UNCLASSIFIED

AD NUMBER

ADB275662

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Jul 2001. Other requests shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott St., Ft. Detrick, MD 21702-5012.

AUTHORITY

USAMRMC ltr, 18 Apr 2003

THIS PAGE IS UNCLASSIFIED

AD______________

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 2001

TYPE OF REPORT: Annual Summary

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

DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, Jul 01). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20020215 085

NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

Award Number: DAMD17-99-1-9199
Organization: Georgetown University Medical Center

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

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

Carole B Christian ____________________

1-25-02 ____________________

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2001	Annual Summary (01 Jul 00 - 30 Jun 01)

4. TITLE AND SUBTITLE
Cell Cycle Regulation of Estrogen and Androgen Receptor

5. FUNDING NUMBERS
DAMD17-99-1-9199

6. AUTHOR(S)
Elisabeth D. Martinez

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University Medical Center
Washington, DC 20057

E-Mail: martine@georgetown.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

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

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, Jul 01).
Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

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 regulation is maintained by cancer-inducing non-steroidal agents. We have found that the androgen receptor (AR) has optimal activity in G0 cells. The receptor shows moderate activity in cells along S phase. However, it has been found that the AR losses its activity at the G1/S boundary. Androgen upregulation of receptor protein is seen in both G0 and G1/S arrested cells, although the final receptor levels are consistently higher in G0 cells. Serum starved cells consistently show 10-20 fold induction of estrong receptor activity. Treatment with hydroxyurea after starvation obliterated all ER activity while the same drug treatment on asynchronous cells reduced estrogen activation to 3-4 fold over basal values. Hydroxyurea treatment of serum starved cells had only a slight effect on estrogen activity. Non-steroidal activators tested with our cells induced ER or AR two or three fold or not at all. Through these studies we are gaining understanding of how estrogen and androgen receptors are regulated and how their deregulation may contribute to the onset of tumorigenesis and hormone independent growth.

14. SUBJECT TERMS Breast Cancer, cell cycle, androgen receptor, non-steriodal activators, L cells, antiandrogens, antiestrogens, androgens, estrogens	15. NUMBER OF PAGES 21 / 16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover... ......

SF 298.. 2

Table of Contents.. 3

Introduction... ...4

Body.. 4

Key Research Accomplishments... 8

Reportable Outcomes...9

Conclusions...10

References...10

Appendices...11 on

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 the July 2000 annual report and in the present report.

Aim1: Our first aim is to measure the activity of steroid-activated ER and AR during the cell cycle. To this purpose, four tasks were assigned in the original Statement of Work under Aim 1. Tasks A and B were completed in the first year of funding and corresponding results were submitted in the July, 2000 annual report. Task C was partially accomplished during the first year. Here we summarize the results obtained under Aim 1 task C up to June, 2000 and present work done on this task since then. During the first year of funding, we found that the androgen receptor had high activity during the G0 phase of the cycle (while cells are serum starved) and good activity when cells were arrested throughout S phase by treatment with a variety of S arresting drugs including aphidicolin, hydroxyurea and thymidine. No activity was detected in the G2 phase of the cycle. In the case of the estrogen receptor (ER), no complete arrests were obtained yet phases were enriched by drug treatment. Estrogen receptor expressing cells with a cell cycle distribution of 50% G0/G1, 40% S phase and 10% G2/M showed a ten fold induction of ER activity when treated with estradiol. As the S phase fraction increased to 60% (with consequent decrease in G0 cells to 25% and some increase in G2/M to 15%), for example, induction decreased to about 2 or 3 fold over no hormone controls. These results have been confirmed and furthered over the last 12 months. Indeed, it is clear that the androgen receptor (AR) has optimal activity (30 to 100 fold hormone induction) in serum starved G0 cells. The receptor is active in cells that are arrested anywhere along S phase, showing good activity (10 to 50 fold hormone

induction). In these cells, the AR is active from the mouse mammary tumor virus promoter as well as from the natural androgen responsive probasin promoter (Fig. 1). However, it has been found and confirmed in several well-controlled experiments that the androgen receptor losses its activity in cells arrested at the G1/S boundary. Even at high hormone concentrations, no more than a three fold activation of the AR can be observed while cells undergoing the same treatment show a 10-20 fold activation of the glucocorticoid receptor under the same conditions (Fig 2). The effects of the antiandrogen cyproterone acetate (CA) were also studied through the cell cycle. CA acts as a relatively potent antiandrogen but at high concentrations it can acquire agonistic activity. CA did not acquire agonistic activity in any phase of the cycle beyond its basal effects. This discounts the possibility (at least in these cells) that this antiandrogen may have androgenic effects in rapidly growing cells that contain a high S phase fraction.

Estrogen receptor studies have also been continued. Serum starved cells consistently show 10-20 fold induction with nanomolar concentrations of hormone. This is the highest detected level of activity that we have seen. In these cells, the antiestrogen ICI 182-780 (ICI) inhibits 70-100% of the activity of the estrogen induced receptor. Cells growing in 3% or 10% serum, regardless of whether or not they were starved just prior to serum addition, show a relatively constant 5-7 fold induction of activity when estrogen treated. The decrease in the presence of serum may be due to steroid binding proteins present in the serum that lower the effective hormone concentration. However, cells growing in 3% serum that were deprived of glutamine gained activity over cells growing in the presence of glutamine, showing a clean 10 fold induction of ER activity. This suggests that the effect seen on steroid receptor activity in the presence of serum is not fully due to serum binding factors. Interestingly, also in the case of the ER, treatment with hydroxyurea after starvation (for G1/S arrest) obliterated all ER activity while the same drug treatment on asynchronous cells (to arrest cells along S phase) reduced estrogen activation to 3-4 fold over basal values. Hydroxyurea treatment of serum starved cells had only a slight effect on estrogen activity, with 7-9 fold activation of the receptor in the presence of the drug compared to 10 fold activation in its absence, showing that the drug itself does not have gross effects on receptor function. ICI showed its characteristic inhibitory effect on estrogen activity in all cases and did not gain agonistic activity in any cell cycle phase. The ER results are summarized in Fig. 3.

Furthermore, as proposed in the Statement of Work Aim1, C, levels of AR protein have been measured during the cell cycle. In the absence of hormone, receptor levels are low. Nanomolar dihydrotestosterone (dht) treatment clearly upregulates the receptor protein. This upregulation is seen in both G0 arrested and G1/S arrested cells, although the final receptor levels are consistently higher in G0 cells (Fig 4). Androgens are known to

regulate AR protein levels. Whether the lower receptor levels in the G1/S cells represent the cause of the reduced activity or its consequence is not yet clear. We favor the following interpretation: bound hormone stabilizes the receptor protein, thus in the presence of hormone receptor levels increase in all stages of the cell cycle to some extent. Yet a further increase in receptor protein requires a transcriptionally active AR: while this complex is present in the G0 cells, it is not functional in the G1/S cells. Thus the latter group does not reach optimal, self-induced receptor levels. ER protein levels have not yet been measured.

In Aim 1 task D, we proposed to measure the K_d and EC_{50} values of the ER and AR at different cell cycle stages if results from task C warranted this. Although detailed dose response curves at different cell cycle phases have not been performed, we deemed it necessary to investigate the possibility that the G1/S inactivity of the receptors would be caused by a transient decrease in affinity for hormone. We thus repeated the cell cycle arrests inducing the cells with 100- fold more hormone than in original experiments. The AR remained transcriptionally inactive in G1/S cells even in the presence of 100 nM androgens. The ER behaved similarly. Thus, we are quite confident that the G1/S effect is not simply the result of a small decrease in sensitivity for hormone. Rather, it is possible that cofactors that are necessary for receptor activity are unavailable or inactivated at the G1/S boundary.

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 levels two or three fold or not at all. Even these low activities 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. This makes the accomplishment of tasks C, D and E impossible as the receptors are not activate enough to make measurements possible. In addition, a further complication exists. As the AR expressing cells used in these studies also express endogenous glucocorticoid receptor (GR), and since both receptors can activate transcription from the MMTVLTR promoter that is stably integrated into these cells, it would be hard to know if a non-steroidal activator was acting through the AR or the GR. Thus, a new cell line was developed derived from E8.2 cells which do not express the GR but are otherwise identical to the parental line L929 (1). These E8.2 cells were stably transfected with two distinct probasin luciferase constructs: one containing a portion of the androgen responsive region of the probasin promoter, the other containing three copies of the minimum androgen responsive sequences. These cells lines respond to

androgens and not to glucocorticoids as expected thus making specificity studies possible (Fig 5). They offer the advantage of a natural androgen responsive promoter and an alternate reporter gene (luciferase instead of CAT). Preliminary studies on the effects of non-steroidal activators on these cells are currently underway, yet to date no good activation has been detected.

We are presently investigating whether the cells can be sensitized to respond better to the non-steroidal activators. It has been reported that treatment of cells with trichostatin A, a specific inhibitor of histone deacetylase, results in histone hyperacetylation and consequently chromatin relaxation (2). Under these conditions, transcriptional pathways should be more active than under condensed chromatin conditions. We therefore propose to test the effects of select non-steroidal ER and AR activators in cells pretreated with trichostatin A as well as the effects of antagonists under these conditions. If good activation is obtained in the presence of trichostatin A, tasks A-E can be re-evaluated; if, however, only poor activation or no activation of the receptors is obtained, we will focus on Aim 3 for the remainder of the grant period. Indeed, recent progress in the field has underscored the importance of Aim 3.

Aim 3: In the third Aim, we proposed to identify the cell cycle regulatory molecules involved in the control of estrogen and androgen receptor activity. Cell cycle regulators that affect steroid receptor action have recently been identified and include cyclin E, cyclin D1, the retinoblastoma protein (Rb) (3-7) and E2F (personal communication, Olga Rodriguez and Mark Danielsen). Preliminary work under tasks A and B of this Aim has began in collaboration with others in our laboratory. Under task A, we have measured the activity of the ER and the AR in two related cell lines: CV-1 and COS-7. CV-1 cell lines were originally established from the kidney of an African green monkey and contain no endogenous steroid receptors. COS-7 cells were derived from CV-1 cells by transformation with Large Tumor antigen (Tag) and express high levels of this oncogene. Tag is known to bind to and thus reduce available pools of proteins such as Rb. We therefore measured the activity of ER and AR in CV-1 cells (which contain high levels of available Rb) vs. COS-7 cells. We find that the activity of the AR on MMTV based promoters is high in CV-1 cells with only barely detectable activity in COS 7 cells (Fig 6). The ER, however, seems to be moderately active in COS cells. Whether transient coexpression of Rb or other proteins bound by Tag restores AR activity or increases ER activity has not been yet measured by us. Work underway towards task B, includes the development of a cell line that overexpresses the cell cycle regulatory transcription factor E2F. This cell line is derived from L929 mouse fibloblasts and is thus particularly well matched for our studies.

KEY RESEARCH ACCOMPLISHMENTS

- Three new cell lines have been developed from E8.2 cells and have been characterized for their level of androgen receptor activity and the receptor's transcriptional response to agonists and antagonists.
- Two cell lines overexpressing different levels of the E2F transcription factor and cell cycle regulator have been developed and are being characterized in collaboration with others.
- It has been established that the androgen receptor is active in L929 derived cell lines during the G0/G1 phase of the cell cycle from both MMTVLTR and probasin promoters.
- It has been established that the androgen receptor is active in cells progressing through S phase from both MMTVLTR and probasin promoters.
- It has been established that the androgen receptor lacks detectable activity in cells arrested at the G1/S boundary and that only very low activity is present in these cells even when exposed to hormone concentrations 100-1000 times greater than the EC50.
- It has been established that the estrogen receptor shows highest activity when the cells are treated by serum starvation and are mainly in G0/G1.
- It has been established that the estrogen receptor shows reduced activity in cells treated with S phase arresting drugs and no activity when treated for G1/S boundary arrest.
- It has been established that the androgen antagonist cyproterone acetateand the estrogen antagonist ICI 182-780 do not gain measurable agonistic activity during any tested phase of the cell cycle (G0, G1/S or S).
- 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.
- It has been established that Cd does not induce the estrogen or androgen receptors in asynchronously growing or G0 arrested L929 derived cell lines unless micromolar concentrations of the metal are used. This induction is low (2-3 fold) and not consistently seen.
- It has been established that forskolin, EGF, IGF-1 and KGF do not activate the androgen receptor in L929 derived cells asynchronously growing or arrested at G0.
- It has been shown that AR has poor activity from an MMTVLTR promoter in COS 7 cells which express high levels of the Rb binding oncogene Large T antigen.

REPORTABLE OUTCOMES

Abstracts and publications (Jan 2000 to date)

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.

List, H.J., Smith, C.L., **Martinez, E.**, Harris, V., Danielsen, M. and Riegel, A.T. (2000) Effects of anti-androgens on chromatin remodeling and transcription of the integrated mouse mammary tumor virus promoter. *Experimental Cell Research 260, 160-165.*

Martinez, E. and Danielsen, M. (2000) Androgen Receptor activation by an antiproliferative drug in the absence of androgens. 82nd Annual Meeting, The Endocrine Society, Toronto, Canada.

Martinez, E. and Danielsen, M. (2000) An antiproliferative drug activates the Androgen Receptor. Keystone Symposia on Nuclear Receptors, Steamboats, CO.

Development of cell lines

Five cell lines have been developed since July, 2000

E8.2-PBLUC: E8.2 cells stably expressing luciferase under the control of a minimal probasin promoter showing moderate-high androgen receptor activity.

E8.2-ARR3LUChi: E8.2 cells stably expressing luciferase under the control of three copies of the androgen responsive region of the probasin promoter showing high androgen receptor activity.

E8.2-ARR3LUClow: E8.2 cells stably expressing luciferase under the control of three copies of the androgen responsive region of the probasin promoter showing low androgen receptor activity

E8.2-E2F#9: E8.2 cells stably overexpressing hi levels of the E2F transcription factor (collaboration)

E8.2-E2F#20: 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 not obtained for lack of funds)

Presentations:

-Poster presentation 5/01: Androgen receptor transcriptional activity is regulated through the cell cycle in mouse fibroblasts. EMBO workshop on Nuclear Receptors: structure and function, Sicily, Italy.

-Oral presentation 10/00: Regulation of Androgen Receptor transcriptional activity. Data presentation series. Department of Biochemistry and Molecular Biology, Georgetown University School of Medicine, Washington, DC.

CONCLUSIONS

Through these studies we are gaining understanding of how estrogen and androgen receptors are regulated and how their deregulation may contribute to the onset of tumorigenesis and to the development of hormone independent growth. We have found that the activity of the androgen and estrogen receptors is indeed regulated throughout the cell cycle with no activity of the androgen and estrogen receptors in cells treated for arrest at the G1/S boundary and low activity of the estrogen receptor in cells which are mainly in S phase. Furthermore, our data indicate that non-steroidal activators of the receptors may act in a cell type and promoter dependent manner as we do not detect receptor activation by growth factors in our system. We will continue to analyze the regulation of receptor activity as outlined by the original proposal, taking into consideration recent developments in the field and the issues discussed in the body of the proposal.

References:

1. Housley, PR and Forsthoefel AM. (1989) Isolation and characterization of a mouse L cell variant deficient in glucocorticoid receptors. Biochem Biophys Res Commun 164 (1), 480-7.
2. Minucci, S et al. (1997) A histone deacetylase inhibitor potentiates retinoid receptor action in embryonal carcinoma cells. Proc Natl Acad Sc USA 94, 11295.
3. Knudsen, K et al. (1999) D-type cyclins complex with the androgen receptor and inhibit its transcriptional transactivation ability. Cancer Res 59 (10), 2297-2301.
4. Yamamoto, A et al. (2000) Cyclin E as a coactivator of the androgen receptor. J Cell Biol 150 (4), 873-880.
5. Lu, J and Danielsen, M. (1998). Differential regulation of androgen and glucocorticoid receptors by retinoblastoma protein. J Biol Chem 273 (47), 31528-31533.
6. Zwijsen, RM et al. (1997) CDK-independent activation of estrogen receptor by cyclin D1. Cell 88(3), 405-415.
7. Yeh, S et al. (1998). Retinoblastoma, a tumor suppressor, is a coactivator for the androgen receptor in human prostate cancer DU145 cells. Biochem Biophys Res Commun 248(2), 361-367.

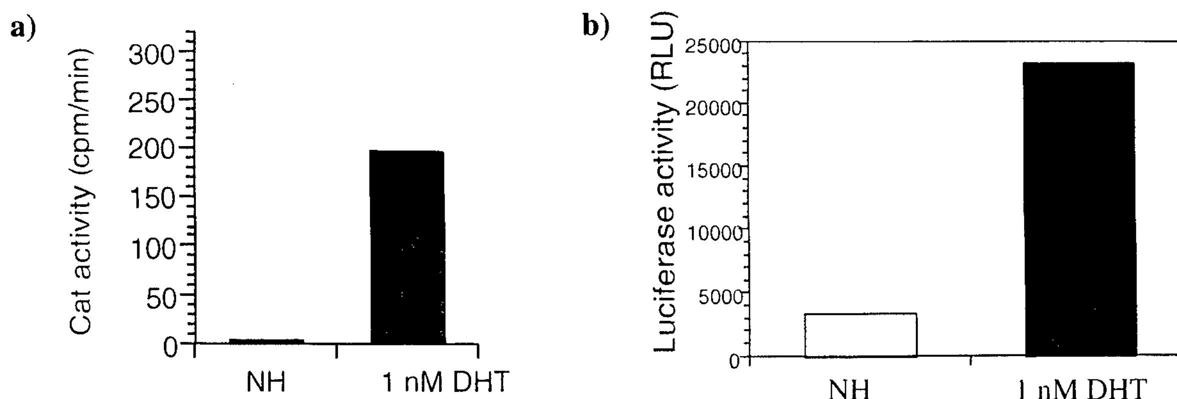


Figure 1: (a)L 929 cells stably transfected with pMMTVCAT were cultured in DMEM supplemented with 3% calf serum (CS). Cells were serum starved (grown in 0.1% CS) for 48 hrs to induce entry into G0. During an additional 24 hrs of starvation, cells were treated with 1 nM DHT or untreated. Cells were separated into three groups: aliquots for CAT assay were resuspended in Tris buffer; aliquots for FACS analysis were resuspended in citrate buffer and cells for western analysis (Fig 4) were lysed in modified RIPA buffer. All samples were stored frozen until analyzed. CAT activity was measured using ^{3}H Acetyl CoA. CAT activity is expressed as cpm/min. (b) L 929 cells stably transfected with a probasin-luciferase reporter construct (ARR3-Luc) were cultured in DMEM supplemented with 3% CS and treated as in (a). Cells were harvested for luciferase assays in Promega's cell culture lysis reagent. Luciferase activity was measured using a luminometer and luciferin substrate. Activity is expressed in relative light units.

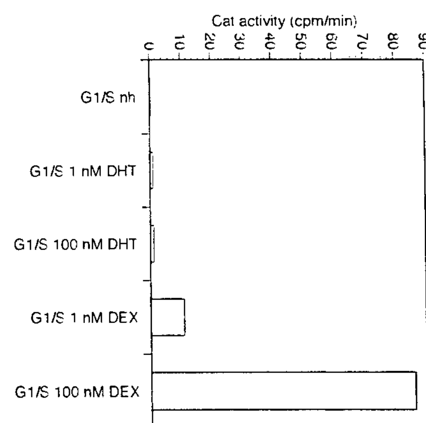


Figure 2: L 929 cells stably transfected with pMMTVCAT were cultured in DMEM supplemented with 3% calf serum (CS). Cells were serum starved (grown in 0.1% CS) for 48 hrs to induce entry into G0. After this starvation, cells were exposed to 1-2 mM hydroxyurea in 10% serum for arrest at the G1/S boundary. During an additional 24 hr exposure to hydroxyurea, cells were treated with 1 nM DHT, 100 nM DHT, 1 nM DEX, 100 nM DEX or untreated. Cells were harvested and assays carried out as in Fig. 1.

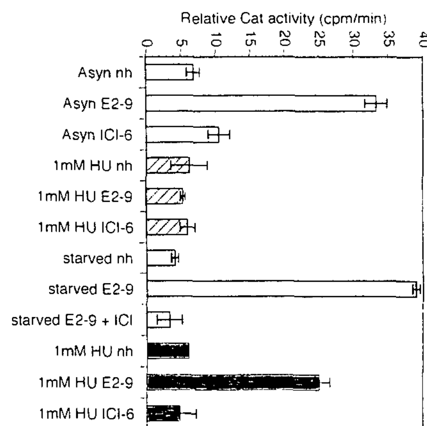


Figure 3: E 8.2 cells stably transfected with pMERECAT and expressing ER were cultured in DMEM supplemented with 3% calf serum (CS). *White bars*: asynchronous cells growing in 3% CS were induced with 1 nM estrogen (E2), 1 μM ICI or uniduced (nh). *Hatched bars*: Cells were serum starved (grown in 0.1% CS) for 48 hrs to induce entry into G0. After this starvation, cells were exposed to 1-2 mM hydroxyurea in 10% serum for arrest at the G1/S boundary. During an additional 24 hr exposure to hydroxyurea, cells were hormone treated as indicated. *Gray bars*: Cells were serum starved (grown in 0.1% CS) for 48 hrs to induce entry into G0. During an additional 24 hrs of starvation, cells were treated with 1 nM E2 +/- ICI or untreated. *Black bars*: cells growing in 3%CS were exposed to 1-2 mM hydroxyurea for 48 hrs. During an additional 24 hr exposure to hydroxyurea, cells were hormone treated as indicated. All cells were harvested in Tris buffer and assays carried out as in Fig. 1.

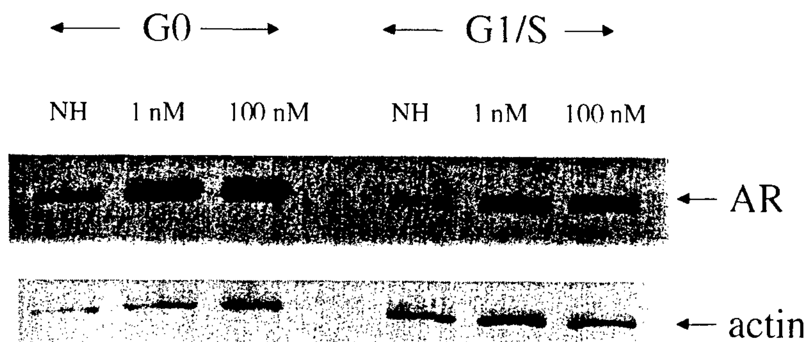


Figure 4: Aliquots of L929 cells from Figure 1 and 2 were harvested in modified RIPA buffer and subjected to Western analysis. Samples were diluted and protein quantified using the Bradford assay. Equal total protein was loaded in each lane. Membranes were blotted with PA1-111A, a polyclonal antibody that recognizes the N-terminus of the AR. AR immunoreactive bands were visualized using an anti-rabbit HRP-conjugated secondary antibody and Amersham's ECL reagents. Actin is shown as a loading control.

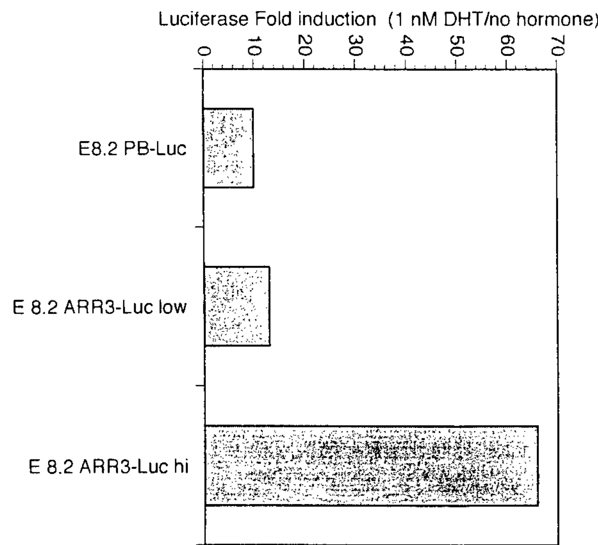
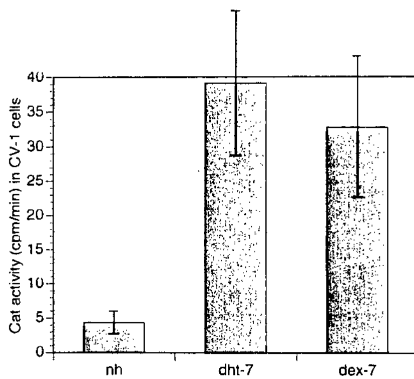


Figure 5: E8.2 cells were stably transfected with one of two probasin-luciferase constructs as described in "Reportable outcomes". Neomycin resistance was use as a selection marker. Three clones were analyzed that show ten fold or higher induction of luciferase activity when treated with 1 nM DHT. Luciferase assays were performed as in Fig. 1 b.

a)



b)

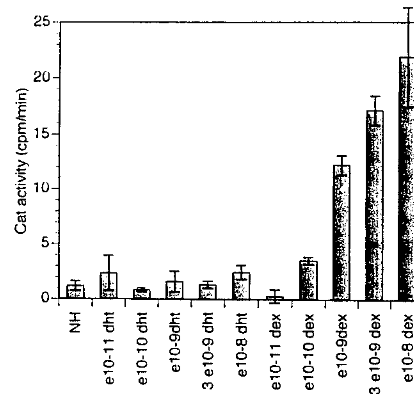


Figure 6: CV-1 cells (a) or COS-7 cells (b) were transiently transfected through liposomal mediation (Dosper reagent) with expression vectors for the AR, GR and a CAT reporter gene. Cells were induced with 100 nM DHT or DEX (a) or with increasing concentrations of hormone (b) for 24-48 hrs. Cells were harvested in Tris buffer and CAT activity measured as in Figure 1 a.

Androgen receptor transcriptional activity is regulated through the cell cycle in mouse fibroblasts

Elisabeth Martinez and Mark Danielsen
Department of Biochemistry and Molecular Biology
Georgetown University School of Medicine
Washington, DC USA

Androgens are necessary for the development, growth, and maintenance of sexual organs such as the prostate. This control of cell proliferation is progressively lost in the cancerous state leading to steroid-independent tumor growth in, for example, advanced prostate cancer. In an effort to understand how androgens regulate cell proliferation in normal and disease states, investigators have studied the effects of androgen treatment on cell cycle regulators in tissue culture models. They have found that upon androgen withdrawal, androgen dependent cells upregulate cdk inhibitors, inactivate cyclin/cdk complexes and arrest at G0. Recently, it has been reported that the androgen receptor (AR) itself interacts with the cell cycle machinery and that its transcriptional activity is affected by these regulatory factors. While transient overexpression of Rb or cyclin E enhances AR transcriptional activity, cyclin D1 overexpression inhibits this activity. Given the above, we became interested in investigating whether these effects are relevant in a normal cell cycle when regulators are not overexpressed.

To determine the net effect of cell cycle regulators on the AR, we measured receptor activity in asynchronous, G0, G1/S, and G2/M arrested L929 cells which endogenously express the AR. We found that the activity of the receptor is modulated throughout the cell cycle but the pattern shown cannot be predicted simply by what is known about the levels of expression or activation of the retinoblastoma protein or cyclins D and E. The AR consistently exhibits highest transcriptional activity in G0 or quiescent cells. There is a marked decreased in activity at the G1/S boundary but this activity is regained in mid and late S phase. To date, no activity has been detected in the G2/M phase of the cycle, paralleling what is known for the glucocorticoid receptor (GR). In contrast to the GR, however, only the AR loses activity at the G1/S boundary, with GR remaining at least moderately active. The modulation of AR activity throughout the cell cycle is independent of both the promoter and the reporter systems used. Additionally, the drugs used to induce cell cycle arrest do not, on their own, affect receptor activity nor receptor protein levels. To further understand the mechanisms of androgen action as well as the specificity of androgenic responses, we plan to measure the hormone binding and DNA binding properties of the AR at the G1/S boundary and to determine what type of regulatory event may be responsible for switching the receptor on and off.

This work was supported by a DOD predoctoral fellowship (DAMD17-99-1-9199) to EM.

Experimental Cell Research 260, 160–165 (2000)
doi:10.1006/excr.2000.5018, available online at http://www.idealibrary.com on IDEAL®

Effects of Antiandrogens on Chromatin Remodeling and Transcription of the Integrated Mouse Mammary Tumor Virus Promoter

Heinz-Joachim List,* Catharine L. Smith,† Elisabeth Martinez,‡ Violaine K. Harris,* Mark Danielsen,‡ and Anna T. Riegel*,[1]

*Departments of Oncology and ‡Biochemistry and Molecular Biology, Vincent T. Lombardi Cancer Center, Georgetown University, Washington, D.C. 20007; and †Laboratory of Receptor Biology and Gene Expression, National Institutes of Health, Bethesda, Maryland 20892

Inhibition of the ligand-activated androgen receptor (AR) by antiandrogens plays an important role in the treatment of various hyperandrogenic disorders including prostate cancer. However, the molecular mechanisms of antiandrogen activity *in vivo* remain unclear. In this study we analyzed the effects of cyproterone acetate (CPA), flutamide (F), and hydroxyflutamide (OHF) on transcriptional activation and chromatin remodeling of the genomically integrated mouse mammary tumor virus (MMTV) promoter. This promoter has provided an excellent model system to study the impact of steroid hormones on transcriptional activation in the context of a defined chromatin structure. The MMTV hormone response element is positioned on a phased nucleosome, which becomes remodeled in response to steroids. We utilized this model system in mouse L-cell fibroblasts that contain a stably integrated MMTV promoter. In these cells, dihydrotestosterone (DHT) induced a large increase of AR protein levels that correlated with transcriptional activation and chromatin remodeling of the MMTV promoter. Coadministration of DHT and CPA or DHT and OHF in these cells inhibited the increase of AR levels, which resulted in a strong blockage of transcriptional activation and chromatin remodeling of the MMTV promoter. In contrast, F had no significant influence on these activities. We conclude that a major portion of the antiandrogenic effects of CPA and OHF *in vivo* are mediated by the reduction of AR levels.
© 2000 Academic Press

Key Words: **androgen receptor; cyproterone acetate; hydroxyflutamide; flutamide; chromatin; transcription; MMTV promoter.**

INTRODUCTION

Androgens play an important role in male development and the maintenance of the male sexual phenotype. The cellular response to androgens is mediated through the androgen receptor (AR), which belongs to a large group of intracellular receptors, the nuclear receptor superfamily [1, 2]. The AR regulates the expression of target genes by binding to a hormone response element (HRE) located in the enhancer or promoter of a target gene after binding to androgen agonists such as testosterone or its reduced metabolite dihydrotestosterone (DHT). Conversely, synthetic androgen antagonists such as cyproterone acetate (CPA), flutamide (F), or hydroxyflutamide (OHF), some of which are used for the treatment of hyperandrogenic disorders, can block AR-mediated pathways. The effects of these antagonists on transcriptional activation mediated by the androgen receptor has been intensively studied utilizing the transiently transfected mouse mammary tumor virus (MMTV) promoter as a model system [3–5]. However, the exact mechanisms by which antiandrogens exert their inhibitory effect on the AR in the context of chromatin has not been identified.

A useful model system to study the interaction of the AR with a promoter in the context of chromatin has been the activation of the integrated MMTV long terminal repeat (LTR) by steroid hormones [6, 7]. The HRE, which is responsible for the transcriptional response to androgens, glucocorticoids, progestins, and mineralocorticoids, is located on nucleosome B of a phased array of nucleosomes positioned along the LTR [8, 9]. During glucocorticoid stimulation, the region encompassing nucleosome B becomes DNase I hypersensitive and accessible to restriction enzymes, indicating changes in the chromatin structure of this promoter [8, 10, 11]. This remodeling event is accompanied by recruitment of transcription factors such as NF1 and Oct 1 to the MMTV promoter downstream of the HRE [12, 13]. Interestingly, although the glucocorticoid receptor (GR) and the AR can efficiently stimulate these events, the progesterone receptor (PR) and mineralocorticoid receptor (MR) are substantially less effective [14–17], leading to the idea that restriction of HRE function by chromatin structure might play a role in steroid-specific responses.

[1] To whom correspondence and reprint requests should be addressed at Department of Oncology, Vincent T. Lombardi Cancer Center, Research Building, E307, Georgetown University, 3970 Reservoir Road, Washington, DC 20007. Fax: (202) 687-4821.

0014-4827/00 $35.00
Copyright © 2000 by Academic Press
All rights of reproduction in any form reserved.

We have previously determined in mouse L-cell fibroblasts that a large portion of the induction of the integrated MMTV promoter is indirect via androgen-induced increases in AR levels [18]. Induction of AR levels also explains some of the synergistic increase in androgen-induced MMTV promoter activity in the presence of the histone deacetylase inhibitor trichostatin A (TSA) [19]. In the present study, we elucidate at which point during androgen induction of MMTV transcription antiandrogens exert their effects. Our results indicate that antiandrogen inhibition of androgen-induced changes in chromatin remodeling and transcription from the MMTV promoter can be exerted by preventing increases in AR protein levels.

MATERIALS AND METHODS

Cell lines. The cell line 29+ was derived from mouse L-cell fibroblasts, which had been selected for spontaneous resistance to glucocorticoids [20]. These parental cells contain no GR, PR, or MR [20] but express the endogenous AR [21]. The plasmid pMMTV-CAT was stably integrated into these cells by cotransfection with pSV2 NEO and selection with G418 (400 μg/ml) (GIBCO/BRL), resulting in the cell line 29+. This cell line harbors approximately 20 copies of the MMTV-CAT integrant [21]. The cells were cultivated in IMEM supplemented with 1% charcoal-stripped FCS, 2 mM glutamine, 1% penicillin/streptomycin, and 400 μg/ml G418. All hormone treatments were carried out in charcoal-stripped serum.

Hormone inductions and CAT assay. For the hormone induction experiments, the cells were treated for 24 h with various concentrations of CPA, F, and OHF in the presence or absence of DHT. Cells were then washed twice with PBS, resuspended in 0.1 M Tris–HCl, pH 7.8, and lysed by three freeze–thaw cycles and the transcriptional activity was measured by CAT assay. The CAT assay was performed by the method of Neumann *et al.* [22]. In short, 50 μl of the protein solution (40 μg) was added to 200 μl of reaction buffer (1.25 mM chloramphenicol, 100 mM Tris–HCl, pH 7.8, 0.125 mM acetyl coenzyme A, 35 pmol [acetyl-^{3}H]coenzyme A (0.1 mCi/ml, DuPont NEN)). The reaction mix was overlayed with 2 ml scintillation fluid (Econofluor-2, DuPont) and radioactivity in the scintillant was determined after 4 h when production rate of the acetylation product was linear.

RNA analysis. 29+ cells were treated with 10 nM DHT and/or various concentrations of CPA, F, and OHF for 24 h. Total cellular RNA was isolated as described previously [16] and subjected to S1 nuclease assay [16]. The CAT-specific probe used in this assay was generated by multiple rounds of Taq polymerase extension from an antisense CAT-oligonucleotide (5′ TCCAGTGATTTTTTTCTCCAT 3′) using *Sst*I-digested pMMTV-CAT [23] as a template. The actin-specific probe and the actin template have been described previously [16]. Extension was carried out in the presence of [α-^{32}P]dATP in a Hybaid Omnigene apparatus for 30 cycles. The full-length extension product was gel purified. After hybridization with RNA samples, S1 nuclease digestion was performed for 1 h at room temperature. Digestion products were separated on an 8% denaturing urea gel that was exposed to Phosphorimager screens. Quantification was carried out using ImageQuant software (Molecular Dynamics).

Analysis of chromatin structure by in vivo restriction enzyme hypersensitivity. The *in vivo* restriction enzyme hypersensitivity assay was performed as described previously [18]. In brief, cells were treated with 10 nM DHT in the presence or absence of 10 μM CPA, 1 μM F, or 1 μM OHF for 24 h. After harvesting the cells, the nuclei were purified, resuspended in 50 μl digestion mix (200 U *Sst*I per 1 × 10^8 cells, in 10 mM bis tris propane, pH 7, 10 mM $MgCl_2$, 1 mM DTT) and incubated for 15 min at 30°C. After proteinase K treatment of the incubation mix the genomic DNA was purified by extraction with phenol/chloroform. The isolated DNA was subsequently analyzed by linear amplification (30 cycles) of the purified DNA with Taq polymerase and a specific primer for the MMTV promoter (position +64 to +81). The genomic DNA (10 μg) was incubated with 5 U Taq polymerase (Boehringer), 1 ng ^{32}P end-labeled primer, and 0.2 mM each of dATP, dCTP, dGTP, and dTTP. The PCR reaction products were purified by extraction with phenol/chloroform, precipitated with ethanol, separated on a 8% polyacrylamide denaturing gel, and analyzed by autoradiography.

Western blot analysis. For the AR Western blots, 29+ cells were treated with 10 nM DHT and/or various concentrations of CPA, F, and OHF for 24 h. At the end of treatment, the cells were washed twice with PBS and harvested with a cell scraper and whole cell lysates were prepared as described previously [18]. Fifty micrograms of protein was electrophoresed on a 7.2% SDS–polyacrylamide gel and then transferred to a nitrocellulose membrane. The membrane was incubated for 16 h at room temperature with 5% nonfat milk in PBST (PBS/0.05% Tween 20) followed by washing once for 15 min and twice for 5 min with PBST. The membrane was then incubated for 1 h at room temperature with primary antibody against the AR (CUS 280, Schering AG), washed as described above, and incubated for 1 h with a secondary antibody–peroxidase conjugate (2000-fold dilution in PBST). After washing, the membranes were incubated for 1 min with ECL detection solution (Amersham) and then exposed to film. Quantification was carried out using densitometry.

RESULTS

Inhibition of Androgen-Induced Transcription of the Integrated MMTV Promoter by Cyproterone Acetate, Flutamide, and Hydroxyflutamide in 29+ Cells

In order to elucidate the effects of CPA, F, and OHF on androgen-mediated transcription in the context of chromatin, we analyzed their effects on transcriptional activation and chromatin remodeling on the integrated MMTV promoter in 29+ L-cell fibroblasts.

First, we established whether these drugs are able to inhibit androgen receptor-mediated activation of the integrated MMTV promoter. We induced these cells with 100 nM DHT for 24 h in the presence or absence of 10 μM CPA, 1 μM F, or 1 μM OHF and measured CAT activity as described under Materials and Methods. As Fig. 1 shows, we observed a six- to sevenfold increase of CAT activity after DHT induction compared to nontreated cells. Induction of the cells with DHT in the presence of CPA or OHF resulted in a 70 or 50% reduction in CAT activity, respectively (Fig. 1). Cotreatment of the 29+ cells with F showed no significant inhibition of DHT-induced CAT activity (see Fig. 1), consistent with its lack of activity in transient assays [3]. We concluded from these data that CPA and OHF are able to inhibit DHT-induced transcription of the integrated MMTV promoter in a manner similar to the inhibition of androgen-induced CAT transcription of the transiently transfected MMTV promoter as shown previously [3, 4].

Surprisingly, when we treated 29+ cells with 10 μM CPA, 1 μM F, or 1 μM OHF in the absence of DHT, no significant increase of CAT activity was observed. Previous studies with these compounds utilizing a tran-

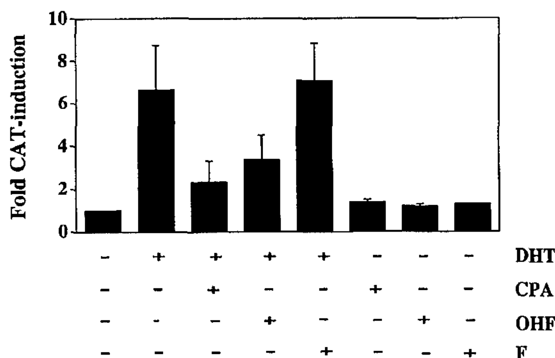


FIG. 1. Inhibition of androgen-induced CAT transcription of the integrated MMTV promoter by cyproterone acetate, flutamide, or hydroxyflutamide. 29+ cells were induced for 24 h with 10 μM CPA, 1 μM F, or 1 μM OHF in the presence or absence of 100 nM DHT and harvested. CAT assays were performed on cell lysates as described under Materials and Methods. Results are representative of three independent experiments.

sient transfected MMTV promoter showed partial androgen receptor agonist activity of CPA and OHF [3, 4]. These differences might be due to a different threshold for CAT activation from the transiently transfected and stably integrated templates or caused by the different cell types used. Since the CAT response is dependent on both CAT mRNA and protein accumulation, we also determined CAT mRNA changes as a more accurate measure of transcriptional activity at the MMTV promoter. CAT mRNA levels were quantitated using a S1 nuclease protection assay after induction of the 29+ cells with 100 nM DHT for 24 h in the presence or absence of 10 μM CPA, 1 μM F, or 1 μM OHF. The repression of the DHT-induced CAT mRNA levels by CPA and OHF was 60–80%, which is similar to the repression we observed when measuring CAT transcription (Fig. 2 compared to Fig. 1). In cells treated with antagonist alone, we detected CAT mRNA levels that were comparable to the CAT mRNA levels in cells without hormone treatment.

Effects of Androgen Antagonists on MMTV Chromatin Remodeling

Next we addressed the question whether the blockage of androgen-induced transcription by androgen antagonists is accompanied by changes in the chromatin structure of the HRE. One possibility is that the inhibition of transcription by CPA or OHF was caused by the inability of the antagonist-bound AR to open chromatin. Another possibility is that the antagonist/AR complex was able to bind to DNA and open chromatin but was not able to activate transcription. To identify the mechanism by which CPA, F, and OHF mediate their antagonist effects, we tested whether these drugs modulate DHT-induced chromatin remodeling of the MMTV promoter. We used a restriction enzyme hypersensitivity assay that we have described previously [18] (outlined in Fig. 3A). This primer extension assay measures the enhanced accessibility of DNA to *Sst*I in the nucleosome B region of the MMTV promoter after hormone treatment (Fig. 3A). We induced 29+ cells for 24 h with 10 nM DHT in the presence or absence of 10 μM CPA, 1 μM F, or 1 μM OHF and measured chromatin remodeling as described under Materials and Methods. We observed a fourfold increase in chromatin remodeling after DHT induction that was reduced to basal levels after cotreatment with CPA or OHF (Figs. 3B and 3C). Consistent with its lack of effect on CAT mRNA transcription, F was not able to inhibit chromatin remodeling. The inhibition of chromatin remodeling by CPA and OHF are consistent with the effects on transcription observed above (Figs. 1 and 2).

Effects of Antiandrogens on AR Levels

In previous studies we showed that the degree of transcriptional activation and chromatin remodeling of the integrated MMTV promoter is correlated with hormone receptor levels [18]. In addition, effects of a histone deacetylase inhibitor (trichostatin A) on transcrip-

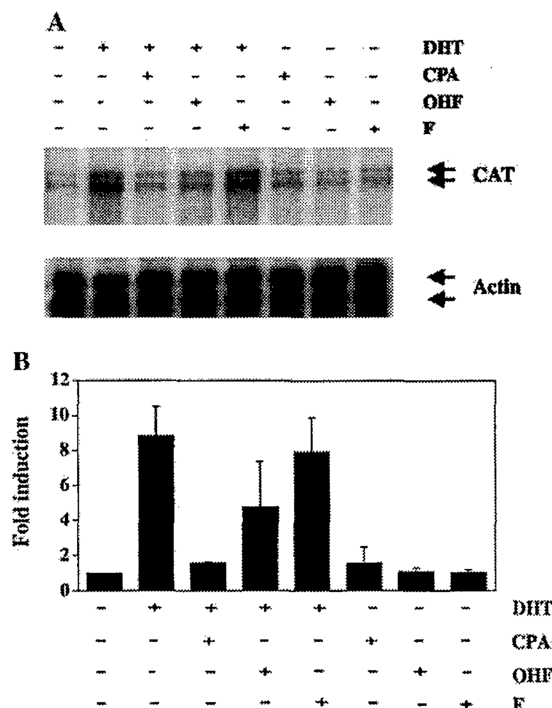


FIG. 2. CAT RNA analysis from 29+ cells. (A) Cells were induced for 24 h with 10 μM CPA, 1 μM F, or 1 μM OHF in the presence or absence of 100 nM DHT. After cell harvest, total RNA was isolated and subjected to S1 nuclease analysis as described under Materials and Methods. (B) Quantification of the S1 analysis. Levels of CAT mRNA were normalized to those of actin mRNA for each sample. Fold inductions for normalized CAT mRNA were calculated relative to the untreated control sample. The mean ±SD from two independent experiments is shown.

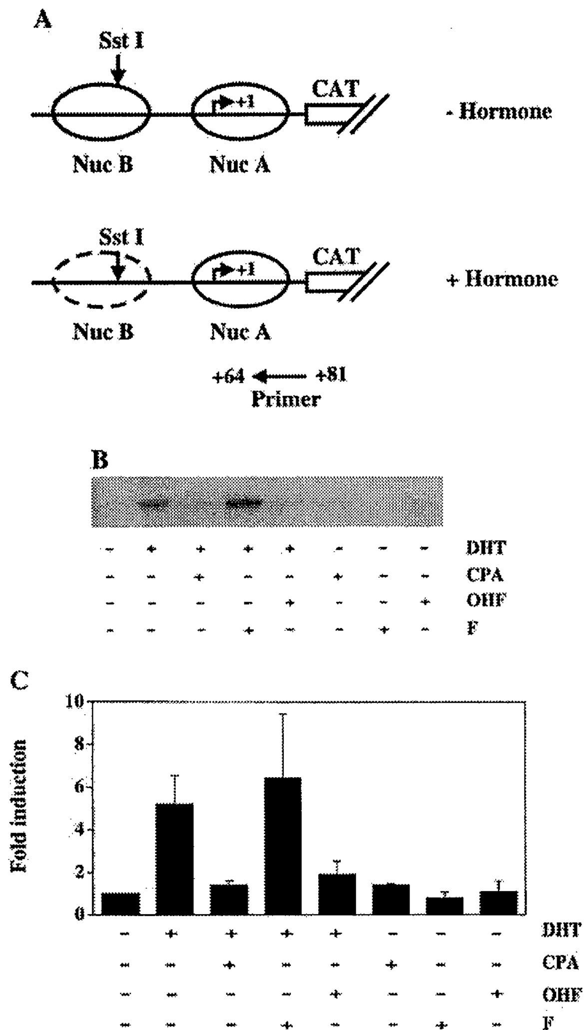


FIG. 3. Chromatin remodeling of the MMTV promoter by the AR. (A) Diagram of the proximal part of the MMTV promoter, indicating the relative positions of nucleosomes A and B [8]. The HREs are all positioned within nucleosome B, which is remodeled and becomes hypersensitive to restriction enzyme (*Sst*I) cleavage during androgen treatment. The cleavage site of the restriction enzyme *Sst*I in nucleosome B is indicated as is the position of the primer used to generate the 190-bp extension product in the *in vivo* restriction enzyme hypersensitivity assay. (B) Chromatin remodeling of the MMTV promoter by the AR in the cell line 29+. Cells were induced for 24 h with 10 μM CPA, 1 μM F, or 1 μM OHF in the presence or absence of 10 nM DHT. Nuclei were isolated and *Sst*I access to the MMTV promoter was determined as described under Materials and Methods. (C) Quantification of the *in vivo* restriction enzyme hypersensitivity assay. The data represent the ratio of the intensity of the primer extension product determined by densitometry for hormone- and/or hormone antagonist-treated cells versus untreated cells at each time point. The mean ±SD from two independent experiments is shown.

tion and chromatin remodeling also correlated well with changes in AR levels in mouse L-cell fibroblasts [19]. We speculated, therefore, that one major mechanism through which CPA and OHF act on MMTV promoter transcription and chromatin remodeling is by changing AR levels. To test this hypothesis, we induced 29+ cells with DHT alone or in combination with CPA, F, or OHF for 24 h and subsequently measured AR levels by Western blot analysis. We found that CPA and OHF inhibited DHT-mediated elevation of AR levels while treatment of the cells with these antagonists alone did not change AR levels significantly. While F alone did not change AR levels it inhibited slightly DHT-mediated elevation, although to a much smaller extent than CPA or OHF (Figs. 4A and 4B). This reduction of AR levels is probably not sufficient to block DHT-induced effects on MMTV transcription and chromatin remodeling as seen above.

Time Course of Antiandrogen Effects on the MMTV Promoter

It has been shown previously [15, 18] that other steroids such as glucocorticoids can cause a rapid, transient increase in MMTV transcription and chromatin remodeling with effects returning to basal levels after 24 h of hormone treatment. It was therefore possible that antiandrogens had similar rapid effects on MMTV at time points prior to 24 h, as shown in Figs. 2–4. We therefore examined AR protein levels as well as transcriptional and chromatin effects in the presence or absence of antiandrogens at earlier time points. After 1 h of androgen or antiandrogen treatment we saw no change in CAT mRNA, chromatin remodeling, or AR levels relative to the control (Figs. 5A–5C; open bars). This was not due to inactive compounds since cells treated in parallel for 24 h were fully responsive to androgens and antiandrogens (Figs. 5A–5C; solid bars). The earliest time point at which we have detected an increase in AR levels after DHT treatment was after 3–6 h and at this time point we saw the same pattern of responses to antiandrogens and androgens as we did at 24 h (not shown). We conclude therefore that at no time point during the antiandrogen treatment did these compounds mimic DHT effects and that their major mechanism of androgen antagonism in these cells is by inhibiting androgen-induced increases in AR levels.

DISCUSSION

In the present study we have determined that antiandrogens can effectively inhibit DHT induction of androgenic responses in mouse L-cell fibroblasts. We have previously observed that a major component of the androgen induced increase in MMTV transcription is due to DHT induction of AR levels, presumably through increased expression of a gene involved in androgen receptor gene expression [18]. In the present study we show that induction of this gene is effectively inhibited by antiandrogens so that the amount of AR in the cells remains very low in the presence of DHT.

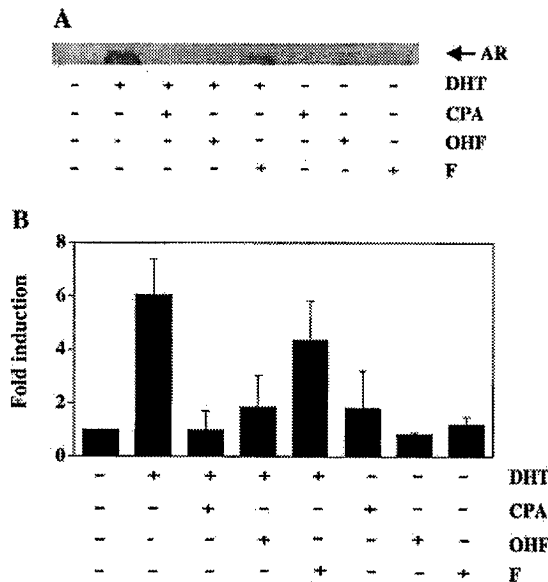


FIG. 4. Changes in the AR protein levels in 29+ cells after treatment with cyproterone acetate, flutamide, or hydroxyflutamide in the presence or absence of DHT. (A) Western blot analysis of the AR from whole cell extracts at 24 h after treatment with 10 μM CPA, 1 μM F, or 1 μM OHF in the presence or absence of 100 nM DHT. (B) Quantification of the AR levels from the Western blots in (A). The mean ±SD from two independent experiments is shown.

Clearly the antiandrogens effectively prevent the increase in AR levels, which is a prerequisite for a robust androgenic response from the MMTV promoter. This suggests that antiandrogens not only inhibit androgen-induced responses directly by antagonizing HRE containing promoters but also indirectly by reducing cellular levels of available AR.

A question that follows from this is whether the androgen antagonist receptor complex directly interacts with the HRE in the context of chromatin. In this regard it has been shown that the antagonist-bound AR can bind DNA *in vitro* and activate transcription from a transiently transfected template [3, 4]. This would suggest that the antiandrogen receptor complex can bind directly to the HRE in the context of chromatin but is unable to recruit the necessary cofactors to accomplish chromatin remodeling or transcription. In this sense antiandrogens used in this study would be analogous to type II glucocorticoid receptor antagonists that form a complex with the GR, bind to the HRE, but are unable to remodel chromatin or induce transcription [24]. In contrast the RU486-bound PR, which is able to bind to DNA and maintain an open chromatin structure, is still not able to activate transcription [25]. The different ability of various steroid hormone receptor antagonist complexes to remodel chromatin or to activate transcription is probably related to their ability to bind different coactivators. For example, it has been shown that CPA or OHF promote the interaction between the AR and its coactivator ARA70 and enhance AR transcriptional activity in cotransfection experiments with AR and ARA 70 or ARA 55 in DU145 cells [26, 27] but not with the AR coactivator ARA 54 [28]. It will be interesting to determine which of the general or androgen-specific cofactors are involved in mediating antiandrogen and androgen effects on chromatin and transcription *in vivo.*

Finally, although inhibitory cross-talk between steroid antagonist and agonist receptor complexes has been described [29], this would be limited in the case of antiandrogens since the absolute amounts of antiandrogen receptor complex in the cell would be maintained at very low levels. However, inhibitory cross-talk may come into play in cells in which the endogenous AR levels are constitutively high.

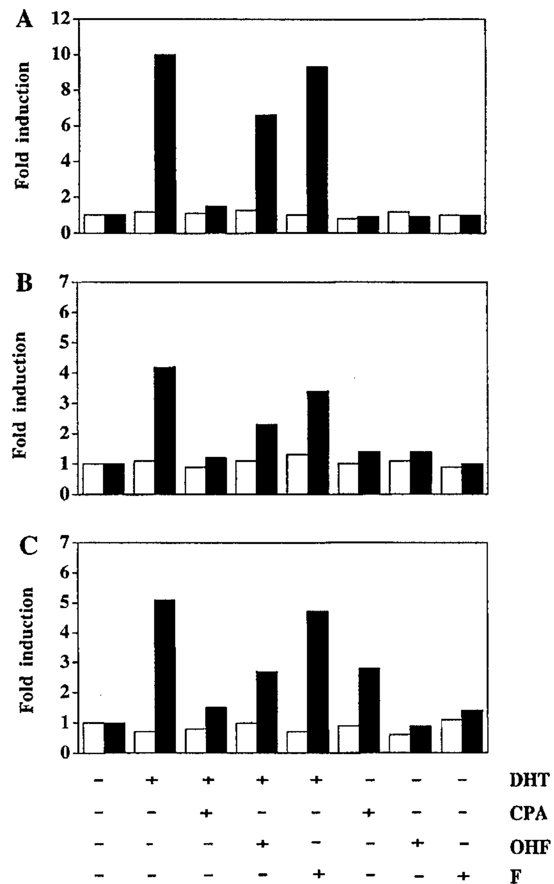


FIG. 5. Comparison of antiandrogen effects in 29+ cells after treatment with cyproterone acetate, flutamide, or hydroxyflutamide in the presence or absence of DHT at different time points. Cells were treated as indicated for 1 h (open bars) or 24 h (solid bars) with antiandrogens and DHT. Concentrations of DHT or antiandrogens were identical to those in previous experiments, as described in the legends to Figs. 2–4. Cells were analyzed for changes in (A) CAT mRNA measured as described in the legend to Fig. 2; (B) chromatin remodeling as described in the legend to Fig. 3; or (C) AR levels as described in the legend to Fig. 4.

We are extremely grateful to Dr. Susan Chrysogelos for reading the manuscript. We also thank Dr. Paul Housley for his generous gift of cell lines for this study. We also thank Dr. Anton Wellstein for helpful discussions. This work was supported in part by grants from the NIH and ACS.

REFERENCES

1. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesoso, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1997). The nuclear receptor superfamily: The second decade. *Cell* **83,** 835–839.
2. Perlmann, T., and Evans, R. M. (1997). Nuclear receptors in sicily: All in the famiglia. *Cell* **90,** 391–397.
3. Kemppainen, J. A., Lane, M. V., Sar, M., and Wilson, E. M. (1992). Androgen receptor phosphorylation, turnover, nuclear transport, and transcriptional activation. *J. Biol. Chem.* **267,** 968–974.
4. Wong, C.-I., Kelce, W. R., Sar, M., and Wilson, E. M. (1995). Androgen receptor antagonist versus agonist activities of the fungicide vinclozolin relative to hydroxyflutamide. *J. Biol. Chem.* **270,** 19998–20003.
5. Kemppainen, J. A., Langley, E., Wong, C.-I., Bobseine, K., Kelce, W. R., and Wilson, E. M. (1999). Distinguishing androgen receptor agonists and antagonists: Distinct mechanisms of activation by medroxyprogesterone acetate and dihydrotestosterone. *Mol. Endocrinol.* **13,** 440–454.
6. Archer, T. K., and Mymryk, J. S. (1995). Modulation of transcription factor access and activity at the MMTV promoter *in vivo. In* "The Nucleus" (A. P. Wolf, Ed.), Vol. 1, pp. 123–150. JAI Press, London.
7. Smith, C. L., and Hager, G. L. (1997). Transcriptional regulation of mammalian genes *in vivo. J. Biol. Chem.* **272,** 27493–27496.
8. Richard-Foy, H., and Hager, G. L. (1987). Sequence specific positioning of nucleosomes over the steroid-inducible MMTV promoter. *EMBO J.* **6,** 2321–2328.
9. Fragoso, G., John, S., Roberts, M. S., and Hager, G. L. (1995). Nucleosome positioning on the MMTV LTR results from the frequency-biased occupancy of multiple frames. *Genes Dev.* **9,** 1933–1947.
10. Zaret, K. S., and Yamamoto, K. R. (1984). Reversible and persistent changes in chromatin structure accompany activation of a glucocorticoid-dependent enhancer element. *Cell* **38,** 29–38.
11. Truss, M., Bartsch, J., Schelbert, A., Hache, R. J. G., and Beato, M. (1995). Hormone induces binding of receptors and transcription factors to a rearranged nucleosome on the MMTV promoter *in vivo. EMBO J.* **14,** 1737–1751.
12. Cordingley, M. G., Riegel, A. T., and Hager, G. L. (1987). Steroid-dependent interaction of transcription factors with the inducible promoter of mouse mammary tumor virus *in vivo. Cell* **48,** 261–270.
13. Lee, H.-K., and Archer, T. K. (1994). Nucleosome-mediated disruption of transcription factor–chromatin initiation complexes at the mouse mammary tumor virus long terminal repeat *in vivo. Mol. Cell. Biol.* **14,** 32–41.
14. Archer, T. K., Zaniewski, E., Moyer, M. L., and Nordeen, S. K. (1994). The differential capacity of glucocorticoids and progestins to alter chromatin structure and induce gene expression in human breast cancer cells. *Mol. Endocrinol.* **8,** 1154–1162.
15. Archer, T. K., Huay-Leng, L., Cordingley, M. G., Mymrck, J. S., Fragoso, G., Berard, D. S., and Hager, G. L. (1994). Differential steroid hormone induction of transcription from the mouse mammary tumor virus promoter. *Mol. Endocrinol.* **8,** 568–576.
16. Smith, C. L., Htun, H., Wolford, R. G., and Hager, G. L. (1997). Differential activity of progesterone and glucocorticoid receptors on MMTV templates differing in chromatin structure. *J. Biol. Chem.* **272,** 14227–14235.
17. Bonovich, M. T., List, H-J., Zhang, S., Danielsen, M., and Riegel, A. T. (1998). Identification of glucocorticoid receptor domanins necessary for transcriptional activation of the mouse mammary tumor virus promoter integrated in genome. *Exp. Cell Res.* **239,** 454–462.
18. List, H.-J., Lozano, C., Lu, J., Danielsen, M., Wellstein, A., and Riegel, A. T. (1999). Comparison of chromatin remodeling and transcriptional activation of the mouse mammary tumor virus promoter by the androgen and glucocorticoid receptor. *Exp. Cell Res.* **250,** 414–422.
19. List, H-J., Smith, C. L., Rodriguez, O., Danielsen, M., and Riegel, A. T. (1999). Inhibition of histone deacetylation augments dihydrotestosterone induction of androgen receptor levels: An explanation of trichostatin A effects on androgen induced chromatin remodeling and transcription of the mouse mammary tumor virus promoter. *Exp. Cell Res.* **252,** 471–478.
20. Housley, P. R., and Forsthoefel, A. M. (1989). Isolation and characterization of a mouse L cell variant deficient in glucocorticoid receptors. *Biochem. Biophys. Res. Commun.* **164,** 480–487.
21. Bonovich, M. T. (1996). "Comparison of the Effects of Glucocorticoid and Mineralocorticoid Receptors on the Mouse Mammary Tumor Virus Long Terminal Repeat Indicates Domains Necessary for Transcriptional Activation of an Integrated Promoter." Ph.D. thesis, Georgetown University.
22. Neumann, J. R., Morency, C. A., and Russian, K. O. (1987). A novel rapid assay for chloramphenicol acetytransferase gene expression. *Biotechniques* **5,** 444–447.
23. Danielsen, M., Northrop, J. P., Jonklaas, J., and Ringold, G. M. (1987). Domains of the glucocorticoid receptor involved in specific and nonspecific deoxyribonucleic acid binding, hormone activation, and transcriptional enhancement. *Mol. Endocrinol.* **1,** 816–822.
24. Mymryk, J. S., and Archer, T. K. (1995). Influence of hormone antagonists on chromatin remodeling and transcription factor binding to the mouse mammary tumor virus promoter *in vivo. Mol. Endocrinol.* **9,** 1825–1834.
25. Mymryk, J. S., and Archer, T. K. (1995). Dissection of progesterone receptor-mediated chromatin remodeling and transcriptional activation *in vivo. Genes Dev.* **9,** 1366–1376.
26. Miyamoto, H., Yeh, S., Wilding, G., and Chang, C. (1998). Promotion of agonist activity of antiandrogens by the androgen receptor coactivator, ARA70, in human prostate cancer DU145 cells. *Proc. Natl. Acad. Sci. USA* **95,** 7379–7384.
27. Fujimoto, N., Yeh, S., Kang, H.-Y., Inui, S., Chang, H.-C., Mizokami, A., and Chang, C. (1999). Cloning and characterization of androgen receptor coactivator, ARA55, in human prostate. *J. Biol. Chem.* **274,** 8316–8321.
28. Kang, H.-Y., Yeh, S., Fujimoto, N., and Chang, C. (1999). Cloning and characterization of human prostate coactivator ARA54, a novel protein that associates with the androgen receptor. *J. Biol. Chem.* **274,** 8570–8576.
29. Fryer, C. J., Nordeen, S. K., and Archer, T. K. (1998). Antiprogestins mediate differential effects on glucocorticoid receptor remodeling of chromatin structure. *J. Biol. Chem.* **273,** 1175–1183.

Received January 25, 2000
Revised version received June 15, 2000

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 18 Apr 03

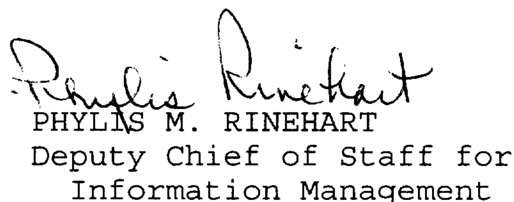
MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:



Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

ADB285846
ADB255323
ADB246514
ADB233740
ADB286395
ADB275662
ADB285859
ADB272667
ADB259790