanism of PR-mediated reorganization of chromatin structure

As mentioned above, the changes in chromatin architecture that we observe in T47D/2963.1 cells require PR binding but are independent of hormone-induced transcription. The reorganization of the region encompassed

by Nuc-B into an open conformation by the PR is rapid and reversible (Fig. 8). Inhibition of PR binding by treatment with ZK98299 resulted in a reorganization of the promoter into a repressive architecture within 1 hr (Figs. 2 and 3), and the open organization could be re-established by treatment with R5020 for an additional hour (Fig. 5B). The ability of the chromatin organization to cycle back and forth between the open and closed states implies that the mechanism of chromatin reorganization is an extremely dynamic process. This short time frame also precludes a requirement for DNA replication in the alteration of chromatin organization.

The above experiments demonstrate that PR binding is required to maintain an open chromatin state. However, they do not distinguish whether the receptor accomplishes the remodeling alone or in concert with NF1 and the other factors that bind within the region encompassed by Nuc-B (see Fig. 3). Alternatively, this process may also involve mammalian homologs of the SWI/SNF complexes required for steroid receptor activation of transcription in yeast [Hirschhorn et al. 1992; Laurent and Carlson 1992; Yoshinaga et al. 1992; Côté et al. 1994] and mammalian cells [Muchardt and Yaniv 1993; Chiba et al. 1994]. In either case, hormone activation of MMTV transcription will provide an avenue for addressing questions concerning the intimate relationship between chromatin remodeling and transcriptional activation *in vivo*.

Materials and methods

Cell culture

Cell line T47D/2963.1 was derived from human T47D breast cancer cells by stable transfection of the chimeric bovine papilloma-based construct pJ83d, carrying the MMTV promoter attached to the bacterial CAT gene [Mymryk et al. 1995]. Cells were grown in Dulbecco's modified Eagle medium containing 10% fetal bovine serum.

CAT assays

Sixty-millimeter plates of subconfluent T47D/2963.1 cells

were treated with R5020 (10^{-9} M) with or without ZK98299 (10^{-7} M) or RU486 (10^{-7} M) for 24 hr. Cells were harvested, lysed in 200 μ l of 0.25 M Tris-HCl (pH 7.8) by three rounds of freezing and thawing. CAT activities were determined by a kinetic assay (Neumann et al. 1987) and were normalized to total protein. Fold inductions were calculated by averaging those for each of the four time periods.

In vivo analysis of restriction enzyme hypersensitivity

Cells were treated with R5020 (10^{-9} M), ZK98299 (10^{-7} M), or RU486 (10^{-7} M) for 1 hr. Nuclei were isolated and digested with restriction endonucleases as described previously (Archer et al. 1991). All samples, with the exception of those cleaved in vivo with *Hae*III, were digested to completion with *Hae*III in vitro to provide an internal standard for accessing the extent of in vivo cleavage and confirm that equivalent amounts of DNA (10–20 μ g) were used for reiterative primer extension analysis using 32 P-labeled primer 22 (specific for the MMTV promoter) and *Taq* polymerase (Archer et al. 1992). (Extended products generated by in vitro digestion with *Hae*III are indicated with an asterisk.) Purified extended products were analyzed on 5% or 7% polyacrylamide denaturing gels.

In vivo analysis of transcription factor loading

Cells were treated with R5020 (10^{-9} M), ZK98299 (10^{-7} M), or both for 1 hr. Isolated nuclei were digested with *Hae*III (1000 U/ml) and exonuclease III (625 U/ml) as described previously (Mymryk and Archer 1994) to detect specific stops corresponding to the 5' boundaries of bound factors. DNA was purified, and single-stranded overhangs were removed with mung bean nuclease and digested to completion with *Hae*III prior to analysis by reiterative primer extension using 32 P-labeled primer 22 and *Taq* polymerase (Mymryk and Archer 1994). Purified extended products were analyzed on 7% polyacrylamide denaturing gels.

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