

## Modulation of Androgen Receptor Transcriptional Activity by the Estrogen Receptor

M. VIJAY KUMAR,\* MARK E. LEO,† AND DONALD J. TINDALL\*†‡

From the \*Departments of Urology Research, †Urology, and ‡Biochemistry/Molecular Biology, Mayo Foundation, Rochester, Minnesota.

**ABSTRACT:** Estrogen/androgen receptor interactions in naturally occurring physiological systems and the effect of their respective steroid hormones on transcriptional activity remain undefined. In an attempt to delineate further the nature of the interaction between these two steroid hormone receptors we have examined the effect of cotransfection of androgen (AR) and estrogen receptor (ER) cDNAs on the expression of the mouse mammary tumor virus long terminal repeat region (MMTV-LTR) linked to the chloramphenicol-acetyltransferase (CAT) reporter gene. In QT6 cells, which contain neither AR nor ER, cotransfection of AR cDNA with the MMTV-LTR-CAT reporter, resulted in transactivation only in the presence of dihydrotestosterone (DHT). Treatment with  $10^{-8}$  M each of estradiol-17 $\beta$  ( $E_2$ ), dexamethasone, or progesterone did not enhance CAT activity, whereas treatment with the androgens DHT and mibolerone resulted in 87% and 89% CAT activity. Transfection of increasing concentra-

tions of ER cDNA in the presence of 100 ng of AR cDNA and  $10^{-8}$  M each of DHT and  $E_2$  showed a dose-dependent decrease in CAT activity as compared to the response with DHT alone. Cotransfection of AR and ER cDNA in the presence of  $10^{-8}$  M DHT and increasing concentrations of  $E_2$  resulted in a dose-dependent decrease in CAT activity. When cells were treated with increasing concentrations of DHT with  $10^{-8}$  M  $E_2$  no significant increase in CAT activity was observed. In GC cells, which contain endogenous ER but no AR, cotransfection of AR cDNA and treatment with  $E_2$  and DHT, also reduced DHT-induced CAT activity. Thus, we conclude that the  $E_2$ /ER complex is capable of inhibiting transcriptional activity of the AR.

Key words: Androgen receptor, estrogen receptor, modulation, transcription, QT6 cells, GC cells.

J Androl 1994;15:534-542

Androgen and estrogen receptors are found simultaneously in multiple, naturally occurring organ systems and tissues. The interaction between these receptors and their effect on one another may be responsible for a variety of physiological effects including normal development and pathological change. However, the precise interaction between these two receptors at the molecular level has been largely undefined.

One interesting system involves the interaction between androgen receptor (AR) and estrogen receptor (ER) in the pathogenesis of the clinical entity of benign prostatic hyperplasia. Though it is widely believed that estrogen interaction in this androgenic environment plays a role, it remains unclear as to how alterations in this hormonal environment are responsible for the pathogenesis of the disease (Walsh and Wilson, 1976; Coffey and Walsh, 1990). Another physiological system is the sensitivity of mouse mammary gland development to androgen concentration and the possible role of estradiol interfering with this

response by acting through the ER (Wasner et al., 1983).

Previous studies have shown that expression of one steroid hormone receptor can result in the modulation of steroid-dependent transcriptional activity of another steroid hormone receptor in the same cell line (Meyer et al, 1989; Bansal and Latchman, 1990). This observation has led to the concept of the "interfering receptor" in steroid hormone systems.

The AR is a member of the steroid receptor family, which when bound by either testosterone (T) or dihydrotestosterone (DHT) is capable of regulating protein synthesis in target cells containing androgen-responsive genes. The mouse AR has been cloned (He et al, 1990) and shown to contain two highly conserved regions (Beato, 1989; Wahli and Martinez, 1991), which correspond to the DNA- and steroid-binding domains. The structure of the DNA-binding domain is a unique arrangement of tetrahedrally coordinated zinc ions commonly referred to as zinc fingers. These zinc finger motifs have been shown to be very highly conserved regions across all members of the steroid hormone receptor family, including the estrogen, glucocorticoid, progesterone, and androgen receptors (O'Malley, 1990; Wahli and Martinez, 1991). It has been shown that substitution of the ER DNA-binding domain with that of the glucocorticoid receptor resulted in a "hybrid receptor," which was capable of activating expression of a glucocorticoid response gene and not of an estrogen-

Supported by NIH grants CA 32387, DK 47592, and HD 09140 (to D.J.T.) and a grant from the American Foundation for Urologic Diseases in cooperation with The Connaught Foundation (to M.V.K.). The first two authors (M.V.K. and M.E.L.) contributed equally to this project.

Received for publication January 26, 1994; accepted for publication July 21, 1994.

responsive gene (Green and Chambon, 1987; Green et al, 1988).

In an attempt to characterize the nature of the interaction between AR and ER, we have examined the effect of cotransfection of AR and ER cDNAs and treatment with steroid hormones on the transcriptional activation of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) chloramphenicol-acetyltransferase (CAT) reporter.

## Materials and Methods

### Materials

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, chick serum, penicillin-streptomycin, and fungizone were obtained from Gibco-BRL. DHT was obtained from Steraloids Inc. ICI 164,384 was a gift from Dr. A. E. Wakeling of the ICI Pharmaceuticals, UK. Estradiol-17 $\beta$  (E<sub>2</sub>) and other laboratory reagents were from Sigma Chemical Company. Endogenous steroids were removed from serum by treating with dextran-coated charcoal (He et al, 1991). The construct ARE-tk-CAT, a gift from Dr. M. Tsai, consists of four copies of ARE/PRE/GRE (ARE) cloned upstream of thymidine kinase (tk) promoter. The construct ERE-tk-CAT consists of one copy of ERE cloned upstream of a thymidine kinase promoter in pBLCAT2 vector. This was a gift from Dr. G. Greene. The mouse AR cDNA was cloned in pcDNA1 Neo (In Vitrogen) as described elsewhere (He et al, 1990). The human ER cDNA was a gift from Dr. P. Chambon (Green and Chambon, 1987; Green et al, 1988).

### Cell Culture and Transfection

**QT6 Cells**—Quail fibrosarcoma cells (QT6) were obtained from Dr. Nita Maihle at the Mayo Clinic/Foundation. They are a chemically induced quail fibrosarcoma cell line (Moscovici et al, 1977) and were used because they contain no endogenous AR or ER. The cells were maintained in DMEM, which contained 6% glucose, 5% fetal calf serum, 1% chicken serum (BRL-Gibco), 200 units/ml of penicillin-streptomycin sulfate, and 1  $\mu$ g/ml of fungizone. Cells were grown in 175-cm<sup>2</sup> flasks in a Forma Scientific incubator at 37°C with 5% CO<sub>2</sub>.

Cells were transfected with DNA using the calcium-phosphate technique (Graham and van der Eb, 1973). In brief, the cells at 30–40% confluency were washed and fed with fresh medium and were transfected after 6 hours. Individual DNA samples containing 5  $\mu$ g of MMTV-LTR-CAT (Denison et al, 1989), ARE-tk-CAT, ERE-tk-CAT, 1  $\mu$ g RSV- $\beta$ -galactosidase (Lindzey et al, 1993; Grossmann et al, 1994), and mouse AR cDNA and/or human ER cDNA were mixed together. The total DNA concentration was brought to 15  $\mu$ g per tube using pBluescript vector DNA (Stratagene). Thirty microliters of 2 M calcium chloride and distilled water were added to bring the final volume to a total of 245  $\mu$ l per tube. A 2 $\times$  HBPS buffer solution was made by mixing 2.5 ml of 10 mM HEPES buffer and 90 mM sodium chloride at a pH of 7.1 with 100  $\mu$ l of 70 mM sodium phosphate buffer, pH 7.1. Each DNA sample was then added to 255  $\mu$ l of the 2 $\times$  HBPS solution with constant mixing. After 30 minutes of precipitation, the DNA was layered onto the cells. Cells were

then incubated with the DNA at 37°C in the presence of 5% CO<sub>2</sub>. After 18 hours, the medium was removed, fresh medium was added and treated with appropriate hormones. Cells were grown for 48 hours prior to harvesting.

Cells were washed with 10 ml of phosphate-buffered saline (PBS), and 1 ml of harvest buffer (40 mM Tris-HCl, pH 7.8, 1 mM EDTA, and 150 mM sodium chloride) was added to each plate and allowed to stand for 5 minutes. The cells were detached using a cell lifter (Fisher Scientific), placed in 1.8-ml microfuge tubes on ice, and then centrifuged briefly, resuspended in 150  $\mu$ l of 100 mM Tris-HCl, pH 7.8, containing 0.1% Triton X-100. After 15 minutes, the cells were centrifuged for 10 minutes and the supernatant was assayed for protein (Bradford, 1976),  $\beta$ -galactosidase activity (Rosenthal, 1987), and CAT (Gormon et al, 1982; Neumann et al, 1987) activity.

**GC Cells**—GC cells were derived from rat pituitary glands (Ye et al, 1988) and were provided by Dr. Norman Eberhardt, Mayo Clinic/Foundation. They were used because they are known to contain endogenous ER (Gershengorn et al, 1979). They were grown in DMEM in the presence of 5% calf serum and 5% bovine serum. The cells were harvested using trypsin-EDTA, and 6  $\times$  10<sup>6</sup> cells were seeded into T-175 flasks. After 3 days the cells were washed with PBS and supplemented with DMEM containing 4% stripped fetal bovine serum. The cells were further grown for 3 days with change of medium every day. On day 6 the cells were harvested, washed twice with the medium, and 40  $\times$  10<sup>6</sup> cells per electroporation were resuspended in 400  $\mu$ l of 0.1% glucose in PBS containing 15  $\mu$ g of the reporter vector DNA, 3  $\mu$ g  $\beta$ -galactosidase DNA, and 250 ng cDNA. Resuspended cells were electroporated at 960  $\mu$ FD and 0.35 kV in a Bio-Rad gene pulser. After 7 minutes on ice, cells were resuspended in 1 ml of 4% stripped fetal bovine serum in PBS and incubated at room temperature for 7 minutes. The cells were added to 42 ml of medium, gently mixed, and plated in four 100-mm petri dishes. Dishes were treated with hormones and incubated for 48 hours before harvest.

The cells were washed with PBS and stripped from plates by repeated pipetting of PBS containing 2 mM EDTA. The cells were collected by brief centrifugation in the cold room and were lysed by the addition of 500  $\mu$ l 100 mM Tris-HCl, pH 7.8, containing 0.1% Triton X-100.

### $\beta$ -Galactosidase Activity

$\beta$ -Galactosidase was used as an internal control to determine the efficiency of transfection (Rosenthal, 1987). Cell extract was added to 3  $\mu$ l of 100 $\times$  magnesium chloride (100 mM MgCl<sub>2</sub> and 4.5 M  $\beta$ -mercaptoethanol), 50  $\mu$ l of *O*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) 4 mg/ml, and 0.1 M sodium phosphate, pH 7.0, up to a volume of 300  $\mu$ l and then incubated at 37°C. The enzyme reaction was stopped by the addition of 500  $\mu$ l 1 M sodium carbonate. The optical density at 420 nm was measured in a Beckman DU-64 spectrophotometer.

### CAT Assays

CAT activity was assayed using a thin-layer chromatography (TLC) method (Gormon et al, 1982) or a liquid phase extraction assay (Neumann et al, 1987). For the TLC technique cell extracts were added to 20  $\mu$ l of 40 mM acetyl-CoA, 1  $\mu$ l of [<sup>14</sup>C]-chlor-

amphenicol, and 70  $\mu$ l 1 M Tris-HCl, pH 7.8, and adjusted to a final volume of 150  $\mu$ l with distilled water, incubated at 37°C for 4 hours and then extracted using 1 ml of ethyl acetate. Ethyl acetate was evaporated to dryness and the pellet was resuspended in 30  $\mu$ l of ethyl acetate. Aliquots (10  $\mu$ l) were subjected to TLC on 20-cm  $\times$  %wcm silica gel TLC plates (EM Industries) in a mixture of chloroform/methanol (95:5) mixture for 2 hours. Subsequently, the products were visualized by autoradiography by exposure to X-Omat film (Kodak) for 18 hours using an image intensifier at -70°C. The percent acetylated form was determined by analysis of each plate using the AMBIS spectrophotometer (Automated Microbiology Systems Inc., San Diego) and was expressed as acetylated counts per minute (cpm)/total cpm for each individual lane of the plate.

For the liquid phase extraction method, cell extracts were brought to a volume of 250  $\mu$ l with 100 mM Tris-HCl at pH 7.8 in small scintillation vials. To that mixture, a solution containing 205  $\mu$ l of a premix of 1.25 mM chloramphenicol and 100 mM Tris-HCl, pH 7.8, and 0.1  $\mu$ Ci [<sup>14</sup>C]-acetyl CoA was added. The contents were mixed and then overlaid with 3 ml of Econofluor cocktail (National Diagnostics Inc.). Samples were then counted at timed intervals in a Beckman scintillation counter.

## Results

### *Determination of Optimal AR cDNA Concentration for Inducing Transcriptional Activity*

In order to establish a concentration of AR cDNA that would produce a maximum CAT response, increasing amounts of the AR cDNA (10 ng, 50 ng, 75 ng, 100 ng, 150 ng, and 200 ng) were cotransfected with the MMTV-LTR-CAT construct into QT6 cells, and CAT activity was measured. A dose-dependent increase in androgen-induced MMTV-LTR-CAT activity was observed between 10 ng and 75 ng AR cDNA, reaching a plateau between 100 ng and 150 ng (79% and 77% conversion, respectively). A further increase in AR cDNA (200 ng) resulted in a decrease in CAT activity (55%) (Fig. 1). Cells that were transfected with only the MMTV-LTR-CAT exhibited basal activity (10%). These results demonstrated a remarkable sensitivity of the system to the amount of AR cDNA transfected.

### *Steroid Specificity of the MMTV-LTR-CAT Construct*

Previous studies have shown that the MMTV-LTR sequence contains response elements capable of binding different steroid hormone-receptor complexes including glucocorticoid, progesterone, and androgen (Cato et al, 1987). For the purposes of this study, it was important to determine whether the response of the MMTV-LTR-CAT construct is specific to androgens when the AR cDNA is present.

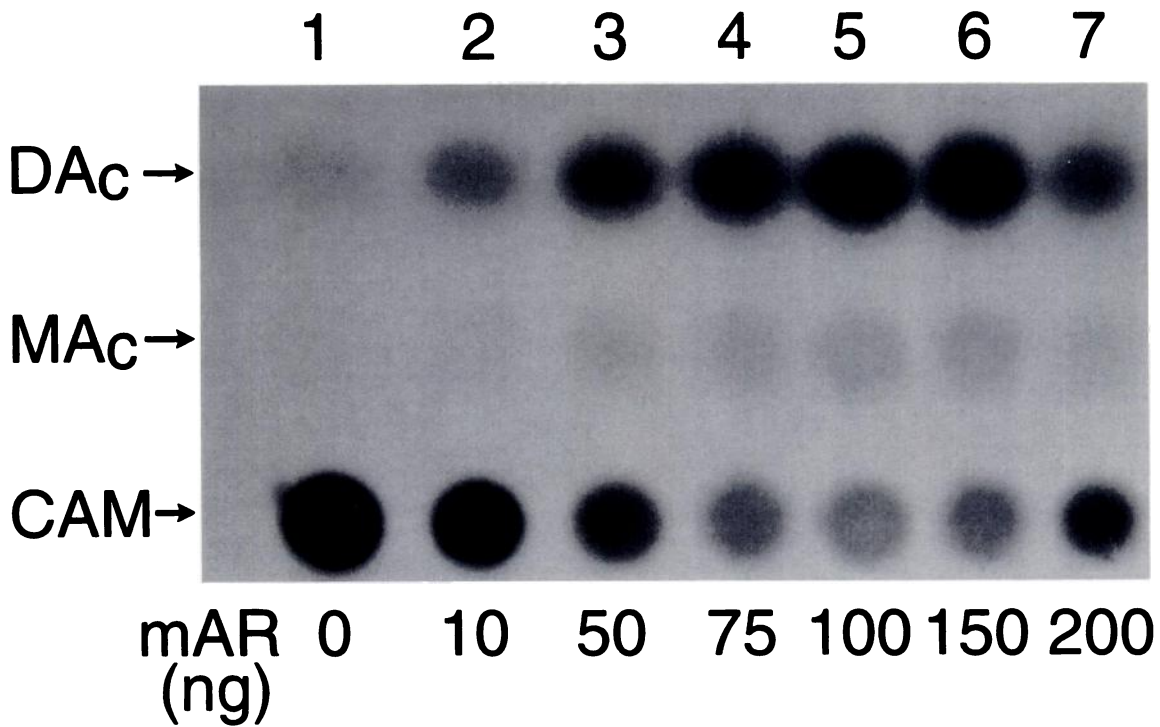
The AR cDNA (100 ng) was cotransfected with the MMTV-LTR-CAT reporter construct into QT6 cells, and

the cells were treated with equimolar ( $10^{-8}$  M) concentrations of E<sub>2</sub>, progesterone (P), dexamethasone (DEX), DHT, and the synthetic androgen mibolerone (MIB) (Fig. 2). Treatment with the androgens DHT and MIB resulted in 87% and 89% increase in CAT activity, whereas treatment with E<sub>2</sub>, P, and DEX resulted in CAT activity similar to that of control cells (20% conversion), which were treated with ethanol alone (Fig. 2). The results demonstrate that the reporter system used in these experiments is specific to androgens and that E<sub>2</sub> at the concentrations used had no effect on the AR itself.

### *Effect of ER cDNA on Androgen-Induced CAT Activity*

In order to determine whether the ER could modulate DHT/AR-induced transcription activity, increasing concentrations of ER cDNA (50 ng/plate to 1,000 ng/plate) were cotransfected with AR cDNA at a concentration of 100 ng/plate, along with the MMTV-LTR-CAT construct. Cells were then treated with either DHT ( $10^{-8}$  M) alone or DHT and E<sub>2</sub> ( $10^{-8}$  M each) together. The results showed that the DHT-induced CAT activity was reduced in a dose-dependent manner in the presence of E<sub>2</sub> and ER (Fig. 3A). Inhibition of AR-induced transcription activity ranged from 43% with 50 ng ER cDNA to 87% with 1  $\mu$ g ER cDNA. However, when the cells were treated with DHT and E<sub>2</sub> in the absence of transfected ER cDNA, the CAT activity was similar to cells treated with DHT alone, suggesting that the decrease in CAT activity in the presence of E<sub>2</sub> is mediated through the ER. Moreover, treatment of the cells with E<sub>2</sub> alone (data not shown) did not induce CAT activity over a wide range of ER cDNA concentrations (50 ng/plate to 1,000 ng/plate), demonstrating that the E<sub>2</sub>/ER alone cannot activate transcription of the MMTV-LTR.

Controls in this experiment (not shown) included the MMTV-LTR-CAT construct alone, the MMTV-LTR-CAT construct with ER cDNA, and the MMTV-LTR-CAT construct in the presence of AR cDNA, and cells were treated with ethanol,  $10^{-8}$  M E<sub>2</sub>,  $10^{-8}$  M DHT, or  $10^{-8}$  M E<sub>2</sub> and  $10^{-8}$  M DHT together. Transfection of MMTV-LTR-CAT alone resulted in basal CAT activity with all treatments, confirming that endogenous AR is not present in QT6 cells. When ER cDNA was cotransfected with MMTV-LTR-CAT and treated with  $10^{-8}$  M E<sub>2</sub>,  $10^{-8}$  M DHT, or  $10^{-8}$  M E<sub>2</sub> and  $10^{-8}$  M DHT, the CAT activities were similar to that of the ethanol-treated controls, further confirming that ER does not bind to MMTV-LTR-CAT cDNA and induce transactivation. When the AR was cotransfected with the MMTV-LTR-CAT construct, there was a significant increase in CAT activity in the presence of DHT but not with E<sub>2</sub>, indicating that E<sub>2</sub> cannot activate AR transcriptional activity at the concentration used. This concept was further supported by the observation that combined treatment of the cells

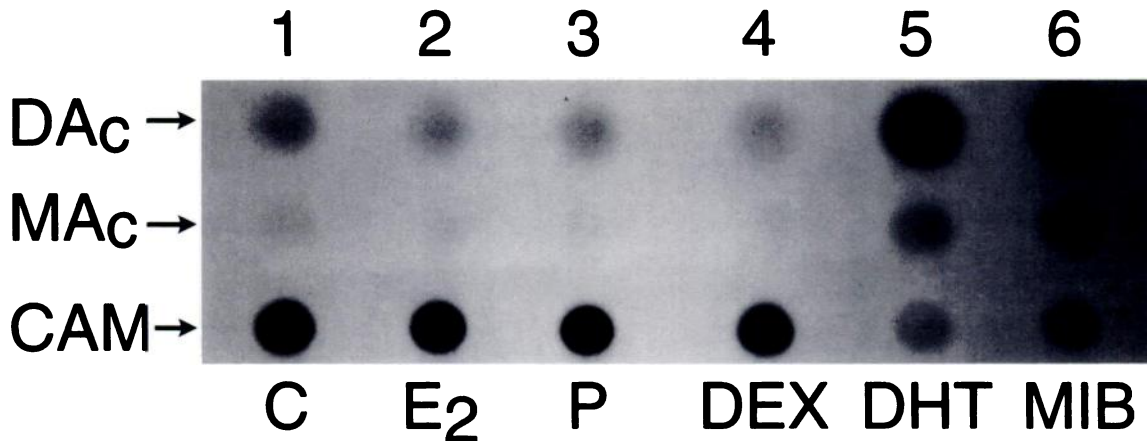


**FIG. 1.** Effect of increasing concentrations of AR cDNA on MMTV-LTR-CAT activity in the presence of androgens. The MMTV-LTR-CAT construct was cotransfected with increasing concentrations of AR cDNA. The control (lane 1) was not transfected with AR cDNA, whereas lanes 2–7 consisted of 10 ng, 50 ng, 75 ng, 100 ng, 150 ng, and 200 ng of AR cDNA. All cells were treated with 10 nM DHT. The figure depicts [<sup>14</sup>C]-chloramphenicol as spots at the bottom of the thin-layer chromatogram with its acetylated forms above. In this assay more of the chloramphenicol is acetylated with increasing CAT activity (Gorman et al, 1982).  $\beta$ -Galactosidase was used as internal control. CAM denotes chloramphenicol, MAc is monoacetyl chloramphenicol, and DAc is diacetylchloramphenicol.

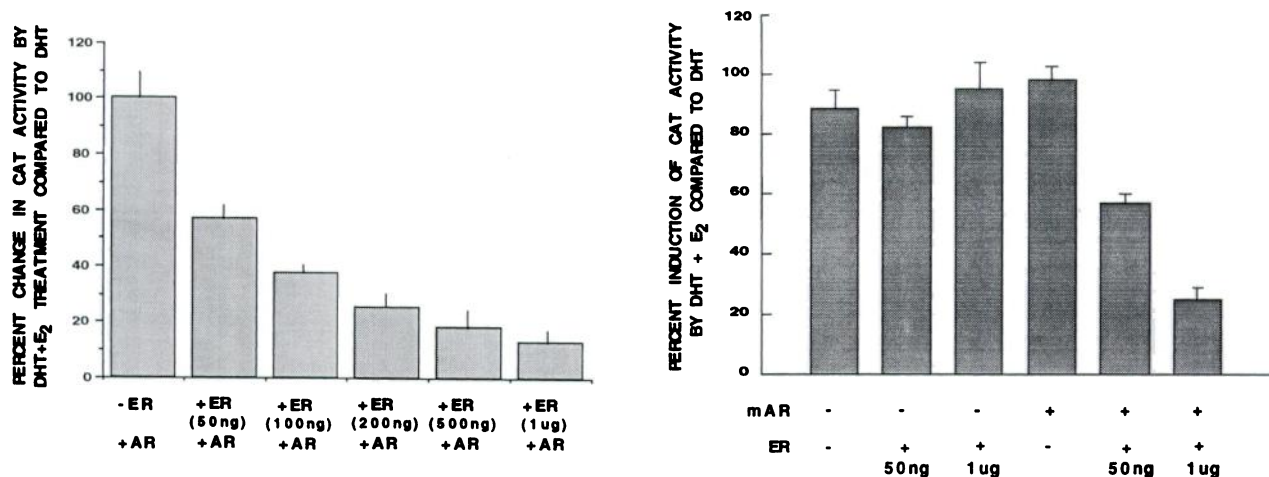
with DHT and E<sub>2</sub> did not change the CAT activity compared to DHT-treated cells.

The MMTV-LTR is a complex region with several response elements. Therefore, in order to determine that the effects seen with MMTV-LTR are indeed due to the

binding of AR to the androgen response element (ARE), an ARE-tk-CAT construct, with four copies of ARE cloned upstream of the thymidine kinase (tk) promoter, was used in transient transfections. The results support those obtained with MMTV-LTR-CAT (compare Fig. 3A and B).



**FIG. 2.** Specificity of androgenic induction of MMTV-LTR-CAT activity. QT6 cells were cotransfected with MMTV-LTR-CAT and AR cDNA and treated with ethanol (C), and 10<sup>-8</sup> M each of estradiol-17 $\beta$  (E<sub>2</sub>), progesterone (P), dexamethasone (DEX), dihydrotestosterone (DHT), and mibolerone (MIB). The figure shows a thin-layer chromatogram of CAT activity as described in the legend to Figure 1.  $\beta$ -Galactosidase was used as internal control. CAM denotes chloramphenicol, MAc is monoacetyl chloramphenicol, and DAc is diacetylchloramphenicol.



**FIG. 3.** Cotransfection of MMTV-LTR-CAT or ARE-tk-CAT with AR cDNA and ER cDNA. (Left) QT6 cells were transfected with 100 ng of AR cDNA alone, or AR cDNA (100 ng) and increasing concentrations (50 ng, 100 ng, 200 ng, 500 ng, and 1,000 ng) of ER cDNA. The MMTV-LTR-CAT construct was transfected as a reporter. The cells were treated with either DHT ( $10^{-8}$  M) alone or DHT and  $E_2$  ( $10^{-8}$  M each) together. The results are expressed as percent change in CAT activity by treatment of the cells with  $10^{-8}$  M each of DHT and  $E_2$  compared to  $10^{-8}$  M DHT alone. CAT activity was measured using the liquid phase extraction method (Neumann et al, 1987) as described in the Materials and Methods. Results are expressed as mean  $\pm$  standard error. The experiments were repeated three times with at least three to four plates per treatment per experiment. (Right) QT6 cells were transfected with AR cDNA (100 ng) or ER cDNA (50 ng or 1  $\mu$ g) either individually or together. An ARE-tk-CAT construct was transfected as a reporter. The cells were treated and processed as described above. Results are expressed as the mean  $\pm$  standard error. The experiments were repeated two times with at least three to four plates per treatment per experiment.

Treatment of cells, cotransfected with ARE-tk-CAT and AR cDNA, with  $E_2$  did not affect the DHT-induced CAT activity. However, when 50 ng/plate of ER cDNA was cotransfected with ARE-tk-CAT and AR cDNA, treatment with DHT and  $E_2$  resulted in a 40% decrease in CAT activity. With an increase in the concentration of transfected ER cDNA to 1  $\mu$ g/plate, the CAT activity decreased to 25% when the cells were treated with DHT and  $E_2$  (Fig. 3B). The negative controls in this experiment included transfection of ARE-tk-CAT alone or in combination with ER cDNA.

In order to determine whether these effects are steroid dependent, equal concentrations of AR and ER cDNA expression vectors (100 ng each) were cotransfected with the MMTV-LTR-CAT construct into QT6 cells, and cells were treated with varying concentrations of either DHT or  $E_2$  (Fig. 4). In one experiment, the cells were treated with a constant concentration of  $10^{-8}$  M DHT with concurrent increasing concentrations of  $E_2$ , ranging from  $10^{-11}$  M to  $10^{-7}$  M  $E_2$ , whereas control cells were treated with DHT alone. The results showed an increase in CAT activity when treated with  $10^{-11}$  M  $E_2$  compared to DHT treatment alone, whereas a dose-dependent decrease in CAT activity was observed between  $10^{-10}$  M and  $10^{-7}$  M  $E_2$  (Fig. 4A). These results suggest that the ER has the capability of interfering with androgen-induced transcriptional activity and physiological concentrations of  $E_2$  are sufficient to affect this change. The increase in CAT activity at the low dose ( $10^{-11}$  M) of  $E_2$  is perplexing and it may be the result of a decreased metabolism of DHT,

since estrogens are known to affect metabolism of androgens (Tindall et al, 1981).

In a parallel experiment, a second set of cells was treated with a constant concentration of  $10^{-8}$  M  $E_2$  in the presence of increasing concentrations of DHT, ranging from  $10^{-11}$  M to  $10^{-7}$  M DHT with control plates containing only  $E_2$ . No significant changes in CAT activity were observed with any of the treatment groups (Fig. 4B).

#### *Effect of AR on ER-Induced Activation of ERE-tk-CAT Construct*

In order to determine whether the decrease in transcriptional activity of AR cDNA in the presence of ER cDNA was due to squelching effects, an ERE-tk-CAT construct was cotransfected with ER and AR cDNA. The results indicate that  $E_2$  treatment of cells transfected with ER cDNA (50 ng or 1  $\mu$ g) resulted in a three- to sixfold induction of CAT activity compared to ethanol-treated controls (Fig. 5). Cotransfection of AR and ER cDNA did not affect the CAT activity, which was similar to the ER cDNA transfected group. When mAR cDNA was cotransfected with the ERE-tk-CAT and treated with either  $E_2$  or DHT, the CAT activity was similar to control. These experiments suggest that the decrease in transcriptional activity of AR may not be due to the squelching of transcriptional factors.

In order to determine further the specificity of the decrease in transcriptional activity of AR in the presence of ER and  $E_2$ , QT6 cells were transfected with ARE-tk-CAT, AR cDNA, ER cDNA, and treated with ethanol, DHT,

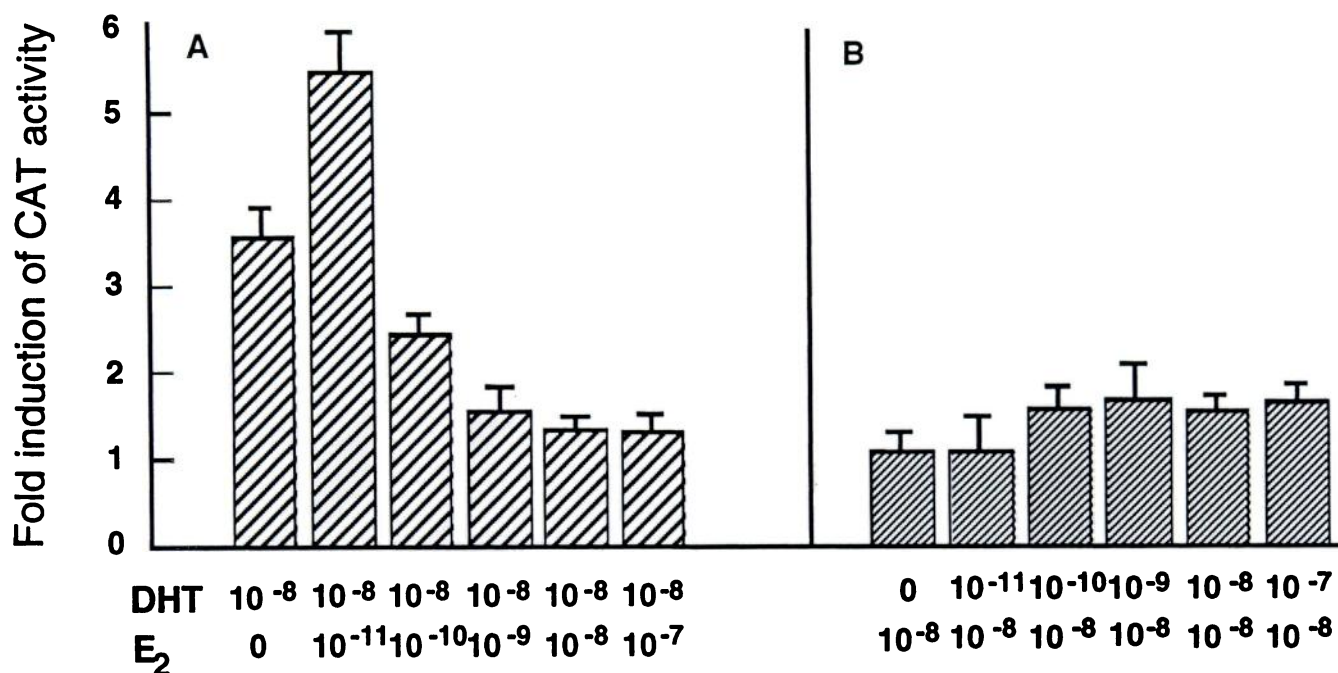


FIG. 4. Transcriptional regulation of MMTV-LTR-CAT cotransfection of AR and ER cDNA. (A) QT6 cells were transfected with 100 ng each of AR and ER cDNA. One group of cells was treated with increasing concentrations of E<sub>2</sub> (0–10<sup>-7</sup> M) in the presence of DHT (10<sup>-8</sup> M). (B) Another set of cells was treated with E<sub>2</sub> (10<sup>-8</sup> M) and increasing concentrations of DHT (0–10<sup>-7</sup> M). CAT activity was measured as described in the legend to Figure 3. Results are expressed as the mean ± standard error. The experiments were repeated three times with at least three to four plates per treatment per experiment.

E<sub>2</sub>, or the antiestrogen ICI 164,384. The results show that in the absence of transfected ER, treatment of the cells with E<sub>2</sub> and antiestrogen did not affect DHT-induced CAT activity. However, when ER cDNA was added, the treatment with E<sub>2</sub> resulted in a significant reduction in the CAT activity (Fig. 6). However, the influence of E<sub>2</sub> was lost when the cells were treated with the antiestrogen ICI 164,384. The negative controls in this experiment included ARE-tk-CAT alone or ARE-tk-CAT cotransfected with ER cDNA. These results confirm that DHT-induced CAT activity was decreased only in the presence of the ER/E<sub>2</sub> complex.

#### Effects of Endogenous ER on Androgen-Induced CAT Activity

In order to determine whether endogenous ER could inhibit the AR transcription activity, GC cells, which contain cytoplasmic ER (Gershengorn et al, 1979) but not AR, were transfected with the MMTV-LTR-CAT construct and AR cDNA and were treated with DHT and DHT + E<sub>2</sub>. Transfection of the MMTV-LTR-CAT construct into GC cells and treatment with DHT did not yield an increase in the CAT activity compared to ethanol-treated controls, confirming that GC cells do not possess endogenous AR, which are capable of transactivating the MMTV-LTR-CAT construct (Fig. 7). However, cotransfection of AR cDNA and subsequent treatment with DHT

resulted in a sixfold increase in CAT activity compared to ethanol-treated cells. When cells transfected with MMTV-LTR-CAT and AR cDNA were treated with E<sub>2</sub> in addition to DHT, the CAT activity was significantly

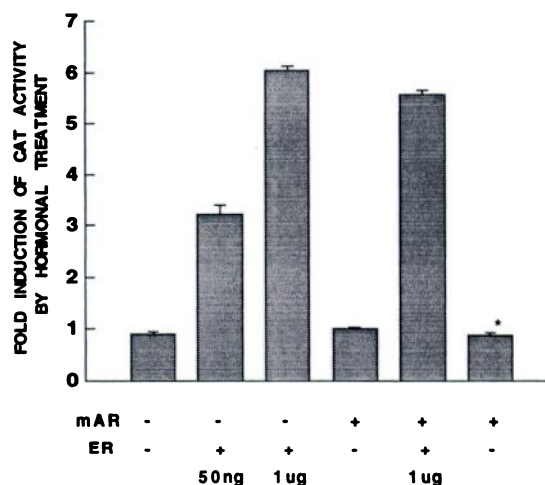


FIG. 5. Transcriptional regulation of ERE-tk-CAT by cotransfection of ER and AR cDNA. QT6 cells were transfected with AR cDNA (100 ng), ER cDNA (50 ng or 1 µg), and ERE-tk-CAT (5 µg). The CAT activity is expressed as fold induction by treatment with E<sub>2</sub> or DHT compared to ethanol treatment. The asterisk (\*) denotes cotransfection of ARE-tk-CAT and AR cDNA and treatment with DHT. All other cells were treated with E<sub>2</sub>. Results are expressed as the mean ± standard error. The experiments were repeated two times with at least three to four plates per treatment per experiment.

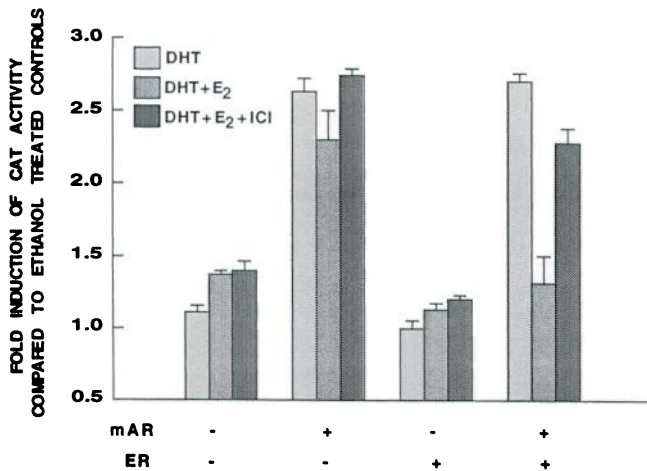


FIG. 6. Transcriptional regulation of ARE-tk-CAT by E<sub>2</sub>. QT6 cells were transfected with mAR cDNA (100 ng), ER cDNA (1 μg), or ARE-tk-CAT (5 μg). The cells were treated with DHT (10<sup>-8</sup> M), E<sub>2</sub> (10<sup>-8</sup> M), or ICI 164,384 (10<sup>-7</sup> M). Results are expressed as the mean ± standard error. The experiments were repeated two times with at least three to four plates per treatment per experiment.

reduced compared to DHT-induced cells, suggesting that the endogenous ER is capable of inhibiting androgen-induced transactivation of MMTV-LTR-CAT (Fig. 7). These experiments confirm the results obtained with QT6 cells, indicating that ER activated by treatment with E<sub>2</sub> is capable of modulating the transcriptional activity of AR.

## Discussion

Interactions between steroid hormones and their receptors play an important role in the mechanism of development, growth, homeostasis, physiologic response, and pathologic disturbance. It is important to consider that if the interaction between AR and ER is to be examined *in vitro*, a model should be chosen that would contain either known concentrations of endogenous receptors or no endogenous AR or ER. This would allow for the precise control of receptor concentrations in relation to each other. In the current study the QT6 cell line was chosen because it offered a functional model for transient cotransfection assays as well as an environment free from endogenous receptor. In addition, the reporter system in such a study should ideally be activated by only one of the receptor-hormone complexes, which offers a unique analysis of the effect of activation of one receptor on another. Although it is possible that cell-specific factors are necessary for gene expression, previous studies have shown the AR could be expressed in cell lines containing no endogenous AR, thus adding credibility to the present model (Rondlet et al, 1990). The MMTV-LTR-CAT con-

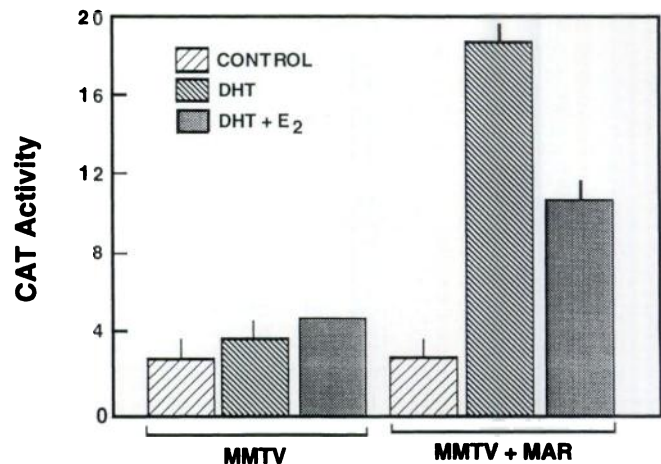


FIG. 7. Transcriptional regulation of MMTV-LTR-CAT by DHT after cotransfection with AR cDNA in GC cells. GC cells were transfected by electroporation with MMTV-LTR-CAT (15 μg) alone or together with AR cDNA (250 ng). The cells were treated with ethanol, DHT (10<sup>-8</sup> M), or DHT and E<sub>2</sub> (10<sup>-8</sup> M each). CAT activity was measured as described in the legend to Figure 3. Results are the mean ± standard error. The experiments were repeated three times with at least three to four plates per treatment per experiment.

struct was used as a reporter, as it has been previously shown that the MMTV-LTR-CAT reporter system was androgen responsive but showed no activity in the presence of E<sub>2</sub>/ER complexes (Cato et al, 1987). The present experiments indicate that the MMTV-LTR-CAT reporter system was specifically regulated by the addition of androgens (DHT or MIB) but not by E<sub>2</sub>, P, or DEX. Furthermore, the specificity of response was confirmed by transfection of the ARE-tk-CAT construct. Thus, the experimental model chosen for this study appears to be ideal for examining the effect of ER on an androgenic response.

Our results indicate that the E<sub>2</sub>/ER complex is capable of inhibiting transcriptional activity of the DHT/AR complex. This effect appears to involve the E<sub>2</sub>/ER complex specifically, as the addition of E<sub>2</sub> alone in the absence of ER was not able to negatively affect transcriptional activation of the MMTV-LTR-CAT and ARE-tk-CAT reporter by DHT. It has been shown previously that E<sub>2</sub> itself can bind weakly to the AR (Tindall et al, 1981; Murthy et al, 1984) and can activate an androgen-responsive gene (Glover and Dabre, 1989). However, these effects were observed at very high hormone concentrations and much greater than those used in the present investigation. In a previous report (Meyer et al, 1989) a similar phenomenon of transcriptional inhibition was shown to occur by coexpression of ER in a dose- and estrogen-dependent manner with progesterone receptor and glucocorticoid receptor. Their concept of the "interfering receptor" resulting in inhibition of hormone-induced transcriptional activity by another receptor is supported by the results of the present study.

There are a number of different possibilities to consider in examining the effect of the "interfering receptor." The model for transcriptional activation of steroid-responsive genes by ligand-activated receptor involves the allosteric conformational change induced by a ligand, which allows access to regions of the receptor for other transcription factors. In addition, the binding of steroid receptor to the proximal promoter region of responsive genes involves stabilization of various transcription factors and results in a conformational change and subsequent initiation of transcription (Evans, 1988; Beato, 1989; Wahli and Martinez, 1991). Ptashne and Gann (1990) described the mechanism of activating factors in the initiation of transcription, which led to the concept of "squenching." Accordingly, the presence of one activator can sequester a protein or transcription factor, making it unavailable to another activator for the purpose of initiating transcription. Because ER was necessary for the inhibitory effect on transcription observed in the present study, the possibility exists that it may be sequestering, or "squenching," some unknown factor necessary for transcription to occur through DHT activation of the AR.

Another possibility for the ER inhibition of transcription is the interaction of the ER with the AR directly. This may involve some type of receptor-receptor complex formation resulting in inactivation or formation of non-productive heterodimers. This dimer formation hypothesis remains a theoretical possibility. Similar interaction has been suggested by using gel retardation assays (Meyer et al, 1989).

In summary, we have shown that the ER is capable of inhibiting transcriptional activation of the MMTV-LTR-CAT and ARE-tk-CAT constructs by AR cDNA in both a dose-dependent and estrogen-dependent manner. Such an inhibition may have important implications in the roles of steroid hormones in development and maintenance of reproductive organs.

## Acknowledgments

We thank Dr. Pierre Chambon for providing the human estrogen receptor (HER) construct. We also thank Dr. M. Tsai for the ARE-tk-CAT construct and Dr. G. Greene for the ERE-tk-CAT construct. The authors are grateful for the skillful technical assistance of Ms. M. D. Blexrud.

## References

- Bansal GS, Latchman DS. Oestrogen enhances the responsiveness of the MMTV-LTR to glucocorticoid in 2R-75-1 human breast cancer cells. *J Steroid Biochem* 1990;36:399-405.
- Beato M. Gene regulation by steroid hormones. *Cell* 1989;56:335-344.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 1976;72:248-254.
- Cato ACB, Henderson D, Ponta H. The hormone response element of the mouse mammary tumor virus DNA mediates the progestin and androgen induction of transcription in the proviral long terminal repeat. *EMBO J* 1987;6:363-368.
- Coffey DS, Walsh PC. Clinical and experimental studies of benign prostatic hyperplasia. *Urol Clin North Am* 1990;17:461-475.
- Denison SH, Sands A, Tindall DJ. A tyrosine aminotransferase glucocorticoid response element also mediates androgen enhancement of gene expression. *Endocrinology* 1989;124:1091-1093.
- Evans RM. The steroid and thyroid hormone receptor superfamily. *Science* 1988;240:889-895.
- Gershengorn MC, Marcus-Samuels BE, Geras E. Estrogens increase the number of thyrotropin-releasing hormones receptors on mammotropic cells in culture. *Endocrinology* 1979;105:171-176.
- Glover JF, Dabre PD. Multihormone regulation of MMTV-LTR in transfected T470 human breast cancer cells. *J Steroid Biochem* 1989;32:357-363.
- Gorman CM, Moffat LF, Howard BH. Recombinant genomes which express chloramphenicol acetyl transferase in mammalian cells. *Mol Cell Biol* 1982;2:1044-1051.
- Graham FL, van der Eb AJ. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 1973;52:456-467.
- Green S, Chambon P. Oestradiol induction of a glucocorticoid-responsive gene by a chimaeric receptor. *Nature* 1987;325:75-78.
- Green S, Kumar V, Theulaz I, Wahli W, Chambon P. The N-terminal DNA-binding 'zinc finger' of the oestrogen and glucocorticoid receptors determines target gene specificity. *EMBO J* 1988;7:3037-3044.
- Grossmann ME, Lindzey J, Kumar MV, Tindall DJ. The mouse androgen receptor is suppressed by the 5'-untranslated region of the gene. *Mol Endocrinol* 1994;8:448-455.
- He WW, Fischer LM, Sun S, Bilhartz DL, Zhu X, Young CYF, Kelley DB, Tindall DJ. Molecular cloning of androgen receptors from divergent species with a polymerase chain reaction technique: complete cDNA sequence of the mouse androgen receptor and isolation of androgen receptor cDNA probes from dog, guinea pig, and clawed frog. *Biochem Biophys Res Commun* 1990;171:697-704.
- He WW, Kumar MV, Tindall DJ. A frame-shift mutation in the androgen receptor gene causes complete androgen insensitivity in the testicular-feminized mouse. *Nucleic Acids Res* 1991;19:2373-2378.
- Lindzey J, Grossmann M, Kumar MV, Tindall DJ. Regulation of the 5'-flanking region of the mouse androgen receptor gene by cAMP and androgen. *Mol Endocrinol* 1993;7:1530-1540.
- Meyer ME, Gronemeyer H, Torcotte B, Bocquel M-T, Tasset D, Chambon P. Steroid hormone receptors compete for factors that mediate their enhancer function. *Cell* 1989;57:433-442.
- Moscovici C, Moscovici MG, Jimenez H, Lai MMC, Hayman MJ, Vogt PK. Continuous tissue culture cell lines derived from chemically induced tumors of Japanese quail. *Cell* 1977;11:95-103.
- Murthy LR, Chang CH, Rowley DR, Scardino PT, Tindall DJ. Physicochemical characterization of the androgen receptor from hyperplastic human prostate. *Prostate* 1984;5:567-579.
- Neumann JR, Morency CA, Russian KO. A novel rapid assay for chloramphenicol acetyltransferase gene expression. *Biotechniques* 1987;5:444-447.
- O'Malley B. The steroid receptor superfamily: more excitement predicted for the future. *Mol Endocrinol* 1990;4:363-369.
- Ptashne M, Gann AAF. Activators and targets. *Nature* 1990;346:329-331.
- Rondlet SE, Wu X-P, Miesfeld RL. Functional characterization of the androgen receptor confirm that the molecular basis of androgen action is transcriptional regulation. *Mol Endocrinol* 1990;4:708-713.
- Rosenthal N. Identification of regulatory elements of cloned genes with functional assays. *Methods Enzymol* 1987;152:704-720.
- Tindall DJ, French FS, Nayfeh SN. Estradiol-17 $\beta$  inhibition of androgen uptake, metabolism and binding in epididymis of adult male rats *in*

- vivo*: a comparison with cyproterone acetate. *Steroids* 1981;37:257-268.
- Wahli W, Martinez E. Superfamily of steroid nuclear receptors: positive and negative regulators of gene expression. *FASEB J* 1991;5:2243-2249.
- Walsh PC, Wilson JD. The induction of prostatic hypertrophy in the dog with androstanediol. *J Clin Invest* 1976;57:1093-1097.
- Wasner G, Hennerman I, Kratochwil K. Ontogeny of mesenchymal androgen receptors in the embryonic mouse mammary gland. *Endocrinology* 1983;113:1771-1779.
- Ye ZS, Foreman BM, Randa A, Pascual A, Hae-Young P, Casanova J, Samuels HH. Rat growth hormone gene expression. *J Biol Chem* 1988;263:7821-7829.

---

The American Society of Andrology wishes to thank the following organizations for their generous support:

#### **Gold Club**

Buckeye Urology and Andrology, Inc.

#### **Silver Club**

National Medical Enterprises  
West Michigan Reproductive Institute, P.C.

#### **Sustaining Sponsor**

Hamilton Thorne Research  
Serono Laboratories, Inc.  
Texas Institute for Reproductive Medicine and Endocrinology, P.A.  
The Upjohn Company

#### **Annual Supporters of the Society**

Genetics & IVF Institute  
Merck U.S. Human Health  
ReproGen  
Southern Illinois University School of Medicine:  
Office of the Dean  
Department of Obstetrics and Gynecology  
Department of Physiology  
Anna Steinberger, Ph.D.