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AUTHORITY
USAMRMC ltr, 18 Apr 2003
Award Number:  DAMD17-99-1-9199

TITLE:  Cell Cycle Regulation of Estrogen and Androgen Receptor

PRINCIPAL INVESTIGATOR:  Elisabeth D. Martinez

CONTRACTING ORGANIZATION:  Georgetown University Medical Center
Washington, DC  20057

REPORT DATE:  July 2002

TYPE OF REPORT:  Annual Summary

PREPARED FOR:  U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland  21702-5012

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THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

Carole B. Christoph

1-17-03
It has been found that the androgen receptor is transcriptionally active in G0, and S-phases of the cell cycle but inactive at G1/S. This activity parallels protein levels although low protein alone is not responsible for loss of activity at G1/S since histone hyperacetylation rescues AR activity at this junction without increasing AR protein levels. It has been further shown that the histone deacetylase inhibitor TSA increases the transcriptional activity of the AR and that methoxyacetic acid synergizes with androgens.
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Appendices

Manuscript 1: Loss of Androgen Receptor Transcriptional Activity at the G1/S transition

Manuscript 2: Short-term effects of Methoxyacetic Acid on Androgen Receptor and Androgen-Binding Protein Expression in Adult Rat Testis
INTRODUCTION

The purpose of our study is to increase our understanding of estrogen and androgen receptor action in tumors of the breast with a view to aid in the development of new hormonal and non-hormonal therapies for the treatment of anti-estrogen resistant tumors. Specifically, we seek to identify the phases in the cell cycle during which steroid-activated estrogen and androgen receptors are normally transcriptionally active and to determine whether this cell cycle regulation of receptor activity is maintained when cancer-inducing non-steroidal agents activate the receptors. Our hypothesis is that the activities of steroid-induced ER and AR are controlled by cell cycle regulators and that cancer-inducing, non-steroidal activators bypass or alter this regulation of receptor activity giving rise to aberrant ER and AR function. Similarly, we predict that disruption of certain cell cycle regulators results in altered control of steroid receptor activity.

BODY

All the work to date has focused on L929 cells and cell lines derived from these as reported in prior reports and in the present report. In addition, as mentioned in the second report, all new data corresponds to studies of the androgen receptor, since estrogen receptor cell lines were not effectively arrested in the different stages of the cell cycle to warrant further investigation.

Aim 1:

Task A: completed
Task B: completed
Task C: completed
Task D: Dose response curves in G0 and G1/S phases of the cell cycle were performed. Although EC50 values were not calculated, the data obtained gave the necessary information: the AR is transcriptionally inactive in G1/S even in the presence of high androgen concentrations. In contrast, the AR is transcriptionally active in G0, even at low androgen concentrations. This data is shown in Figure 5 B and C of Manuscript 1.

Aim 2: With respect to Aim 2, work began corresponding to task A in the original Statement of Work. As reported earlier, non-steroidal activators tested with our cell lines (such as cadmium, forskolin, EGF, KGF and IGF) only induced ER or AR activity two or three fold or not at all. The same was true on the glucocorticoid receptor negative cell lines developed and reported on the second report. Even these low activities of the non-steroidal agents were not consistently detected. As the receptors had been shown by us to have highest activity while serum starved, the non-steroidal activators were tested...
for their ability to induce the transcriptional activity of the receptors in G0 cells. Again, either no activity or very low activity was detected while hormone induction was strong in all cell lines tested. Other investigators have seen transcriptional activation using these nonsteroidal agents, yet other cell lines were used and, importantly, transiently expressed receptors and transiently expressed promoter constructs were studied. Our system shows that these agents do not activate the endogenous receptor on a stably integrated promoter-reporter construct. It is thus possible that chromatin structure may inhibit the action of these non-steroidal compounds. The lack of activity of cadmium, forskolin, EGF, KGF, IGF, etc. makes the accomplishment of tasks A, B, C, D and E impossible as the receptors are not activate enough to make measurements possible.

To complement this lack of information, the effect of several other compounds on the activity of the androgen receptor was studied. These compounds synergize with androgens to over-activate the AR. Indeed, it was found that the deacetylase inhibitor trichostatin A, not only enhances the activity of the AR in all stages of the cell cycle when AR is active, but actually also rescues AR activity at the G1/S transition. This is particularly interesting as it shows that changes in acetylation of histones -as occurs in cancers- can potentially allow for the activation of the AR during phases of the cell during which AR should be off. Additionally, we have shown that methoxyacetic acid can synergize with androgens in activating the AR both in asynchronous and in G0 cells. The data mentioned here is shown in Figure 6 of Manuscript 1 and Figures 8-10 of Manuscript 2.

**Aim 3:**

Task A: nothing new to report
Task B: In collaboration with other members of the laboratory, a new cell line was developed which overexpresses the cell cycle regulatory transcription factor E2F. This cell line is derived from L929 mouse fibroblasts and is thus particularly well matched for our studies. Inspite of the observation that transiently expressed E2F can inhibit the transcriptional activity of the AR on a transient template, we were not able to see any decrease in AR activity in a cell line expressing more than 20 times the normal amount of E2F. Additionally, efforts to develop a cell line with decreased levels of the general factor p300 or its related factor CBP were unsuccessful. Given the results obtained in Aims 1 and 2, and the knowledge gathered from the literature, however, a model was developed of possible proteins that may act in the regulation of the AR during the cell cycle. This model is shown in Figure 7 of Manuscript 1.

Task C: not undertaken
Task D: not undertaken
All the above experimentation was done mainly from June 1, 2001-September 1, 2001. The main task during September 2001-May 2002 was to prepare results for publication, and write up, present and defend my doctoral thesis. My Ph.D. was granted and I graduated in May, 2002.
KEY RESEARCH ACCOMPLISHMENTS

- Several new cell lines have been developed from L929 cells in collaboration with other investigators. These cells overexpress different levels of the E2F transcription factor and have been characterized for their level of androgen receptor activity and the receptor's transcriptional response to agonists.
- It has been established that the level of androgen receptor protein varies through the cell cycle in L929 cells, with lowest levels at G1/S.
- It has been established that androgens stabilize the AR protein in G0, G1/S and S phase cells and that this increase in protein does not correlate with increased transcriptional activity.
- It has been established that the androgen receptor regains activity in G1/S cells when histones are hyperacetylated such as occurs with trichostatin A treatment.
- It has been established that the androgen receptor retains transcriptional activity in G0 cells even in the presence of low hormone concentrations, yet is inactive in G1/S cells even in the presence of high concentrations of androgens.
- It has been established that methoxyacetic acid synergizes with androgens in activating the transcriptional activity of the androgen receptor.
- It has been shown that AR levels in cells arrested at the G1/S boundary are clearly increased by androgens although final receptor levels are lower than in G0 cells.
- A model for the regulation of androgen receptor levels and activity has been developed and put forth.
- A manuscript describing a good portion of the above results has been accepted for publication and is in press. A second manuscript containing a small part of the above results has been accepted and is under revision.
REPORTABLE OUTCOMES

Abstracts and publications (Jan 2000 to date)

**Martinez, E. and Danielsen, M. (2002)** Loss of androgen receptor activity at the G1/S transition. JBC in press.


**Martinez, E. and Danielsen, M. (2001)** Androgen receptor transcriptional activity is regulated through the cell cycle in mouse fibroblasts. EMBO workshop on Nuclear Receptors: structure and function, Sicily, Italy.


**Development of cell lines**
Several cell lines have been developed and characterized since the last report:
**E8.2-E2F#9**, and #3: E8.2 cells stably overexpressing hi levels of the E2F transcription factor (collaboration)
**E8.2-E2F#20**, and #11: E8.2 cells stably overexpressing low levels of the E2F transcription factor (collaboration)

**Funding applied for based on work supported by this award**
Travel grant award to cover partial costs for attending a scientific conference on Nuclear Receptors to present work done on cell cycle regulation of androgen receptor. Award of $1000.00 received to attend Keystone Symposia.
Presentations:
- Seminar series 6/02: Functional interactions between the androgen receptor and cell cycle regulators. Laboratory of Receptor Biology and Gene Expression, NCI, NIH. Bethesda, MD.
- Poster presentation 4/02: Loss of androgen receptor activity at the G1/S transition. Keystone Symposia on Nuclear Receptors, Utah.
- Oral presentation 2/02: Functional interactions between the androgen receptor and cell cycle regulators. Data presentation series. Department of Biochemistry and Molecular Biology, Georgetown University School of Medicine, Washington, DC.
- Oral presentation 9/01: Loss of androgen receptor activity at the G1/S transition. Seminar series, Laboratory of Steroid Biology, NIDDK, NIH.
Loss of Androgen Receptor Transcriptional Activity at the G₁/S Transition*

Received for publication, December 19, 2001, and in revised form, May 17, 2002 Published, JBC Papers in Press, June 7, 2002, DOI 10.1074/jbc.M112134200

Elisabeth D. Martinez and Mark Danielsent†
From the Department of Biochemistry and Molecular Biology, Georgetown University School of Medicine, Washington, D. C. 20007

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 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 binds 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 G₀ 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 G₀, 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 G₀/G₁ and accelerating entry into S phase, by affecting the expression and/or activity of androgen receptor and the ARAP. Androgen receptor-associated protein interacts with androgen receptors and is thought to modulate AR activity and expression (26). We have shown that ARAP associates with AR in vivo and that this association is increased by androgen treatment (27). ARAP interacts with the androgen receptor through a C-terminal region that is required for its activity (28). The ARAP-AR complex is recruited to androgen responsive elements through the recruitment of ARAP-binding proteins (29). Androgen receptor-associated protein may also regulate AR activity at the transcriptional level. For example, ARAP knockdown decreases AR transcriptional activity, and ARAP overexpression increases AR transcriptional activity (30). It has been proposed that ARAP regulates AR transcriptional activity by competitively inhibiting the interaction between AR and its coactivator, SRC-1 (31). Thus, ARAP may play a role in the regulation of AR transcriptional activity and may be a target for small molecule inhibitors of ARAP activity.

* This work was supported by Department of Defense predoctoral fellowship DAMD17-98-1-9159 (to E. M.) and by American Heart Association (Mid-Atlantic) Grant 9951256U (to M. D.). The costs of publication of this article were defrayed in part by the payment of page charge(s). This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Georgetown University School of Medicine, Washington, D. C. 20007. Tel.: 202-687-4169; Fax: 202-687-7186; E-mail: dar@bc.georgetown.edu.

This paper is available on line at http://www.jbc.org 29719

1 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; DEK, deoxymethasone; NH₄, no hormone; TSA, trichostatin A; p300/CBP, p300/CREB-associated factor. Throughout this paper, the term "G₁/S transition," "G₁/S boundary," "G₁/S junction," and "G₁/S" are used interchangeably.
cycloheximide (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 RB-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 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 DEAE dextran (Sigma, St. Louis) according to the manufacturer’s protocol. To obtain the L929-ProbasinLuc cell line, L929 cells were transfected using LipofectAMINE (Invitrogen) with p-286/+28PB-luciferase (32) and pSV2neo (20:1 ratio), according to the manufacturer’s protocol. A 1:200 dilution of sc-1616, a goat polyclonal antibody, was used. A 1:500 dilution of horseradish peroxidase-conjugated secondary antibodies and ECL (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.

**Assays—**Cell extracts were assayed for luciferase activity by adding 10 μl of 2 mg/ml chloramphenicol, and 80 μl of 0.25 mM Tris-HCl, pH 7.8. One ml of organic scintillation mixture Econoflour-2 (Packard Instrument Co.) was overlaid on the reaction mix, and aliquots were placed in a scintillation counter. As the reaction proceeded, the acetylated product is incorporated into the organic phase and is counted (35). Samples were counted for three consecutive cycles in a β counter, and the results were expressed as the increase of the counts produced/min (cpm/min). For luciferase assays, equal amounts of protein from each extract were combined with 10 μl of luciferase assay substrate (Promega) and immediately counted in a luminometer.

**Western Analysis—**Cells were collected, spun, and washed in cold PBS. Cells pellets were dissolved in modified radioimmuno precipitation assay substrate (Promega). In all cases, cells were washed with PBS after hormone treatment and resuspended in ice-cold PBS. Cell pellets were dissolved in modified radioimmuno precipitation buffer (see below). All samples were stored frozen until assayed. Citrate buffer samples were analyzed for DNA content at the Lombardi Cancer Center Flow Cytometry/Cell Sorting Shared Resource by propidium iodide staining in a FACSort (Becton Dickinson) (33).

**CAT Assays/Luciferase Assays—**Cell extracts in 0.25 mM Tris-HCl buffer, pH 7.8, were frozen/thawed three times to lyse the cells. Protein concentrations were measured by the Bradford method (34). For CAT assays, equal amounts of protein from each extract (typically 1 or 2 μg) were added to 2 μl of 10 mM DTT, 0.5 mM MgCl2, and 0.5 μCi [3H]taurocholate (Amersham), and 1 μCi [3H]glycocholate (Amersham), or 5 μCi [3H]cholesterol (Amersham) to each reaction for 2 hours at 37°C. The reaction was stopped by addition of 40 μl of 5% NaOH. The reaction product was resolved by thin layer chromatography on a polyethyleneimine cellulose plate (35). The amount of product was expressed as the amount of 3H-taurine formed per cell or per mg of protein.

**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 such 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 CAT reporter gene. L929 cells that express endogenous AR were stably transfected with the androgen- and glucocorticoid-responsive reporter plasmid 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...
The AR Is Inactive at the G1/S Transition

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 arrests 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 1, in three independent experiments, the transcriptional activity of the AR at the G1/S junction 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 nonspecific 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 nm 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 this phase of the cell cycle (Fig. 3A, left panel). These data demonstrate that the loss of AR function observed at G1/S is not a nonspecific or toxic effect of the drug per se. Indeed, a similar lack of AR inhibition by hydroxyurea is seen in cells arrested along S phase with this drug (Fig. 2D). To test whether the prolonged treatment of G1/S cells (96 h compared with 72 h for G0 and S phase cells; see Fig. 2A) could account for the inactivity of the AR, cells were serum-starved for 72 h and then exposed to hormone during an additional 24 h of starvation. As can be seen in Fig. 3D, the transcriptional activity of the AR was unaffected by the 96-h starvation treatment. Indeed, we have prolonged starvation for an additional 24 h as well as performed 72-h hydroxyurea treatments in serum with no effects on AR activity (not shown).

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...
The AR Is Inactive at the G₁/S Transition

A. Cell Synchronization

<table>
<thead>
<tr>
<th>Time</th>
<th>0</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
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<tbody>
<tr>
<td>G₀</td>
<td>hormone</td>
<td>serum starvation</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>G₁/S</td>
<td>hormone</td>
<td>hydroxyurea + serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>hormone</td>
<td>hydroxyurea</td>
<td></td>
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</tr>
</tbody>
</table>

B. AR Activity

C. AR Activity

D. AR Activity

Fig. 2. The androgen receptor loses transcriptional activity at the G₁/S boundary. L929-MMTV-CAT cells were arrested in G₀, at the G₁/S boundary, or along S phase as detailed under "Experimental Procedures." The cell cycle arrests were maintained during 24 h of hormone treatment. A, diagram of the protocol used to synchronize cells. B, AR transcriptional activity induced by 1 nm DHT was measured by CAT assay in extracts from G₀-arrested cells containing equal amounts of protein (left panel). FACS analysis of the samples from the left panel is shown in the right panels. DNA histograms for both uninduced cells (NH) or for cells induced with hormone (DHT) are drawn. The percentage of cells arrested at the indicated stage of the cell cycle (shown in parenthesis) was calculated using the software program ModFit. Results are representative of at least three independent experiments. C, AR transcriptional activity and FACS analysis of cells arrested at the G₁/S boundary. D, AR transcriptional activity and FACS analysis of S phase-arrested cells.

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

<table>
<thead>
<tr>
<th>Experiment</th>
<th>G₀ activity</th>
<th>G₁/S activity</th>
<th>Decrease</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>45</td>
<td>Undetectable</td>
<td>~100</td>
</tr>
<tr>
<td>2</td>
<td>75</td>
<td>1.6</td>
<td>96</td>
</tr>
<tr>
<td>3</td>
<td>34</td>
<td>2.7</td>
<td>92</td>
</tr>
</tbody>
</table>
CAT message or protein, respectively, in G/S boundary-arrested cells, since glucocorticoid treatment results in CAT activity.

G/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 progestins (37, 38). The results presented above demonstrate that the strong inhibition of transcriptional activity seen on the MMTV promoter at the G/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 G/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 and 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. 5, A 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 (Kd = 1.4 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 Kd, where only 1% of receptors are predicted to be occupied...
The AR Is Inactive at the G1/S Transition

**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 nM CA, 100 nM DEX, or 100 nM cortisol (CORT). Luciferase activity, measured in duplicate samples, is expressed in luminoeter 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 (NH). 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.

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 activators 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/CA 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 G0. 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.
The AR Is Inactive at the G₁/S Transition

**A.**

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**Fig. 5.** AR levels are low at G₁/S but retain their ability to be up-regulated by androgens. L929-MMTVCAT cells were synchronized as described for Fig. 3B. Cells were harvested in trypsin and aliquoted for FACS analysis, Western blotting, and CAT assays. FACS analysis confirmed effective cell cycle arrests (histograms not shown). A, cells for Western analysis (top panel) were suspended in modified radiomune precipitation buffer and separated by electrophoresis as described under "Experimental Procedures." The same amount of protein was loaded in each lane. Membranes were blotted with PA1-111A, a polyclonal antibody that recognizes the N terminus of the AR. Actin was detected with sc-1616, a goat polyclonal antibody. Immunoreactive bands were visualized by chemiluminescence as described under "Experimental Procedures." Bands were quantified (middle panel) using the software package ImageQuant. AR levels are expressed relative to actin control bands. CAT activity (bottom panel) of an aliquot of the cells used in A was determined as described under "Experimental Procedures." The same amount of protein was used for each CAT assay. The relative CAT activity of S phase cells varies between experiments but is within 20–80% of the activity of cells in G₀. B, Western blot (top panel) of an independent experiment showing the up-regulation of AR levels in the presence of androgens in G₁/S-arrested cells at two different hormone concentrations. Quantifications of bands (middle panel) and CAT activity of the samples shown in A (bottom panel) were performed as described above. C, AR activity in G₀ cells in response to decreasing DHT concentrations. AR activity is present in response to 1 nm DHT (Kᵦ = 1.4 nm, where 50% of receptors are occupied). Activity is still measurable even at more than 100-fold below the Kᵦ, where less than 1% of receptors are predicted to be occupied by hormone.

and not due to the decreased transcriptional activity of the AR itself, since TSA increases AR transcriptional activity without increasing receptor levels.

**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 receptor 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 increased 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
The AR Is Inactive at the G1/S Transition

A.

L929-MMTVCAT

Asynchronous cells

G0 cells

G1/S cells

B.

Relative CAT activity

Asynchronous

G0

G1/S

DHT

DHT+TSA

DHT

DHT+TSA

DHT

DHT+TSA

DHT

DHT+TSA

C.

Cell #

DNA content

DNA content

G1/S (93%)

DHT

NH

G1/S (91.5%)

DHT+TSA

TSA

D.

Asym.

G0

G1/S

CAT activity (cpm/min)

NH

DEX

TSA

NH

DEX

TSA

NH

DEX

TSA

NH

DEX

TSA

E.

Relative AR levels

NH

DHT

TSA

NH

DHT

TSA

AR

actin

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 100 nM DHT in the presence or absence of 5 ng/ml TSA and/or 1 μM GA. 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.

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.
The AR Is Inactive at the G1/S Transition

Cell cycle specific AR coregulatory complexes

**G0**
- pCAF is recruited, binds to AR and acetylates AR and nucleosome histones. 
- Hypophosphorylated Rb is part of the AR coactivator complex.

**G1/S**
- Low AR levels, and pCAF displacement by cyclin D1, prevent AR activity. AR and histones are not acetylated. Phosphorylation inhibits Rb function.

**S-phase**
- pCAF is recruited, binds to AR and acetylates AR and nucleosome histones. 
- Cyclin E is part of the AR coactivator complex.

Although AR levels are low, histone hyperacetylation opens nucleosomes and allows partial AR activity in the absence of pCAF.

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**Fig. 7. Model of AR regulation through the cell cycle.** This model suggests possible mechanisms for the specific negative regulation of the AR at the G1/S transition. In general, AR-activated transcription involves the recruitment of coactivators to the promoter, including proteins that remodel chromatin. In G1/S, we propose that the AR-recruited complexes inefficiently remodel chromatin, leading to loss of receptor activity. Additionally, receptor levels are decreased, further contributing to transcriptional inactivity. The model shows how cell cycle specific coactivators and corepressors may bring about this regulation. Left and bottom right panels, in G0, and S phase, when cyclin D1 levels are low, the AR coactivator complexes that are formed include pCAF, providing acetylation activities. Cell cycle-specific coactivators such as hypophosphorylated Rb (in G0) and cyclin E (in S phase) further promote AR activity. Top right and bottom left panels, in G1/S, binding of cyclin D1 to AR prevents the binding and/or action of pCAF, causing a loss in acetylation of histones and/or of AR. Additionally, phosphorylation of Rb at the G1/S junction inhibits its coactivator function. Thus, low amounts of AR that are expressed are transcriptionally inactive. Histone hyperacetylation by TSA obviates the requirement for chromatin-remodeling complexes, partly restoring AR activity in G1/S. Note that the sizes of the diagrams do not represent relative protein dimensions. Sites of interaction between proteins (where known) are not necessarily accurately drawn. The AR is shown as a monomer for simplicity of presentation.

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-κB 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-
The AR Is Inactive at the G1/S Transition

...remodels MMTV chromatin during transcriptional activation (70), whereas the AR generally 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 GR and reversed or compensated for by a cell-specific component 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 the AR (31), it is possible that increased cyclin E levels in S phase compensate for the low levels of hypophosphorylated Rb and, together with decreasing cyclin D1 levels, explain the S phase activity of the AR.

AR activity at the G1/S transition can be partly restored by treating cells with the histone deacetylase inhibitor TSA. This is particularly interesting, since cyclin D1 inhibition of AR activity is also overcome by TSA treatment (72). Taken together, these data suggest that cyclin D1 inhibits AR activity during G1/S by inhibiting an AR-specific acetylation event(s) that can be overcome with the use of TSA. Two acetylation events have been proposed to increase AR activity. One is the recruitment of coactivator-associated histone acetyltransferase activity, leading to chromatin rearrangement (73, 74). The other is the acetylation of specific lysines in the AR by pCAF (17). Mutation of these lysines severely reduces AR activity. Cyclin D has recently been shown to strongly disrupt pCAF-AR interactions (30). Thus, we propose that the activity of the AR at G1/S is due to competition between cyclin D1 and pCAF, leading to the failure of pCAF to be recruited to AR complexes, resulting in decreased histone and AR acetylation. A model incorporating this idea is shown in Fig. 7, where the balance between the permissive effects of RB, cyclin E, and acetylation and the inhibitory effects of cyclin D1 leads to AR activity in G1 and S phase and inactivity at G1/S.

The biological significance of AR down-regulation and inactivity at the G1/S transition is unclear to us, yet it could be advantageous for cells to have a mechanism for controlling the action of growth-promoting or differentiation factors such as the AR at this check point. The functional meaning of this regulation at G1/S may become clear only in situations where it is lacking due to abnormal coregulators or mutations in AR. Aberrant expression or function of AR coregulators, including proteins of the cell cycle machinery and acetylases/deacetylases, is thought to occur in a range of tumors (75–80). This abnormal environment could potentially alter the ability of the AR to modulate its target genes in a proper temporal manner, leading to defects in growth control or differentiation, even in 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 G2 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.
Short-term Effects of Methoxyacetic Acid on Androgen Receptor and Androgen-Binding Protein Expression in Adult Rat Testis

By

Oscar M. Tirado, Elisabeth Martínez, Olga C. Rodríguez, Mark Danielsen, David M. Selva,
Jaume Reventós, Francina Munell, and Carlos A. Suárez-Quian

2Unitat de Recerca Biomedica
Hospital Materno-Infantil Vall d’Hebron
Barcelona, Spain

3Department of Biochemistry & Molecular Biology
and

4Department of Cell Biology
Georgetown University Medical School
3900 Reservoir Road, N.W.
Washington, D.C. 20007

Short Title: Short-term testicular effects of MAA
Key Words: Androgen receptor, spermatogenesis, testis

Corresponding author: Carlos A. Suárez-Quian
Department of Cell Biology
Georgetown University Medical School
3900 Reservoir Road, N.W.
Washington, D.C. 20007
Ph: (202) 687-1282
Fax: (202) 687-1823
Email: suarezc@georgetown.edu

Supported by grant #HD023484 from NICHD to CS, 9951256U from the American Heart Association to MD and DAMD17-99-1-9199 from the DOD to EM.
ABSTRACT

Spermatogenesis entails the differentiation of a diploid, immature and round spermatogonium into a haploid, mature and streamlined spermatozoon. This developmental process depends upon the action of pro-survival and pro-apoptotic factors that are likely to be elaborated both from within the germ cells themselves, as well as from the supportive element of the seminiferous epithelium, the Sertoli cell. Chemical agents can disrupt the balance between survival and apoptosis and thus give rise to reduced counts of spermatozoa, or oligospermia. One such agent that renders significant germ cell apoptosis at specific stages of the cycle of the seminiferous epithelium is methoxy acetic acid (MAA), the active metabolite of a commonly used solvent, methoxyethanol. Although MAA gives rise to apoptosis of pachytene spermatocytes, it is not known whether MAA exerts solely a direct effect on germ cells, or whether it also affects other testicular cell types such as the Sertoli cells. In the present investigation, we test the hypothesis that MAA has direct effects on Sertoli cells that in turn may contribute to pachytene spermatocyte apoptosis seen with MAA treatment. We report that AR immunohistochemistry in MAA treated rats revealed that the stage specific expression of AR protein in Sertoli cells was significantly altered. In MAA-treated animals high AR expression was found in Sertoli cells coincident with the MAA-induced apoptosis of late-stage pachytene spermatocytes. The altered expression of AR in MAA-treated animals was also seen using seminiferous tubules harvested by Laser Capture Microdissection (LCM). In addition to effects on AR expression, androgen-binding protein (ABP) mRNA levels were also altered in a stage-specific manner. Using a different system, mouse Sertoli cell lines TM4 and MSC-1, positive for either AR or ABP, respectively, we demonstrate a direct effect of MAA on ABP protein and mRNA expression in the MSC-1 cell, but did not detect any effect on AR protein or mRNA.
expression in TM4 cells. Finally, using mouse fibroblasts that express endogenous AR and were stably transfected with two AR promoter/reporter systems, MMTV-CAT and probasin-luciferase, respectively, we examined the ability of MAA to potentiate DHT activation of AR. Results demonstrate that although MAA did not activate AR directly, it did potentiate DHT activation of the AR two- to four-fold. Our results demonstrate that MAA alters the expression level of AR and ABP in vivo and increases AR transcriptional activity in tissue culture cells. We suggest that the abnormal spermatogenesis generated by MAA is at least partly due to direct effects on Sertoli cells. Whether MAA elicits a pro-apoptotic signal from Sertoli cells, or diminishes a pro-survival signal required by germ cells, downstream to altering AR and ABP expression, in a stage-specific fashion, however, remains to be determined.
INTRODUCTION

Methoxyacetic acetic acid (MAA) is the principal toxic metabolite of the ethylene glycol ether 2-methoxyethanol (2-ME), a toxic known to exert transient, but catastrophic effects on testicular histology and function. Administration of MAA to male rodents either by gavage or via intraperitoneal injection at a non-lethal dose, for example, leads to significant infertility [1]. Similarly, in humans, adult males show an increased prevalence of oligospermia and azoospermia after environmental exposure to 2-ME [2]. Given the ubiquitous presence of 2-ME and MAA in paints and industrial solvents, there is a potential for a widespread, negative impact on male reproductive health [3-4]. Indeed, the National Institute of Occupational Safety and Health (NIOSH) estimated that there were 168, 180 employees exposed to 2-ME in 1983 [5].

The testicular cells primarily afflicted following exposure to MAA appear to be primary spermatocytes [5-12]. As early as 12 hours post MAA exposure, for example, nearly 50% of primary spermatocytes undergo apoptosis in a stage-specific fashion. Either at early (II-V) or late (XII-XIV) stages of the cycle of the seminiferous epithelium, a single i.p. injection of MAA induces apoptosis in nearly all pachytene spermatocytes residing at these cell associations, and by 24 hours significant cell loss is apparent [Tirado et al., manuscript submitted]. The dramatic and rapid effect of MAA on pachytene spermatocytes suggests that these cells are direct targets for MAA. This hypothesis is hard to test, however, since pachytene spermatocytes rely on Sertoli cells \textit{in vivo} and cannot be cultured \textit{in vitro}.

Primary cultures of immature rat Sertoli cells also respond directly to treatment of MAA and generate novel Sertoli cell products [13]. Similarly, in seminiferous tubule culture models Src is elevated in the Sertoli cells after incubation with MAA [14]. These observations suggest that Sertoli cells \textit{in vivo} also respond directly to MAA and, in turn, may be involved in the
regulation of apoptosis of primary spermatocytes. In this scenario, recently reviewed by Boekelheide and colleagues, germ cell apoptosis is a direct response to toxic insult on Sertoli cells and may entail regulation of both pro-survival and pro-apoptotic factors [15].

Altering androgen or ABP levels also induces apoptosis of spermatocytes. For instance, abolition of androgens by destroying Leydig cells with EDS, results in a decrease in intratesticular androgen levels, a concomitant diminution in AR immunostaining [16] and is accompanied by significant germ cell apoptosis [17-20]. We have also demonstrated that altered expression of ABP in a mouse transgenic model is associated with significant apoptosis of pachytene spermatocytes [21]. In the present study, we test the hypothesis that MAA brings about apoptosis in pachytene spermatocytes at least partly by altering the level of AR and ABP expression in Sertoli cells. Our results show that ABP levels are indeed altered in a Sertoli tissue culture system and that aberrant expression occurs in a stage specific fashion in Sertoli cells in vivo. In the case of the AR, there is significant stage specific disregulation of expression in Sertoli cells in vivo. In a mouse Sertoli cell line, however, no MAA effect on AR protein nor mRNA levels were detected.
MATERIALS AND METHODS

Animals and treatments

Thirty-seven adult male Sprague-Dawley rats, weighing approximately 400g, were
maintained under standard conditions. Thirty-two rats were treated with a single i.p. injection of
MAA (650 mg/Kg body weight, Sigma-Aldrich, Steinheim, Germany), buffered to pH 7.4 in
0.9% saline solution and sacrificed after three hours (n=5), six hours (n=5), nine hours (n=5),
twelve hours (n=5), twenty-four hours (n=3), three days (n=3), five days (n=3), or seven days
(n=3) after toxin administration. Five rats were used as controls and were treated with saline
solution. Animals were killed by CO₂ asphyxiation. One testis from each animal was fixed in 4%
paraformaldehyde for 24 h and subsequently embedded in paraffin. The other testis was minced,
immediately frozen and used for DNA and RNA extractions. Ten of the thirty-seven rats were
used for LCM experiments (two controls and two sacrificed at 3, 6, 9, and 12h). Rats were killed
as before and testes removed immediately and frozen in liquid nitrogen.

TUNEL staining

TUNEL assay was performed as we previously described [21]. Briefly, dewaxed and
rehydrated sections were treated with 20 µg/ml proteinase K for 15 min and with 3% hydrogen
peroxide for 5 min. After incubation with terminal deoxynucleotidyltransferase (TdT) buffer (25
nM Tris HCl, 200 mM cacodylate acid, and 200 mM KCl) for 15 min, sections were treated with
0.05 U/µl TdT (Roche Molecular Biochemicals, Mannheim, Germany) and 0.5 nM biotin-16-
deoxy (d)-UTP (Roche Molecular Biochemicals) in TdT buffer at 37°C for 90 min and with 300
mM NaCl and 30 mM sodium citrate at room temperature for 15 min. After washing and
incubating with 2% BSA, sections were exposed to avidin-biotin complex (ABC, Vector
Laboratories Inc., Burlingame, CA), diluted 1:25, at 37°C for 45 min, and the peroxidase reaction was visualized with diaminobenzidine and hydrogen peroxide.

Immunohistochemistry

Immunostaining of testicular sections for AR was performed as described previously [22]. Six-μm sections were cut and AR immunolocalized using a polyclonal antibody provided by Dr. Gail Prins (Univ. Illinois) and employing a biotin-streptavidin-immunoperoxidase method, including epitope retrieval. At the completion of the epitope retrieval, the sections were allowed to cool for approximately 20 min. Primary antibody was applied to the sections and allowed to incubate overnight at 4°C. At this point the sections were treated exactly as described in the instruction manual supplied by the manufacturer’s immunostaining kit for AEC as the chromogen (Zymed, Burlingame, CA). Hematoxylin counterstaining of the sections was performed briefly for 30-60 seconds and coverslipped. As controls, additional sections were treated as follows: 1) primary antibody was omitted; 2) normal rabbit sera was used instead of primary antibody; 3) dilutions of primary antisera were performed to quench positive staining as a function of specific antibody concentration; and 4) antibody was pre-adsorbed with specific immunopeptide and then used for immunostaining. The sections were photographed using a Zeiss Axiophot microscope fitted with 63x objectives and images recorded on Kodak Elite Chrome 100 ASA film set at 50 ASA. For publication, photographs were scanned at 300 dpi using a HP scanner and the final prints made using Adobe Photoshop imaging software and an Epson 740 color printer.
Messenger RNA isolation and analysis of its expression by RT-PCR

RNA was obtained from total testis and from microdissected tubules by means of guanidium thiocyanate/phenol-chloroform extraction [23] and by “Micro RNA isolation kit” (Stratagene, La Jolla, CA), respectively. One μg of total RNA isolated from tissues was reverse transcribed using 200U of Superscript II Rnase H- Reverse Transcriptase (Gibco-BRL, Bethesda, MD) in a 20 μl reaction volume, in the presence of 25 g/ml Oligo (dT), first strand buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂), 0.01 M DTT and 10 mM of each dATP, dGTP, dCTP, and dTTP. The RNA and Oligo dT mix were heated at 70°C for 10 min, then cooled to 4°C, the other reagents were added and the reverse transcription performed at 42°C for 50 min.

ABP PCR primers were designed using Oligo 4.0 software from National Biosciences, INC. (Plymouth, MN), based on Genbank published sequences. For rat ABP, a 954-bp product was amplified using an upper primer designed specifically against rat exon 1 (GAGAAGGGAGAGGTGGCCT) and a lower primer that specifically recognized exon 7 (GCTCAAGGCTACTTTGAATAC). In addition, a second primer set was used to perform the PCR of microdissected staged-tubules that rendered a 246 bp product. This second primer set consisted of upper primer (CAGCAAACCCTCTTCCTCC) from exon 1 and lower primer (TTCCATCCACCCCATAGCAGCAG) from exon 2. PCR primers for AR were designed using Primer Express version 1.0 software from Perkin-Elmer; upper primer (CTCCAGGATGCTCTACTTTGCA) hybridized to a sequence of exon 5 and the lower primer (ACACACTGGCTGTACATCCGAG) hybridized to a sequence of exon 6, rendering an 87 bp product. The primer set used to amplify L19 by PCR was (AATCGCCAATGCCAACTCTCG) for upper and (CCCTTCCCTTCCCTATGCCC) for lower. Amplification was carried out in a 2400 Perking Elmer thermocycler (Applied Biosystems, Foster City, CA) and consisted of 40
cycles of amplification. Denaturation was performed at 94°C for 15 sec, annealing at 59°C for both AR and ABP, and extension at 72°C for 45 sec. PCR products were separated on a 2% agarose gel and quantified by the Molecular Analyst/Macintosh data analysis software using a Bio-Rad Image Analysis System (Bio-Rad Laboratories, Inc., Hercules, CA). The products of amplification were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to supplier's instructions and sequenced using an Abi Prism 310 genetic analyzer (Perkin-Elmer Corporation).

Laser capture microdissection

The strategy to target and harvest stage-specific seminiferous tubules by LCM was published previously [24]. In the present study, either control testis or testes from MAA-treated rats were removed from the animals and placed in ice-cold 30% sucrose until they sunk to the bottom of a scintillation vial (approximately 4 h). Next, testes were immersed in Tissue-Tek and frozen in liquid nitrogen. Six to seven micron sections were cut in a cryostat and attached to glass slides. Sections were fixed for 30 sec in 70% ethanol and then stained for hematoxylin and eosin by conventional means. Laser capture microdissection was performed using a PixCell II apparatus (Arcturus, Inc.), essentially as described in detail previously [24]. Fifty tubule cross sections at specific stages (III-IV, VII-VIII, or X-XIII) were pooled and total RNA extracted using a MicroRNA extraction kit from Stratagene (Cedar Creek, Texas). From fifty tubule cross-sections harvested by LCM, there was sufficient total RNA to perform five RT-PCR reactions for AR and ABP as described in the above section. The quality of the RNA prepared from the harvested tubules by LCM was confirmed by performing an isolated harvest of 50 tubules,
preparing the sample as for the experimental tubules, and running the sample on a 2% agarose
gel and staining with ethidium bromide to visualize the 28S and 18S bands.

Sertoli cell lines

MSC-1 and TM4 mouse Sertoli cell lines were a gift from Dr. Leslie Heckert (University
of Kansas, MO). MSC-1 cells were grown in Dulbecco Modified Eagles medium (DMEM)
containing 5% bovine calf serum at 37°C in a saturated atmosphere of 5% CO₂ [25]. TM4 cells
were grown in a 1:1 mixture of Ham’s F12 medium and Dulbecco modified Eagle medium with
1.2 g/L sodium bicarbonate and 15 mM HEPES (92.5%) horse serum (5%) and fetal bovine
serum (2.5%) [26].

Protein extraction and Western blot analysis

Primary cultures of Sertoli cells TM4 or MSC-1 were lysed with RIPA buffer containing
protease inhibitors (1mM phenylmethylsulfonylfluoride, 10 mg/ml aprotinin, and 10 mg/ml
leupeptin) and the lysates were centrifuged at 13,000 x g, at 4°C, for 30 min. The protein content
of the supernatant was determined by the Bradford assay (Bio-Rad Laboratories). Equal amounts
of protein (30 µg) from either TM4 or MSC-1 cells were resolved by 10 % SDS-PAGE and
transferred to nitrocellulose membranes. After blocking, the membranes were incubated at 4°C
overnight with 2 µg/ml of the PG21 rabbit polyclonal anti-mouse AR antibody (TM4), or with
anti-ABP antisera diluted approximately 1:400 (A gift of Dr. Neal Musto [27]). Next, the blots
were incubated for 1 h at room temperature with HRP-conjugated secondary antibody (1/2000).
Peroxidase activity was analyzed with the SuperSignal West Pico Chemiluminescent substrate
kit from Pierce according to the manufacturer’s instructions. The AR and ABP content in TM4 and MSC-1, respectively, were determined densitometrically.

Cell culture

L929 MMTV-CAT cells containing integrated copies of the androgen-inducible construct MMTV-CAT and L929 probasin-luciferase cells containing integrated copies of the androgen-inducible construct probasin-luciferase have been described previously [Martinez and Danielsen, submitted manuscript]. Cells were cultured in DMEM supplemented with 3% calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. For the development of the L929-MMTV-CAT stable cell line, L929 cells were transfected using Dosper liposomal reagent (Boehringer Mannheim) with pMMTV-CAT and pSV2neo (20:1 ratio), according to the manufacturer’s protocol. To obtain the L929-ProbasinLuc cell line, L929 cells were transfected using Lipfectamine 2000 reagent (Life technologies) with p-286/+28PB-luciferase [28] and pSV2neo (20:1 ratio), according to the manufacturer’s protocol. In both cases, the cells were split 48 hours after transfection and selected in growth media supplemented with 400 mg/L G418 sulfate (Cellgro). Single clones were picked with sterile pipette tips and expanded. Clones were screened for chloramphenicol acetyl transferase (CAT) or luciferase activity after 24-hour hormone induction. Single clones showing low basal reporter activity and at least five fold activation with DHT were used for further studies (Clones L929-MMTVCAT #31 and L929-ProbasinLuc 2.9 were used in this study).
CAT and luciferase assays

Stably transfected L929 cell cultures were treated with hormone and/or MAA for 24 h and harvested in 0.25 M Tris-HCl, pH 7.8. For CAT assays, equal amounts of protein from each cell extract were combined with radiolabeled acetyl coenzyme A and chloramphenicol in Tris buffer and the reaction mixture was overlaid with organic scintillation fluid, as previously described [28]. The acetylated product becomes incorporated into the organic phase and is counted in a scintillation counter. For luciferase assays, cell extracts were combined with Luciferase Assay Substrate (Promega) in glass tubes and immediately counted in a luminometer to detect production of luminescence.
RESULTS

MAA induces apoptosis in pachytene spermatocytes

Rats were treated with a single i.p. injection of MAA and testes harvested 9 h later and processed with TUNEL staining to determine the degree of apoptosis (Fig 1). In random sections examined at low magnification, TUNEL staining was easily detected in some tubules and appeared to be present in all pachytene spermatocytes of the seminiferous epithelium of TUNEL positive tubules. In contrast, other tubule profiles appeared completely devoid of any TUNEL staining. As we reported previously [Tirado et al., manuscript submitted], after a short-term exposure of MAA (6-9 h) from an intraperitoneal injection, all pachytene spermatocytes at stages II-IV and stages XII-IV became TUNEL staining positive (Fig. 1, C). However, at no times did large numbers of pachytene spermatocytes residing at stages V-IX exhibit robust TUNEL staining. Thus, a distinct marker of MAA toxicity is the specific TUNEL staining characteristics of the pachytene spermatocytes as a function of the cycle of the seminiferous epithelium.

Testicular AR immunohistochemistry in testis

Specific AR immunostaining in adult rat seminiferous tubules is amply documented and known to reside within the nuclei of Sertoli and peritubular myoid cells, the latter forming the walls of the tubules [29]. While the intensity of the staining in the myoid cell nuclei is constant, in the Sertoli cell nuclei it varies as a function of the cycle of the seminiferous epithelium (Fig. 2, Control). At earlier stages (II-III) the staining is weak and often difficult to discern with certainty. However, as spermiation approaches (stages VII-VIII), staining intensity becomes more robust and reaches its maximum level coincident with those tubules in which sperm release occurs. It is precisely in these later stages that the first signs of germ cell loss are detected due to
androgen deprivation following experimental insult [30]. In subsequent stages (IX-XIV), AR staining in Sertoli cell nuclei cannot be detected [16, 29].

MAA treatment led to a dramatic alteration in the expression of the AR in Sertoli cells (Fig. 2). This effect was clearly seen after only six hours of treatment (the earliest time point examined) where there was an increased expression of AR in Stage IV cells, compared to controls (compare B' to B). Concomitant with the increased expression in stage IV cells there was a decreased expression of AR in stage VIII Sertoli cells (compare C' to C). By 12 h of MAA treatment, expression of AR in stage VIII Sertoli cells had decreased and there was an increase in expression in both early (stage I-II), and late (stage XII) cells. The robust AR staining in stage IX and later Sertoli cell nuclei (Fig 2 D'') was particularly interesting since AR expression in these cells has not been reported before in rodents under any experimental conditions.

AR and ABP mRNA expression in MAA treated rats

In the experiments described above, the cyclic expression of AR protein in vivo was altered by MAA treatment. To determine whether this alteration in AR immunostaining intensity as a function of the cycle of the seminiferous epithelium reflected an overall change in AR expression in the whole testis, AR mRNA levels were quantified in the testes of rats treated with MAA for 3 to 24 hours. As can be seen in Fig. 3, overall AR mRNA levels did not change during this time course. In contrast, ABP levels were significantly higher in testicular extracts of rats 6 hours post MAA treatment, and remained elevated during the duration of the experiment (Fig. 3).
AR and ABP mRNA levels in stage-specific tubules harvested by LCM

The lack of overall changes in AR levels as seen in total testicular extracts probably reflects the developmental changes in AR expression, i.e. some cells in the testis produce more AR in response to MAA while others produce less. For that reason, we examined AR mRNA expression in isolated, staged tubules using LCM (Fig. 4). Tubule cross sections were examined first at high magnification (Fig. 4, B), staged using the scheme of Leblond and Clermont [31] and then all the cells found within the specific cross section collected by LCM. The remaining void in the tissue section indicated the degree to which a particular tubule section was successfully harvested by LCM (Fig. 4, C). No attempt was made to capture seminiferous tubules free of the peritubular myoid cells. Approximately 10-15 tubule cross sections, all at the identical stage of the cycle of the seminiferous epithelium, were collected onto one cap (Fig. 4, D) and then up to 50 captured tubules were pooled for RNA isolation. The quality of the RNA extracted from 50 tubule cross sections was examined using agarose gel electrophoresis and ethidium bromide staining of the 28S and 18S bands (Fig. 4, E).

Relative AR and ABP mRNA levels were determined in the stage-specific tubules harvested by LCM from either control or MAA treated rats at times indicated in figure 5. In control tubules, the relative AR mRNA expression levels as a function of the cycle of the seminiferous epithelium were similar to the protein level expression revealed by immunohistochemistry. Moderate levels were present in stages III-IV, maximum levels were present at stages VII-VIII, and minimal levels were detected in late stages (X-XIII). Presumably, the low level of AR mRNA detected in the later stages was due to the contribution of peritubular myoid cells that were collected along with the seminiferous epithelium using LCM. The pattern of ABP mRNA levels in control tubules as a function of the cycle varied significantly from that
observed for AR mRNA. In early (III-IV) and late (X-XIII) stages, ABP mRNA levels were relatively high, whereas no ABP mRNA was detected in stages VII-VIII.

MAA treatment had a very rapid effect on AR mRNA levels in all stages of the seminiferous tubule. By 3 hours, AR mRNA levels had decreased in stages VII-VIII, but had significantly increased in the other two groups of stages III-IV and X-XIII. At later times post-MAA treatment, AR mRNA levels returned to control levels in the early stages (III-IV), but remained low during the middle stages (VII-VIII) and high at the later stages (X-XII). The changes in mRNA levels upon MAA treatment indicate that at least part of the effect of MAA treatment is an alteration in the rate of AR transcription. However, there was not a complete correlation of changes in AR mRNA levels with the changes in protein levels seen previously. It is likely therefore that MAA also changes the turnover of AR protein.

Expression levels of ABP mRNA were also significantly affected by MAA administration to rats. As early as 3 hours post MAA treatment, ABP mRNA levels at stages III-IV had decreased, remained low for the next 3 hours, but nearly regained normal levels by 12 hours. In contrast, at stages VII-VIII and X-XIII, ABP mRNA increased 3 hours post-MAA treatment and levels failed to return to normal by 12 hours.

**AR and ABP mRNA and protein levels in cell lines**

In the experiments described above, we showed that MAA has rapid and dramatic effects on Sertoli cells in vivo. However, in such a system it is hard to determine which effects are due to direct effects on Sertoli cells and which are mediated through other cell types. To begin to address this, we examined the effects of MAA on the expression of AR and ABP in Sertoli cell lines.
ABP mRNA and protein expression were examined in the MSC-1 cell line. Increases in both ABP mRNA and protein levels were clearly seen by 6 hours and increased to the 12 hour time point (Fig. 6, 7). Thus, Sertoli cells in culture can respond to MAA indicating that in vivo at least some of the effects of MAA are due to a direct interaction with Sertoli cells. Since MSC-1 cells do not produce AR, we turned to the TM4 cell line to examine effects of MAA on AR protein and mRNA levels. Treatment of cells with MAA had no effect on AR protein or mRNA levels even after 12 h of treatment (Fig. 6, 7). This result is perhaps not surprising since even in vivo only some cells at specific stages of the cycle show altered AR expression levels.

Potentiation of DHT activation of AR by MAA

Since no effect on AR expression levels was detected in TM4 cells treated with MAA, we evaluated whether MAA could affect the activity of the AR. L929 cells expressing endogenous AR and stably transfected with a MMTV promoter-CAT reporter system were exposed to MAA in the presence or absence of androgens. AR transcriptional activity was measured in CAT assays. We found that 5 mM MAA potentiated androgen induction of AR activity 3 to 4 fold (Fig. 8). This effect was fully blocked by the antiandrogen cyproterone acetate, indicating that the MAA effect is mediated by the AR. To determine if MAA could potentiate androgen action at androgen concentrations that elicit a sub optimal transcriptional response, cells were treated simultaneously with MAA and with increasing concentrations of androgen. MAA was able to potentiate DHT at all concentrations tested, suggesting that this effect does not require the presence of high amounts of androgens (Fig. 9). Although a potent enhancer of DHT, MAA showed no androgenic activity of its own.
To evaluate if MAA potentiation of androgens could be seen on a natural androgen-responsive gene, we treated mouse fibroblasts L929 cells stably transfected with a probasin promoter-luciferase reporter construct with MAA in the presence or absence of androgens. Again, MAA potentiated the effects of DHT 2 to 4 fold, and this enhanced activity was fully inhibited by cyproterone acetate (Fig. 10). On the probasin promoter, MAA also lacked the ability to elicit a transcriptional response in the absence of androgens. These data demonstrate that MAA increases the transcriptional activity of the AR, suggesting that alteration of the AR function by MAA can contribute to its deleterious effects on spermatocytes.
DISCUSSION

MAA is a well-known endocrine disrupter that leads to decreased fertility due to the induction of apoptosis in pachytene spermatocytes. We have found that MAA has direct effects on Sertoli cells both in vivo and in vitro raising the possibility that part of the toxicity of MAA is due to its action on this cell type. Specifically, we have shown that MAA alters the expression profile of both the AR and ABP in Sertoli cells in a differentiation-specific manner. In addition, studies on an androgen-responsive transcription system show that MAA potentiates the transcriptional activity of the AR. Taken together, these data provide evidence that altered expression and transcriptional activity of the AR may play a role in MAA toxicity.

Apoptosis in Pachytene Spermatocytes

The fact that the toxicity of methoxyacetic acetic acid is specific for pachytene spermatocytes and induces their death by apoptosis is well established [5,11]. Although the molecular mechanism of apoptosis in these cells presumably occurs similarly to other cell types [32-33], the spermatocyte-specific signals that lead to the triggering of apoptosis are not known. Our results show that these signals are stage-specific, since MAA induces apoptosis, indicated by TUNEL staining at both early (II-IV) and late stages (XII-IV), but not at intermediate stages (V-IX) of the cycle of the seminiferous epithelium. The dramatic effects of MAA on Sertoli cells in a stage-specific manner indicate that the apoptotic signals that kill spermatocytes either arise in Sertoli cells or severely influence this cell type.

One possibility is that MAA modulates AR action in Sertoli cells and that this leads to apoptosis in spermatocytes. This hypothesis is at least partly consistent with the apoptotic cell death of spermatocytes seen in animals with reduced AR function. This induction of cell death is
seen with both chemical destruction of Leydig cells [17-20] and with altered expression of ABP [21]. This hypothesis is also consistent with the alteration in AR levels in MAA-treated animals occurring at early and late stages, the very stages that show the greatest amount of apoptosis. The mechanism by which MAA induces pachytene spermatocyte apoptosis, however, is likely to be different than that induced by EDS. EDS-induced apoptosis requires almost three days for the effects to first be detected and preferentially occurs at stages VII-VIII [18], the stages not affected by MAA. Further, the EDS treatment diminishes all AR immunostaining [16], whereas MAA effects were observed in a stage-specific fashion.

MAA probably has many effects on gene expression in the testis. However, a comprehensive analysis of the transcriptional profile of the testis after treatment with MAA has not yet been accomplished. In a previous study, we did examine the expression of the estrogen receptor β in MAA-treated rat testis. We found that short-term administration of MAA to adult rats lead to enhanced expression of estrogen receptor protein and mRNA in pachytene spermatocytes destined to die by apoptosis [Tirado et al., manuscript submitted]. Whether this enhanced estrogen receptor β expression is due to a direct effect of MAA on the spermatocytes or whether it is mediated by another cell type such as Sertoli cells is open to question. Regardless of the answer to this question, it is clear that MAA has numerous effects on a number of cell types in the testis and that a detailed analysis of these effects is required for an understanding of the pachytene spermatocyte cell death that occurs upon MAA treatment.

Changes in AR and ABP levels

In vivo, altered levels of AR mRNA and protein in Sertoli cells were seen at the earliest time points examined (3 and 6 hours respectively). As discussed above, these changes in AR
levels precede apoptosis in pachytene spermatocytes, as measured by TUNEL staining, by 3 to 6 hours (Fig. 1). This timing is consistent with AR changes in Sertoli cells contributing to spermatocyte cell death. In many tissues, the activity of the AR is controlled by fluctuating serum androgen levels. This is not the case in the testis since the seminiferous tubules are bathed in a very high concentration of testosterone that is thought to saturate the receptor. Therefore, perhaps the most efficient way to control AR activity in the testis is to control the level of the protein. The rapid changes in AR levels seen in MAA-treated animals would be expected to give rise to a rapid change in AR activity. Our mRNA and protein data indicate that MAA alters both the turnover rate of the AR as well as the level of mRNA. Indeed, one aspect of AR action that is not completely understood is the control of AR protein and mRNA levels by androgen itself.

Sertoli cell lines were used to test whether MAA directly altered AR and ABP mRNA and protein, respectively. Results were clear in that both ABP mRNA and ABP protein expression were elevated in the MSC-1 cells after incubation with MAA, but MAA did not alter AR mRNA or AR protein in the TM4 cells. One possible explanation of these results is that the regulation of AR expression in Sertoli cells requires the presence of germ cells, whereas ABP expression is germ cell-independent. Hence, MAA exhibited the ability to alter the expression of AR in vivo, but failed to change its expression in vitro when a cell line model was used that lacked the germ cell factor(s). Another explanation may be that MAA will exhibit an effect on AR expression in Sertoli cells only within the context of the cycle of the seminiferous epithelium. Although the use of cell lines is not fully adequate [34-35], these cell lines in particular, were used as a first approximation to try to understand the mechanism of action of MAA on Sertoli cells, and to our knowledge these are the two best characterized Sertoli cell lines available to investigators [25-26]. Furthermore, in our hands, MSC-1 did not express AR,
whereas TM4 did not express ABP. Thus, out of necessity, we had to employ both cell lines to examine the effect of MAA on AR and ABP. Given these limitations, it is clear that additional work needs to be performed to further elucidate the mechanism by which MAA directly influences Sertoli cells, but as a cell line model both TM4 and MSC-1 cells may serve as useful tools to approximate toxic effects on Sertoli cells.

**MAA potentiates AR transcriptional activity**

It is clear that MAA alters the developmental expression of AR in Sertoli cells in vivo in a stage-specific manner. However, we are currently unable to determine the effects of MAA on AR transcriptional activity in this tissue. We therefore used L929 cells that express endogenous AR and contain the androgen-inducible constructs MMTV-CAT or probasin-luciferase. In both cell types, MAA potentiated the effects of DHT but had no effect on the AR in the absence of hormone. We therefore believe that the increased transcriptional activity of the AR is not due to interaction of MAA with the AR itself, but rather is due to some other event such as altered coactivator expression or modification of chromatin.

In a recent review, Boekelheide speculated that testicular toxics could possibly exert a deleterious effect on spermatogenesis by modulating one of three possible Sertoli cell activities and gave examples of each. Toxics could depress pro-survival factors, increase pro-apoptotic factors, and both depress pro-survival and increase pro-apoptotic factors [15]. Given the lack of understanding of how androgens regulate specific transcriptional activity in spermatogenesis [36-38], it is not possible at this time to categorize MAA into one of these three categories, although it is likely that MAA exerts an effect on Sertoli cell activity. Our present observations of AR and ABP increase in Sertoli cells in vivo in response to MAA, for example, are similar to the
previously reported increased clusterin [11] and Src immunostaining in Sertoli and dying pachytene spermatocytes after treatment with MAA [14]. Given that clusterin is implicated in apoptotic cell death [11] and that its expression appears to be androgen regulated [39], ongoing experiments will test whether MAA causes a stage-specific increase of clusterin mRNA. Finally, the more specific role of ABP in the apoptotic process is also not clear. Whereas in the ABP transgenic mouse excess ABP was associated with increased pachytene spermatocyte apoptosis [21], in the present experiment ABP mRNA levels were elevated in the stages in which apoptosis was not observed. Nevertheless, the fact that ABP is a Sertoli cell product that may serve to maintain intra tubular androgen homeostasis [40], and that its mRNA levels were significantly altered in a stage-specific fashion, may suggest that ABP also participates in the apoptotic signaling of pachytene spermatocytes as predicted by Boekelheide [15]. In this latter case, however, AR is unlikely to be implicated, since there is no evidence to suggest that ABP mRNA expression is under androgen regulation [41].

That a toxic exerts an effect on Sertoli cell function, however, does not preclude that it may also directly compromise germ cell activity. As discussed above, we have previously shown that ERβ expression is significantly altered in pachytene spermatocytes in response to MAA [Tirado et al., manuscript submitted]. Whether this increased ERβ expression is secondary to the MAA alteration of Sertoli cell activity, or an independent effect, remains to be determined. Furthermore, as we previously demonstrated, Sertoli cells are not the exclusive AR-expressing cells in the testis; since peritubular myoid cells, Leydig cells, and arteriolar smooth muscle cells are also AR-positive [22, 29]. Given that MAA altered the expression of AR, as well as potentiated androgen activity, it is possible to speculate that some chemical agents that exhibit
testicular toxicity may exert their deleterious effects on a vast number of testicular cells previously unrecognized as their targets.
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FIGURE LEGENDS

Fig. 1. Stage-specific pachytene spermatocyte apoptosis after MAA treatment. TUNEL staining of pachytene spermatocytes from a testis removed from a rat nine hours after treatment with a single *i.p.* injection of MAA. In A, a survey image at low magnification, the heterogeneous TUNEL staining of pachytene spermatocytes as a function of the cycle of the seminiferous epithelium is evident. In B and C, staged tubules from the same section as shown in A indicate TUNEL negative pachytene spermatocytes at stage VII and TUNEL positive pachytene spermatocytes at stage XII. Stages III-IV (not shown at higher magnification) also exhibit similar TUNEL staining intensity of pachytene spermatocytes as present at stage XII. Magnification A = 125x; B and C = 600x.

Fig. 2. AR immunohistochemistry in control and MAA-treated testis. The distribution of AR immunostaining in Sertoli cells as a function of the cycle of the seminiferous epithelium in control and testes from MAA treated rats is shown. Roman numerals of rows indicate the approximate stage of the cycle, whereas columns are grouped into time after MAA exposure. In control testis (A-D), maximal AR immunostaining is evident in Stage VIII Sertoli cells (Vornberger et al., 1994), and there is varying AR staining intensity at other stages. Sertoli cells residing in stages IX-XIV do not immunostain for AR (Vornberger et al., Bremner et al., 1994). As early as six hours post MAA treatment, the relative AR immunostaining intensity is altered, maximal staining intensity becomes apparent at earlier stages (B’) and stage VIII staining becomes diminished (C’). The altered AR immunostaining intensity in Sertoli cells as a function of the cycle is maintained nine hours post MAA treatment (A”-D”). At 12 hours post MAA treatment, stage XII Sertoli cells and higher become AR positive (D’”). In D to D”, the Sertoli
cell nuclei were interpreted to be AR negative, since in the absence of the hematoxylin counterstain their presence could not be discerned. Magnification of all images = 500x.

Fig. 3. ABP and AR mRNA expression in total testis after MAA. Control and testes from MAA treated rats were probed for the relative level of ABP and AR mRNA expression. A sample gel used to quantify the expression is shown in A and the relative levels of the ABP and AR mRNA expression post MAA are shown in B. AR mRNA expression was not changed, whereas ABP mRNA were significantly elevated at 12 and 24 hours post MAA treatment. Each experiment was repeated in triplicate. Asterisk indicates significance at p < 0.05.

Fig. 4. Acquisition of stage-specific tubules by LCM. The steps taken to harvest stage-specific seminiferous tubules are illustrated in A-D. At low magnification (A), tubules are targeted and the identity of the stage verified at higher magnification (B) using the scheme of Clermont and Leblond (1952). Next, the targeted tubule is acquired by LCM and the void remaining in the tissue section imaged (C). The harvested tubule that is affixed to the cap can also be imaged (D). No attempts were made to capture seminiferous epithelium devoid of the peritubular myoid cells. Approximately 10-15 tubules, all at the same stage of the cycle, were collected onto one cap, and caps were then extracted for total RNA using the same solution so as to pool all of the RNA from approximately 50 tubules into one aliquot. One fifth of an aliquot of total RNA harvested from 50 staged-tubules was used to prepare the gel presented in E.

Fig. 5. Stage-specific ABP and AR mRNA expression in tubules obtained by LCM. The relative ABP and AR mRNA in control and testes from MAA treated rats is shown in panels A-D. Each
upper panel is a representative profile of gel run using 50 staged tubules. All experiments were repeated in triplicates. In control, staged tubules, maximal AR mRNA expression is present in stage VII-VIII tubules. The low AR mRNA expression evident in stage XII tubules may correspond to peritubular myoid cells. At 3, 6, and 12 hours post MAA, maximal AR mRNA expression in stage VII-VIII tubules diminishes, whereas it increases in stages III-IV and X-XII.

In contrast to the AR mRNA expression, ABP mRNA expression in control testis was maximal in stages X-XII, and no expression was detected in stages VII-VIII (panel A). In response to MAA, ABP mRNA increased in the middle stages (VII-VIII) and decreased at the later stages (X-XII).

Fig. 8. AR and ABP mRNA expression in TM4 and MSC-1 cells. The relative AR and ABP mRNA expression in TM4 and MSC-1 cell lines, respectively, is presented in panels A and B. In panel A, a sample from one of three different experiments performed to calculate significance is shown. Asterisk represents significance at $p < 0.05$.

Fig. 7. Western Analysis of AR and ABP in TM4 and MSC-1 cells. AR and ABP protein levels in TM4 and MSC-1 cell lines post MAA treatment are presented in panels A and B. The upper panel is a representative sample from three separate experiments. Asterisk indicates significance at $p < 0.05$.

Fig. 8. Potentiation of DHT by MAA on MMTV-CAT in L929 cells. MAA alone had no DHT activity, but in combination with DHT at 1 nM exhibited the ability to potentiate its activity 3-4 fold. 1 μM cyproterone acetate completely inhibited the DHT potentiation effect of MAA.
Fig. 9. Dose response curve of MAA potentiation of DHT on MMTV-CAT in L929 cells. 5 mM MAA exhibited the ability to potentiate DHT activation of AR at all tested doses of DHT.

Fig. 10. Potentiation of DHT effects by MAA using probasin-luciferase system in L929 system. 5 mM MAA exhibited the ability to potentiate DHT activation of AR 2-3 fold, and this activity was inhibited by cyproterone acetate (CA). At lower doses of MAA (0.1 – 1 mM), no significant potentiation of DHT activity was observed.
Figure 1
Figure 2
Figure 3

A

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B

AR and ABP mRNA in Testis

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Figure 3
Figure 5
Figure 6

AR & ABP mRNA Expression in Sertoli Cell Lines Post MAA
Western Analysis of AR & ABP in TM4 and MSC-1

Figure 7
MAA Effects on DHT Induction of MMTV-CAT in L929 Cells

Figure 8
Dose Response of MAA Potentiation of DHT Induction of MMTV-CAT L929 Cells

Figure 9
MAA Effects on DHT Induction of Probasin-Luciferase in L929 Cells

Figure 10
MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for Information Management
ADB285846
ADB255323
ADB246514
ADB233740
ADB286395
ADB275662
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ADB272667
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