Loss of Androgen Receptor Transcriptional Activity at the G₁/S Transition*

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Androgens are essential for the differentiation, growth, and maintenance of male-specific organs. The effects of androgens in cells are mediated by the androgen receptor (AR), a member of the nuclear receptor superfamily of transcription factors. Recently, transient transfection studies have shown that overexpression of cell cycle regulatory proteins affects the transcriptional activity of the AR. In this report, we characterize the transcriptional activity of endogenous AR through the cell cycle. We demonstrate that in G₀, AR enhances transcription from an integrated steroid-responsive mouse mammary tumor virus promoter and also from an integrated androgen-specific probasin promoter. This activity is strongly reduced or abolished at the G₁/S boundary. In S phase, the receptor regains activity, indicating that there is a transient regulatory event that inactivates the AR at the G₁/S transition. This regulation is specific for the AR, since the related glucocorticoid receptor is transcriptionally active at the G₁/S boundary. Not all of the effects of androgens are blocked, however, since androgens retain the ability to increase AR protein levels. The transcriptional inactivity of the AR at the G₁/S junction coincides with a decrease in AR protein level, although activity can be partly rescued without an increase in receptor. Inhibition of histone deacetylases brings about this partial restoration of AR activity at the G₁/S boundary, demonstrating the involvement of acetylation pathways in the cell cycle regulation of AR transcriptional activity. Finally, a model is proposed that explains the inactivity of the AR at the G₁/S transition by integrating receptor levels, the action of cell cycle regulators, and the contribution of histone acetyltransferase-containing coactivators.

Androgens play a key role in the differentiation of male-specific tissues during mammalian development. In the adult, there is a continued requirement for androgens for the maintenance of some of these tissues (1). Androgen withdrawal leads, for instance, to increased apoptosis and regression of the prostate gland (2). This androgen dependence is retained in prostate cancer, where androgens are necessary for the onset and early development of the disease (3). In newly diagnosed cases of prostate cancer, androgen ablation is the primary therapy used (4, 5), yet with time, androgen-independent tumors arise in individuals who undergo this therapy (6, 7). This has led to an intense investigation of the molecular mechanisms involved in androgen signaling.

The actions of androgens are mediated by the androgen receptor (AR), a transcription factor that belongs to the nuclear hormone receptor superfamily. In the absence of androgens, the AR protein is primarily cytosolic and is found complexed with heat shock proteins that keep it inactive (8). Upon binding to androgens, the receptor undergoes a conformational change that releases it from this inhibitory complex (9). AR then localizes to the nucleus, where it binds as a dimer to androgen response elements found on the promoters of target genes (10). The ability of the AR to modulate gene transcription is enhanced by the recruitment of coactivators and possibly by the release of corepressors (11, 12). Coactivators can provide enhanced interactions with the basal transcriptional machinery through activation domains of their own. They also contribute intrinsic or associated histone acetyltransferase activities, thus allowing for chromatin remodeling (13). We have previously shown that activation of AR brings about such nucleosome rearrangements on the mouse mammary tumor virus (MMTV) promoter and that this remodeling correlates with transcriptional activity (14). We have also reported that the hyperacetylation of histones enhances the ability of the AR to remodel chromatin and modulate transcription (15) and that anti-androgens inhibit chromatin remodeling, consequently blocking AR transcriptional activity (16). Thus, the functions of AR require the activity of histone acetylases. The AR itself seems to also be the target of acetylation, and its transcriptional activity may be enhanced in vivo by this modification (17).

The rate of mammalian cell growth is largely determined by the length of the G₁ phase of the cell cycle. Progression from G₀ phase through the G₁/S transition and into S phase is governed by the action of cyclins and cyclin-dependent kinases (CDKs) on the retinoblastoma protein (Rb) (18, 19). Cyclin D1-CDK4 complexes in middle to late G₁, and then cyclin E-CDK2 complexes in G₁/S and early S phase phosphorylate Rb, diminishing its ability to bind and repress the S-phase-promoting factor E2F (20–25). It is known that androgens influence growth, shortening the length of G₁/S and accelerating entry into S phase, by affecting the expression and/or activity of "G₁/S transition," "G₁/S boundary," and "G₁/S" are used interchangeably.

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‡ Throughout this paper, the terms "G₁/S transition," "G₁/S boundary," and "G₁/S" are used interchangeably.

‡ The abbreviations used are: AR, androgen receptor; MMTV, mouse mammary tumor virus; CDK, cyclin-dependent kinase; Rb, retinoblastoma protein; GR, glucocorticoid receptor; CAT, chloramphenicol acetyltransferase; FACS, fluorescence activated cell sorting; DHT, dihydrotestosterone; DEX, dexamethasone; NH, no hormone; TSA, trichostatin A; p/CAF, p300/CBP-associated factor.
cyclins and CDKs (3, 26). Recently, it has been demonstrated that some cell cycle regulatory proteins can, in turn, influence AR transcriptional activity by acting as AR coregulators. These include the retinoblastoma protein, and cyclins D1 and E, molecules that show altered expression in many human cancers.

Our laboratory and others have reported that expression of the retinoblastoma protein restores AR function in Rh-deficient cells (27, 28). Additionally, Knudsen et al. (29) and Reutens et al. (30) have shown that overexpression of cyclin D1 (and to a lesser extent cyclin D3) inhibits AR function in a CDK-independent manner. Furthermore, Yamamoto et al. (31) determined that cyclin E overexpression, independently of its association with CDK2, results in the positive regulation of AR activity. Generally, these experiments used transient transfection techniques to introduce into cells expression vectors of both the AR and the cell cycle regulator under study and measured transcriptional effects on transient templates (27–31). This approach results in overexpression of the cell cycle regulators throughout the cell cycle rather than the phase-specific expression found in normal cells. We have taken a more physiological approach by investigation of the regulation of the transcriptional activity of endogenous AR on integrated promoters during the cell cycle.

In this report, we show that the transcriptional activity of endogenous AR varies through the cell cycle. We demonstrate that the AR is transcriptionally active in G0, loses over 90% of its activity during the G1/S transition, and then regains the ability to enhance transcription in S phase. We show that this transient negative regulation at the G1/S transition is specific for the AR, since the related glucocorticoid receptor (GR) maintains transcriptional activity at this boundary. The down-regulation of AR protein that we observe at G1/S may partially explain the lack of transcriptional activity. However, chemical inhibition of histone deacetylases rescues AR activity during G1/S without increasing the level of AR protein, suggesting that regulation of AR activity during the cell cycle also involves acetylation/deacetylation pathways.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Development of Stable Cell Lines—**L929 cells (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 3% calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. For the development of the L929-MMTVCAT stable cell line, L929 cells were transfected using DKLiposome reagent (Invitrogen) according to the manufacturer’s protocol. To obtain the L929-ProbasinLuc cell line, L929 cells were transfected using LipofectAMINE 2000 reagent (Invitrogen) with pM-TMTVCAT and pSV2neo (20:1 ratio), according to the manufacturer’s protocol. In both cases, the cells were split 48 h after transfection and selected in growth media supplemented with 400 μg/liter G418 (Cellogro). Single clones were picked with sterile pipette tips and expanded. Clones were screened for chloramphenicol acetyltransferase (CAT) or luciferase activity after a 24-h hormone induction. Single clones showing low basal reporter activity and at least 5-fold activation with DHT were used for further studies (clones L929-MMTVCAT #31 and L929-ProbasinLuc 2.9 were used in this study).

**Cell Cycle Arrestrs and Fluorescence-activated Cell Sorting Analysis—**L929-MMTVCAT and L929-ProbasinLuc cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 3% calf serum. All cell cycle arrests were carried out on ~80% confluent cells (300,000 cells/well of a 24-well plate incubated overnight or the equivalent density on larger surface areas) according to the methods shown in Fig. 2A. After plating and overnight growth, cells were serum-starved (grown in 0.1% calf serum) for 48 h to induce entry into G0. (For control experiments, G0 cells were cultured in 1% or 2% hydroxyurea for the second 24 h of starvation and during hormone induction as illustrated in Fig. 3D). During an additional 24 h of serum deprivation, cells were treated with steroids or left untreated. For G1/S arrests, cells were starved for 48 h as above. After this starvation, cells were exposed to 1 or 2 μM hydroxyurea in 10% serum for 24 h and for an additional 24 h in the presence or absence of hormone. Cells growing in serum were arrested along S phase by treatment with 1 or 2 μM hydroxyurea for 48 h. Cells were then induced with steroids for 24 h in the presence of hydroxyurea. In all cases, cells were washed with PBS after hormone treatment and harvested in 0.25 M Tris-HCl, pH 7.8 (when only CAT or luciferase assays were performed) or trypanblue and collected (when additional transfection was utilized above). DNA was isolated for FACS or Western analysis as was described (21, 22).

**Western Analysis—**Cells were collected, spun, and washed in cold PBS. Cell pellets were dissolved in modified radiolabeled precipitation buffer (20 μM Tris-HCl pH 7.8, 140 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 0.5% Nonidet P-40) supplemented with 0.66 mg/ml Pefabloc (In Vitrogen), 3.3 μg/ml leupeptin, and 1 μg/ml diithiothreitol. Typically, 30 μg total protein was loaded in each lane of a 4–20% gradient SDS-polyacrylamide gel and separated by electrophoresis. Protein was transferred to nitrocellulose membranes and confirmed by Ponceau Red staining. After blocking for at least 2 h in 5% milk, 0.2% polyvinyl pyrrolidone, membranes were blotted with the corresponding first antibody. For AR detection, 4 μg/ml PA1-111A, a rabbit polyclonal antibody that recognizes the N terminus of the AR (Affinity Bioreagents) was used. A 1:200 dilution of sc-1616, a goat polyclonal antibody, was used to probe for actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoreactive bands were visualized using anti-rabbit or anti-goat horseradish peroxidase-conjugated secondary antibodies and ECL reagents (Amersham Biosciences). Immunoreactive bands were quantified using the software package ImageQuant.

**RESULTS**

**Construction of a Cell Line with an Integrated Reporter That Responds to Both Androgens and Glucocorticoids—**Several reports over the past few years show the involvement of G0, G1/S, and S phase cell cycle regulators in the control of androgen receptor activity. Generally, these experiments used transient transfection techniques to introduce into cells expression vectors of both the AR and the cell cycle regulator under study and measured transcriptional effects on transient templates (27–31). Although some studies provide important information on the interaction of cell cycle regulators and the AR, they do not distinguish between effects seen due purely to overexpression and those that reflect interactions that occur during normal cell growth. Our approach was to investigate the regulation of the transcriptional activity of endogenous AR on integrated promoters during the cell cycle. To do this, we developed a cell line with an integrated AR-responsive and CAT reporter gene. L929 cells that express endogenous AR were stably transfected with the androgen- and glucocorticoid-responsive reporter pMMTV-CAT. The resulting clones were expanded and characterized. A cell line was established from a representative clone and is referred to here as L929-MMTVCAT. The presence of functional AR in these cells is shown in Fig. 1A (left panel), where
treatment with androgens (1 nM dihydrotestosterone (DHT)) resulted in over 30-fold induction of CAT activity. This transcriptional activity was fully blocked by the anti-androgen cyproterone acetate, demonstrating the involvement of the AR in this response. Since L929 cells are known to also express endogenous GR, we measured transcriptional activity in response to glucocorticoids. CAT activity was induced over 20-fold in the presence of dexamethasone (DEX). This induction of MMTVCAT was due to the action of the GR, since the antiglucocorticoid ZK 98.299 (ZK) blocked the response (Fig. 1A, right panel).

The Androgen Receptor Loses Transcriptional Activity at the G1/S Boundary—To optimize the androgen response for cell cycle studies, a time course of AR activation was performed in serum-starved L929-MMTVCAT cells. Androgens clearly induced measurable CAT activity after 24 h (Fig. 1B). This time point was used in cell cycle experiments, since we observed that cells lose synchrony during prolonged arrest (data not shown). To measure the transcriptional activity of the AR in G0, G1/S, or S phase, cells were arrested prior to receptor activation, and cell cycle blocks were maintained during hormone treatment as described under “Experimental Procedures” and outlined in Fig. 2A. To ensure effective cell cycle arrest throughout the length of the experiments, we performed FACS analysis on arrested cells both prior to (data not shown) and after hormone induction as well as on uninduced controls (Fig. 2, B–D, right panels). Importantly, we observed that 24-h androgen treatment had no discernible effect on cell cycle distribution (compare NH histograms with DHT histograms, in Fig. 2, for example). This was expected, since the growth of L929 cells is affected negatively by glucocorticoids and positively by androgens only under chronic long-term exposure (36).

As seen in Fig. 1A, we found that unsynchronized cells growing in the presence of 3% serum routinely showed 20–30-fold induction of CAT activity in response to 1 nM DHT. AR consistently had the highest activity in serum-starved G0 cells, inducing CAT activity up to 100-fold in the presence of DHT (Fig. 2B, left panel). In contrast, the AR showed no detectable activity after treatment with 1 nM DHT, in cells arrested at the G1/S boundary (Fig. 2C). AR regained transcriptional activity when the cells were released from G1/S arrest (not shown) or were blocked along S phase by direct treatment with hydroxyurea without prior serum starvation (Fig. 2D). These data indicate that there is a transient regulatory event that prevents AR transcriptional activity at the G1/S boundary. The anti-androgen cyproterone acetate inhibited DHT-induced activity in G0 cells and did not show any agonistic activity in cells synchronized at the G1/S boundary (data not shown). As seen in Table I, in three independent experiments, the transcriptional activity of the AR at the G1/S boundary was decreased 92–100% compared with its activity in G0. This shows that at the G1/S transition AR function is strongly and consistently inhibited.

To ensure that the inactivity of the AR in cells arrested at the G1/S transition was not the result of nontarget actions of the arresting drug, we tested the effects of hydroxyurea on AR activity during G0. L929-MMTVCAT cells were prearrested in G0 by serum starvation for 24 h. During the next 24 h, the cells were exposed to 2 mM hydroxyurea with continued serum starvation. In the final 24 h of treatment, cells were induced with androgens during continued exposure to hydroxyurea and serum starvation (Fig. 3B). AR transcriptional activity in G0 cells was unaffected by the presence of hydroxyurea, giving androgen inductions within the range usually obtained with cells in G0. This shows that at the G1/S transition AR function is strongly and consistently inhibited.

The Glucocorticoid Receptor Is Transcriptionally Active in G1/S Cells—To test whether there was a general shut down of transcription or translation at the G1/S boundary or whether this regulation was specific to the androgen pathway, we arrested cells at the G1/S boundary and then treated them with either glucocorticoids or androgens. Treatment of cells synchronized at the G1/S boundary with 100 nM DEX or 1 nM DHT for...
24 h did not alter their distribution along the cell cycle (Fig. 3C). GR was transcriptionally active in cells arrested at the G1/S transition, inducing CAT activity over 20-fold, yet no AR activity was detected in androgen-treated cells in the same experiment (Fig. 3A, right panel). These data show that there is a preferential negative regulation of the AR over the GR at the G1/S transition. They also demonstrate that there is not an inherent deficiency in the transcription or the translation of the

| Experiment | G0 activity | G1/S activity | Decrease |
|------------|-------------|---------------|----------|
| 1          | 45          | Undetectable | ~100     |
| 2          | 75          | 1.6           | 98       |
| 3          | 34          | 2.7           | 92       |
CAT message or protein, respectively, in G1/S boundary-arrested cells, since glucocorticoid treatment results in CAT activity.

G1/S Regulation of Transcription from an Androgen-specific Promoter—The MMTV long terminal repeat is a promiscuous promoter that not only responds to androgens and glucocorticoids but also to mineralocorticoids and progesterins (37, 38). The results presented above demonstrate that the strong inhibition of transcriptional activity seen on the MMTV promoter at the G1/S boundary is specific for the AR. To evaluate whether a similar temporal regulation of AR is observed on promoters that respond only to the AR, we obtained a luciferase reporter construct, driven by the androgen-responsive region of the natural probasin promoter (32). An L929 cell line with integrated copies of this construct was developed as outlined under “Experimental Procedures.” We then tested the specificity of this promoter in our cells (referred to here as L929-ProbasinLuc cells). Treatment with 1 nM DHT for 24 h resulted in a 3–5-fold induction of luciferase activity in asynchronous cultures. Cyproterone acetate inhibited this transcriptional activity, confirming the involvement of the AR (Fig. 4A). Both synthetic (dexamethasone) and natural glucocorticoids (cortisol) completely lacked the ability to induce transcription at this promoter (Fig. 4A).

We then evaluated the regulation of transcription from this androgen-specific promoter during the cell cycle. We found that luciferase activity was induced 3–5-fold in response to 1 nM DHT in G0 cultures (Fig. 4, B and C, leftmost panels). However, there was almost no induction in cells arrested at the G1/S transition (Fig. 4, B and C, middle panels). As seen in the case of the MMTV long terminal repeat, AR retained transcriptional activity on the probasin promoter in cells arrested along S phase (Fig. 4, B and C, rightmost panels). Thus, in cells blocked at the G1/S boundary, the AR is transcriptionally inactive not only on promiscuous promoters but also on promoters which are specifically androgen-responsive.

AR Protein Levels Decrease at G1/S but Retain Their Ability to Be Up-regulated by Androgens—To determine whether there was a correlation between AR transcriptional activity and the levels of AR protein during the cell cycle, cells were arrested in G0, at the G1/S boundary, and along S phase as in Fig. 3 and treated with androgens or left untreated. Cells were harvested, one aliquot was used to determine CAT activity, and another aliquot was used for Western analysis. As can be seen in Fig. 5A, AR levels are regulated across the cell cycle, with the lowest levels occurring at G1/S (Fig. 5A, top middle panels), when AR transcriptional activity is at its lowest (Fig. 5A, bottom panel). Hormone treatment results in increased levels of AR in all stages of the cell cycle examined, including G1/S. The reproducibility of the hormone induction of AR levels in G1/S cells is shown in Fig. 5B. Stabilization of the AR protein in the presence of androgens has been shown to occur in L929 and other cells previously (14, 39), but this is the first demonstration that it occurs in G0, in G1/S, and in S phase and that the stabilization itself does not correlate with the transcriptional activity of the AR. Despite the increase in AR protein seen with androgens, DHT-treated G1/S cells still only contain 20–25% of the receptor levels present in DHT-treated G0 cells (Fig. 5A and B, middle panels).

It has been shown previously that the transcriptional activity of steroid receptors closely correlates with the number of bound receptors based upon the Michaelis-Menten equation adapted for ligand-receptor interaction (40, 41). Therefore, to determine whether the decrease in total receptor levels fully accounts for the loss of transcriptional activity of the AR seen in G1/S-arrested cells, cells in G0 were treated with decreasing concentrations of DHT, and transcriptional activity was determined (Fig. 5C). Substantial transcriptional activity was found in response to 1 nM DHT, where 45% of receptors are occupied \( K_d = 1.4 \text{ nM} \) (27), where 50% of receptors are theoretically occupied by DHT), and to DHT levels 10-fold below this, where only 10% of receptors are occupied. Indeed, measurable transcriptional activity was detected at DHT levels 100-fold below the \( K_d \), where only 1% of receptors are predicted to be occupied...
by hormone. These data indicate that although decreased receptor levels at G1/S may play a significant role, they do not fully account for the almost complete loss of transcriptional activity of the AR at this stage of the cell cycle.

Histone Hyperacetylation Rescues AR Activity in Cells Arrested at the G1/S Boundary without Increasing AR Protein Levels—It is known that steroid receptor action is mediated by the recruitment of histone acetyltransferase-containing coactivators that bring about chromatin remodeling by acetylating histones (42–46). We have previously shown that chromatin remodeling is a necessary step in AR transcriptional activity (14) and that the hyperacetylation of histones facilitates this process, whereas anti-androgens prevent it (15, 16). For these reasons, we decided to test the hypothesis that AR complexes are unable to induce chromatin remodeling during the G1/S transition and that this inability partly accounts for the lack of AR transcriptional activity. If this is true, inhibition of histone deacetylases should restore partial AR activity. To evaluate this, we chemically blocked L929-MMTV-CAT cells at the G1/S boundary and then treated them simultaneously with androgens and with trichostatin A (TSA), an inhibitor of histone deacetylases (47), or with either one alone. Cells induced with androgens showed no more than 2–3-fold induction of CAT activity over background levels (Fig. 6A, right panel). This represents a greater than 90% inhibition of AR activity compared with the corresponding DHT-treated G0 samples shown in Fig. 6A (middle panel). In contrast, AR activity was induced more than 20-fold in cells co-treated with androgens and TSA, whereas TSA alone had no effect (Fig. 6A, right panel). This enhanced transcriptional activity of the AR was not the result of cells progressing through G1/S and entering S phase, since cells remained arrested at the G1/S boundary during treatments, as shown by FACS analysis (Fig. 6C). Furthermore, when the effects of TSA on DHT induction were measured in other stages of the cell cycle and compared, it was clear that TSA preferentially enhanced DHT action at the G1/S transition (Fig. 6B). In the presence of TSA, androgen induction levels increased almost 10-fold in G1/S cells, compared with 3–5-fold increases in asynchronous cells and in G0 cells (Fig. 6A, left and middle panels). In all cases, this enhanced activity was fully blocked by CA, demonstrating that it was mediated through the AR (Fig. 6A, all panels). In contrast, the effects of TSA on DEX induction of the GR remained constant throughout the cell cycle, showing no preferential enhancement in G1/S (Fig. 6D).

Since G1/S cells have decreased levels of AR protein, one possible explanation for the rescue of transcriptional activity in these cells by TSA would be an induction of AR levels by TSA. This was not the case, however, since treatment of G1/S cells with TSA partly restored AR transcriptional activity in response to DHT without altering receptor levels, as shown in Fig. 6E. These data show that the reduced levels of AR found at G1/S are capable of activating transcription in TSA-treated cells. Indeed, when only 1% of the receptors present in G0 are occupied with hormone, AR activity is maintained (Fig. 5C), further demonstrating that a low number of activated receptors can be transcriptionally functional in G1. This suggests that a transient regulatory event involving acetylation/deacetylation pathways prevents AR from activating transcription during the G1/S transition. In addition, these data indicate that the reduced levels of AR protein seen at G1/S are the result of a regulatory event at the level of AR expression and/or stability.

**Fig. 4.** G1/S regulation of transcription from an androgen specific promoter. A, asynchronously growing L929-ProbasinLuc cells containing integrated copies of an androgen-responsive probasin reporter construct were induced for 24 h with 1 nM DHT, 1 nM DHT plus 1 μM CA, 100 nM DEX, or 100 nM cortisol (CORT). Luciferase activity, measured in duplicate samples, is expressed in luminescent light units (RLU). B, L929-ProbasinLuc cells were arrested in G0 (as in Fig. 3B) or in G1/S or along S phase (as in Fig. 2A). AR transcriptional activity was measured by luciferase assays as described under “Experimental Procedures.” C, FACS analysis of cells used in B. The insets show DNA histograms for uninduced cells (NI). The percentage of cells arrested at the indicated stages of the cell cycle is shown in parenthesis. The results shown here are representative of between two and three independent experiments. The RLU values vary from experiment to experiment, but the induction levels are consistent across experiments.
DISCUSSION

This is the first report to measure the transcriptional activity of endogenous AR during the cell cycle. We have demonstrated that the AR is fully active in G0-arrested mouse L929 cells and inactive in cells blocked at the G1/S boundary and that it regains transcriptional activity in cells arrested along S phase. We have shown that this transient negative regulation at the G1/S transition preferentially affects the AR, since the related GR is active in these cells. Androgens were able to up-regulate receptor protein during G1/S boundary arrest, demonstrating that at least one androgenic function remains intact. AR protein levels were found to be regulated through the cell cycle, with the lowest levels present at G1/S. This down-regulation of AR protein may partly explain the lack of AR activity in these cells. However, the partial recovery of AR activity in cells at the G1/S transition treated with TSA, without a concomitant increase in AR levels, indicates that this low level of AR can be active in the context of hyperacetylated histones and that decreased AR levels are not the only reason for AR inactivity in G1/S. Thus, the inactivity of the AR at G1/S seems to be the result of two regulatory events: down-regulation of receptor levels and transient inactivation of the receptor's transcriptional activity. The second but not the first effect can be rescued by inhibiting deacetylases with TSA, providing evidence for the involvement of acetylation/deacetylation pathways in the cell cycle regulation of AR transcriptional activity. An earlier study reported that exogenously expressed AR was transcriptionally active on a transient template in cells treated with hydroxyurea and simultaneously induced with androgens (31). Although these authors termed this a G1/S arrest, it most closely resembles what we term an S phase arrest, since they did not perform a prior G0 synchronization. For this reason, the data from the two studies do not disagree.

The AR is not the only transcription factor outside the family of cell cycle control proteins whose regulation is cell cycle-dependent. The closely related GR, for example, has been shown to be transcriptionally inactive in G1/M in many cell types (48–50). During this part of the cycle, it has been reported that the pattern of GR phosphorylation is altered and that these...
changes may prevent GR from being properly retained in the nucleus (50). Phosphorylation also regulates the activity of other transcription factors through the cell cycle. MEF, a member of the ETS family, for example, is controlled by cyclin A-dependent phosphorylation that restricts its activity to G1 (51). The DNA binding ability of the Cut homeodomain transcription factor is mainly seen during S phase. In this case, cell cycle regulation is the result of increased transcription of the cut gene and of dephosphorylation of the Cut protein by the Cdc25A phosphatase during S phase (52). We cannot rule out an involvement of phosphorylation in the cell cycle control of AR; however, the CDK-independent effects of cyclin D1 and cyclin E on receptor activity suggest that mechanisms other than phosphorylation are at play (29, 31). Indeed, the partial reversal of G1/S inhibition of AR by TSA suggests an involvement of histone acetylation in the response. Regardless of the mechanism of regulation, it is of particular interest that there is a class of transcription factors that affects the dynamics of the cell cycle by controlling the expression of proliferative/differentiation genes and that these transcription factors are regulated by the molecules whose activities define the phases of the cell cycle.

**Fig. 6.** **Histone hyperacetylation rescues AR activity in G1/S-blocked cells.** The effect of trichostatin A on the activity of the AR during the cell cycle was determined by analyzing duplicate samples for CAT activity. Cells were arrested as in Fig. 2. The results are representative of at least two independent experiments. A, AR transcriptional activity in asynchronous, G0-blocked, or G1/S-blocked L929-MMTVCAT cultures after 24-h induction with 1 nM DHT in the presence or absence of 5 ng/ml TSA and/or 1 μM CA. The same amount of protein was used in each assay. B, the results in A are redrawn to show the increase in DHT-induced AR activity in the presence of TSA during the cell cycle (DHT activity is set to 1 in each case). C, FACS analysis of G1/S-arrested cells treated as in A. D, GR transcriptional activity in asynchronous, G0-blocked, or G1/S-blocked L929-MMTVCAT cultures after 24-h induction with 100 nM DEX in the presence or absence of 5 ng/ml TSA. The same amount of protein was used in each assay. E, Western blot of cells arrested at G1/S and treated as in A. Western analysis (left panel) was performed as in Fig 5. Bands were quantified (right panel) using the software package ImageQuant. AR levels are expressed relative to actin control bands.
Down-regulation of AR protein levels during the G1/S transition may be one mechanism by which cells modulate the transcriptional activity of the receptor, since androgen sensitivity in various tissues and cell lines has been correlated with receptor protein levels. The factors that control androgen receptor expression are poorly understood, however, and seem to be highly tissue- and cell type-specific. It has been shown that NF-\(\kappa\)B and NF1 negatively regulate AR gene expression (53, 54), whereas c-Myc and Sp1 increase AR expression (55, 56). Androgens themselves regulate AR expression at several levels, and this regulation has been only partly characterized. Androgens have been observed to have a variety of effects in vivo and in tissue culture according to cell type. These effects include down-regulating steady state levels of AR mRNA (57, 58), stabilizing the AR message (59, 60), and increasing or decreasing the rate of transcription (60, 61). In general, AR protein levels are increased by androgens regardless of the effect on mRNA levels. This increase in AR protein is brought about either by stabilization of the protein as measured by longer receptor half-life (62–64) or indirectly by increased translation as a result of altered mRNA levels or potentially by a combination of both effects (65–67). The present study shows that this basic function of androgens is maintained throughout the cell cycle but is not in itself sufficient to elicit a measurable AR transcriptional response.

The finding that the AR is inactive at G1/S on both the MMTV and the probasin promoters implies that this regulation is a general feature of AR action. The MMTV long terminal repeat is a promiscuous promoter responsive to androgens, glucocorticoids, mineralocorticoids, and progestins (37, 38). In contrast, the probasin promoter is AR-specific. Specificity for AR on the probasin promoter has been associated with the arrangement of one of its two androgen response elements as a direct repeat rather than as the inverted repeats found on MMTV (32, 38, 68). It has been suggested that the exact manner of AR dimer formation, AR N- and C-terminal interactions, and recruitment of coactivator complexes may be different on AR-specific direct repeats compared with general steroid-responsive inverted repeats (69). Regardless of the differences that may indeed exist, the mechanism(s) responsible for AR inactivity at the G1/S transition are at work in both cases. The preferential inactivation of the AR over the GR on MMTV at the G1/S boundary further suggests that this temporal regulation may contribute to transcriptional specificity, reducing or abolishing the androgen responsiveness of some genes while maintaining their glucocorticoid responsiveness.

The observation that histone hyperacetylation restores AR activity at the G1/S junction (Fig. 6) suggests that histone modifications may repress transcription in a manner that can be overcome by GR-recruited protein complexes but not by AR complexes during this transition. This possibility is particularly appealing, since the dynamics of chromatin remodeling at the MMTV promoter have been shown to differ in response to glucocorticoids and androgens (14). The GR rapidly and tran-
siently remodels MMTV chromatin during transcriptional activation (70), whereas the AR gradually induces chromatin remodeling over time (14), suggesting that distinct complexes mediate these two remodeling events. Any coregulator required by AR but not by GR may be modified at the G1/S transition, altering its activity. This could affect the AR directly by post-translational modifications and/or indirectly through chromatin remodeling defects or other inhibitory events. These inhibitory activities may be prevented in G0 and reversed or compensated for in S phase by cell cycle-specific components of AR coactivator complexes.

A number of cell cycle-specific proteins have the potential for regulating AR activity during the cell cycle. These include Rb, cyclin D, and cyclin E. Hypophosphorylated Rb has been shown to be an essential AR coactivator in some cell lines but is not required by GR (27, 71). Low levels of hypophosphorylated Rb are consistent with the inactivity of the AR and the activity of the GR at the G1/S transition but do not explain the presence of AR activity in S phase, since hypophosphorylated Rb levels remain low throughout this stage (20, 23). The loss of hypophosphorylated Rb in late G1 and G2/S is due to the increased activity of cyclin D1-CDK4 complexes at these points of the cell cycle. Interestingly, cyclin D1 strongly inhibits the AR (29, 30). Thus, at the G1/S transition, these two separate but interrelated events may conspire to decrease AR activity (Fig. 7). In S phase, there is decreasing cyclin D-CDK4 activity and increased cyclin E-CDK2 activity (24). Since cyclin E activates AR at this checkpoint, the functional meaning of this is particularly interesting, since cyclin D1 inhibition of AR activity at the G1/S transition can be overcome with the use of TSA. Two acetylation events have been proposed to increase AR activity. One is the presence of wild type AR. It will be interesting to evaluate if AR mutants found, for example, in benign hyperplasias or tumors of the prostate bypass cell cycle regulation, being active during the G1/S transition and/or inactive in G0 or S phase. (For a summary of AR mutants, see Refs. 81 and 82). If such mutants are identified, it would be important to also evaluate their ability to interact with cell cycle-specific AR coregulators. Additionally, it is possible that nonsteroidal activators of the AR (83–86) may bypass G1/S control or further restrict the action of AR during the cell cycle. Whether G1/S regulation of AR activity is necessary for proper control of growth or differentiation in androgen-sensitive tissues awaits further investigation. The development of methods to simultaneously measure AR transcriptional activity and cell cycle position in single cells would greatly facilitate such studies.

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REFERENCES
1. Coffey, D. S., and Pienta, K. J. (1987) Prog. Clin. Biol. Res. 239, 1–73
2. Kajiyama, P., Visakorpi, T., Rantala, I., and Isola, J. (1997) J. Pathol. 183, 55–66
3. Culig, Z., Hobisch, A., Bartsch, G., and Klocker, H. (2000) Urol. Res. 28, 211–219
4. Trachtengarten, J. (1987) in Adrenocorticon of the prostate (Bruce, A. W., and Trachtengarten, J., eds) pp. 173–184, Springer-Verlag New York Inc., New York
5. Leemans, S., and Sonntrapa, S. (1999) J. Med. Assoc. Thai. 82, 192–205
6. Scott, W. W., Menon, M., and Welch, P. C. (1980) Cancer 45, 1929–1936
7. Akakura, K., Bruchovchov, N., Goldenberg, S. L., Rennie, P. S., Buckley, A. R., and Sullivan, L. D. (1992) Cancer Res. 52, 2792–2796
8. Pratt, W. B., and Toft, D. O. (1997) Endocr. Rev. 18, 306–360
9. Ohara-Nemoto, Y., Nemoto, T., Sato, N., and Ota, M. (1988) J. Steroid Biochem. 31, 285–304
10. Jenster, G., Spencer, T. E., Burcin, M. M., Tsai, S. Y., Tsai, M. J., and O’Malley, B. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7879–7884
11. Chen, J. D., and Li, H. (1998) Crit. Rev. Eukaryot. Gene Expr. 8, 169–190
12. Collingwood, T. N., Urnov, F. D., and Wolfe, A. P. (1999) J. Mol. Endocrinol. 23, 255–275
13. Rosenfeld, M. G., and Glass, C. K. (2001) J. Biol. Chem. 276, 36855–36868
14. List, H. J., Lozano, C., Lu, J., Danielsen, M., Wellstein, A., and Riegel, A. T. (1999) Cell 99, 414–422
15. List, H. J., Smith, C. L., Rodriguez, O., Daniesi, M., and Riegel, A. T. (1999) Exp. Cell Res. 252, 471–478
16. List, H. J., Smith, C. L., Martinez, E., Harris, V. K., Daniesi, M., and Riegel, A. T. (2000) Exp. Cell Res. 260, 160–165
17. Fu, M., Wang, C., Reutens, A. T., Wang, J., Angeletti, R. H., Sizun-Noel-Baez, L., Ozyrdo, K., Avantaggiati, M. L., and Pestell, R. G. (2000) J. Biol. Chem. 275, 20853–20860
18. DeCaprio, J. A., Ludlow, J. W., Lynch, D., Furukawa, Y., Griffin, J., Penuca-

Wong, H., Huang, C. M., and Livingston, D. M. (1989) Cell 58, 1085–1095
19. Goodrich, D. W., Wang, N. P., Qian, Y. W., Lee, E. Y., and Lee, W. H. (1991) Cell 67, 293–302
20. Buchovich, K., Duffy, L. A., and Harlow, E. (1989) Cell 58, 1097–1105
21. Chappell, S. P., Hiebert, S. M., Medry, M., Horowitz, J. M., and Nevin, J. R. (1991) Cell 65, 1053–1061
22. Hiebert, S. W., Chappell, S. P., Horowitz, J. M., and Nevin, J. R. (1992) Genes Dev. 6, 177–185
23. DeCaprio, J. A., Furukawa, Y., Ajchenbaum, F., Griffin, J. D., and Livingston, D. M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1789–1798
24. Ellsworth, S. V., and Reed, S. I. (2000) Curr. Opin. Cell Biol. 12, 676–684
25. Weinberg, R. A. (1995) Cell 81, 323–330
26. Gregory, C. W., Johnson, B. R., Jr., Presnell, S. C., Mohler, J. L., and French, F. S. (2001) J. Androl. 22, 537–548
27. Lu, J., and Danielson, M. R. (2001) Biochem. J. Chem. 373, 3152–3153
28. Yeh, S., Miyamoto, H., Nishimura, K., Kang, H., Ludlow, J., Hsiao, P., Wang, C., Su, C., and Chang, C. (1998) Biochem. Biophys. Res. Commun. 249, 361–367
29. Knudson, K. E., Carenee, W. K., and Arden, K. C. (1999) Cancer Res. 59, 2297–2301
30. Reutens, A. T., Fu, M., Wang, C., Albanese, C., McPhaul, M. J., Sun, Z., Balk, S. P., Janne, O. A., Palvimo, J. J., and Pestell, R. G. (2001) Mol. Endocrinol. 15, 797–811
31. Yamamoto, A., Hashimoto, Y., Kohri, K., Ogata, E., Kato, S., Ikeda, K., and Nakaniishi, M. (2000) J. Cell Biol. 150, 873–885
32. Rennie, P. S., Bruchovchov, N., Leco, K. J., Sheppard, P. C., McQueen, S. A., Cheng, H., Sneek, R., Hamel, A., Bock, M. E., MacDonald, B. S., Nickel, B. E., Chang, C., Liao, S., Cattini, P. A., and Matusik, R. (1993) Mol. Endocrinol. 7, 23–36
33. Vindelov, L. L. (1977) Virchows Arch. B Cell Pathol. 24, 237–242
34. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
35. Zhang, S. a. D., M. (2001) in Steroid Receptor Methods (Lieberman, B. A., ed) Vol. 1, pp. 297–316, Humana Press, Totowa, NJ
36. Jung-Testas, I., and Baulieu, E. E. (1985) Exp. Clin. Endocrinol. 86, 151–164
37. Ham, J., Thompson, A., Needham, M., Webb, P., and Parker, M. (1988) Nucleic
