Autologous Down-Regulation of Androgen Receptor Messenger Ribonucleic Acid

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Autoregulation of androgen receptor (AR) mRNA was investigated using Northern blot analysis with AR cDNA fragments as probes. The amount of AR mRNA increased 2- to 10-fold with androgen withdrawal and decreased below control levels after androgen stimulation in rat ventral prostate, coagulating gland, epididymis, seminal vesicle, kidney, and brain, and in a human prostate cancer cell line, LNCaP. In rat ventral prostate, AR mRNA increased 2- to 3-fold within 24 h after castration and remained elevated for 4 days. Treatment with testosterone propionate beginning 24 h after castration reduced ventral prostate AR mRNA 4-fold within 8 h of androgen replacement. Administration of estradiol 24 h after castration had no significant effect on prostatic AR mRNA. Androgens, including testosterone and the synthetic androgen methyltrienolone (R1881), or the antiandrogen cyproterone acetate down-regulated AR mRNA *in vitro* in LNCaP cells, whereas estradiol was without effect. Administration of testosterone propionate to rats with androgen insensitivity did not decrease AR mRNA. Down-regulation of AR mRNA by androgen is therefore a receptor-mediated process which occurs *in vivo* in rat tissues that differ in androgen responsiveness and in cultured human prostate cells. (Molecular Endocrinology 4: 22–28, 1990)

INTRODUCTION

Androgens induce the development and maintenance of male reproductive tract tissues through an interaction with the high affinity androgen receptor (AR) (1). The AR is a ligand-activated, trans-acting regulator of specific gene transcription. Studies on AR have recently been facilitated by the cloning of AR complementary DNA (cDNA) (2–6). The AR has a domain structure characteristic of the steroid receptor family with greatest sequence similarity to the progesterone, mineralocorticoid, and glucocorticoid receptors (2–7). The use of AR cDNA probes in Northern blot analysis has confirmed the presence of AR mRNA in reproductive and nonreproductive tract tissues (8), but little is known about the hormonal regulation of AR mRNA. AR is detectable using conventional ligand binding methods or Northern blot analyses in early-stage human prostatic adenocarcinomas (9), in Dunning R3327 G and H rat tumors (10, 11), and in the LNCaP human metastatic prostate tumor cell line (12, 13).

In this report, we examined the influence of androgen on steady-state AR mRNA levels in rat tissues *in vivo* and on the LNCaP human prostate cancer cell line in culture. Evidence for receptor mediated down-regulation of AR mRNA was obtained using the androgen insensitive, testicular feminized (*Tfm*) rat, which lacks a functional AR protein (14–16). Autologous down-regulation was demonstrated for AR in a manner observed previously for most (17–20), but not all (21) other steroid receptors.

RESULTS

Tissue Distribution and Androgen Control

The tissue distribution and influence of androgen on AR mRNA was examined by Northern blot hybridization. A 10-kilobase (kb) mRNA, shown previously to encode AR (2, 3, 8), was detected in rat kidney, brain, epididymis, and coagulating gland, but not in spleen (Fig. 1, A and C). Steady-state levels of AR mRNA in brain and kidney were considerably lower than reproductive tract tissues even after prolonged exposure to x-ray film. Androgen withdrawal for 4 days (Fig. 1A, lane C and Fig. 1C, *open bars*) resulted in a 1.5- to 3-fold increase in AR mRNA in tissues containing AR. Restimulation with androgen 24 h before tissue removal reduced AR mRNA to the level approaching that of the intact animal. The radioactive bands evident in all tissue extracts at approximately 5 kb result from nonspecific cross-hybridization with ribosomal RNA present in total

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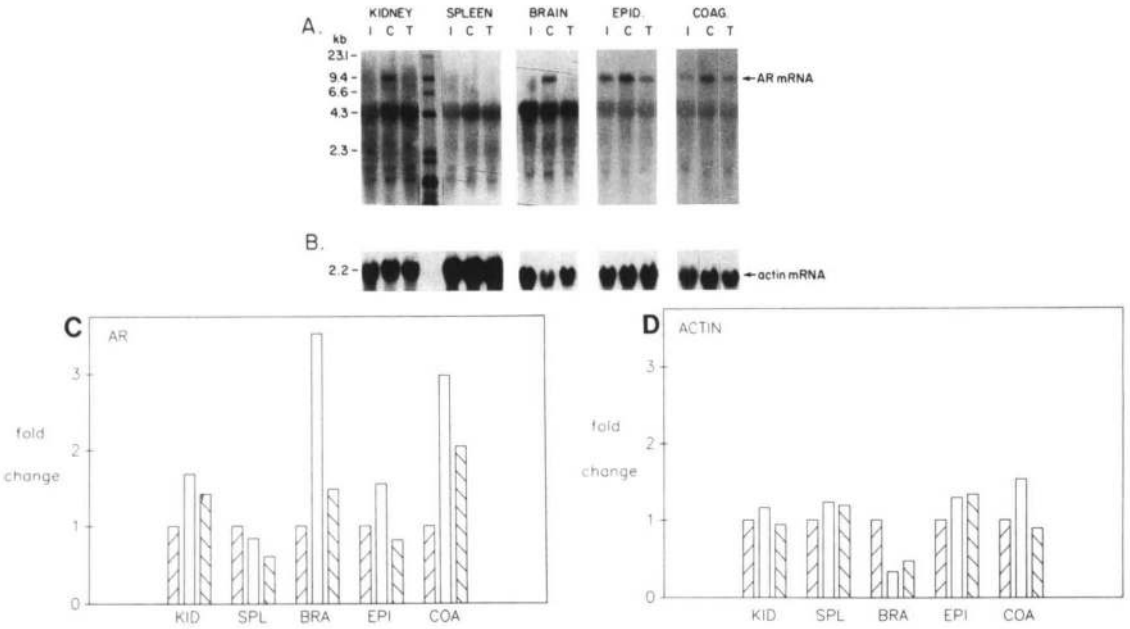

Fig. 1. Northern Blots of Tissue RNA after Androgen Withdrawal and Restimulation

Total RNA was isolated from kidney, spleen, brain, epididymis, and coagulating gland (anterior prostate) from groups of rats which were either intact (I) or used 4 days after castration without (C) or with (T) administration of 2 mg testosterone propionate 1 day before killing. Aliquots of total RNA (20 μg) were analyzed by blot hybridization with an AR cDNA probe (A) as described in *Materials and Methods*. Exposure time for film development was 12 days for kidney, spleen, and brain and 4 days for epididymis and coagulating gland. Molecular weight markers were ^{32}P-labeled *Hin*dIII digested λ DNA and *Hae*III digested phi X 174 DNA. The blot was washed and rehybridized to an actin cDNA probe to control for equivalent RNA loading on the gel (B). Autoradiographs were quantified with an LKB Ultrascan densitometer to determine relative changes in mRNA levels. Data are expressed as the fold change in absorbance units for AR (C) and actin (D). The *bar graph* indicates the fold change in AR mRNA levels relative to untreated intact rats (*hatched bars on left*) for samples obtained 4 days after castration (open bars) and from 4-day castrated, testosterone treated rats (*hatched bars on right*).

RNA extracts (8). Rehybridization with an actin probe showed some variation in a generally high level of actin mRNA expression (Fig. 1, B and D). The uniformity of RNA loading was supported by the constancy of the 5 kb cross-hybridizing ribosomal RNA band, and in most instances, by the actin signal. Inhibitory effects of androgen on actin mRNA were detected predominantly in tissues that show a growth response to androgen, *i.e.* the coagulating gland and ventral prostate (see Fig. 2).

Hormone regulation of AR mRNA was investigated further in rat ventral prostate. Prostate RNA was isolated daily up to 4 days after castration. Additional treatment groups included rats 2 and 4 days after castration administered testosterone propionate or 17B-estradiol 24 h before tissue removal. The amount of AR mRNA increased 2.5-fold relative to untreated intact rats within 1 day of castration as previously shown (8), 3-fold by 3 days, and remained elevated 4 days after castration (Fig. 2, A and C). Administration of testosterone propionate 24 h before tissue removal from rats 2 or 4 days after castration reduced ventral prostate AR mRNA levels to, or below, those of intact animals. Administration of estrogen 2 or 4 days after castration reduced ventral prostate AR mRNA levels to, or below, those of intact animals. Administration of estrogen 2 or 4 days after castration had no detectable influence on AR mRNA. Rehybridization with an actin probe revealed a different mRNA pattern after castration (Fig. 2, B and D). Androgen treatment 2 or 4 days after castration reduced the amount of actin mRNA to that of the untreated control. Estrogen increased actin mRNA above the level of the castrated animal.

Time Course of Down-Regulation

The rate of decline of AR mRNA in ventral prostate after a single injection of testosterone propionate is shown in Fig. 3 (A and C). The first 8 h showed a rapid decrease in AR mRNA which remained low for at least 48 h. Actin mRNA decreased slightly over the same time period (Fig. 3, B and D). Between 8 and 48 h the amount of AR mRNA remained below that of the untreated animal suggesting that a pharmacological dose of testosterone (see Fig. 3E) represses AR mRNA below the physiological range. The initial rapid 5-fold decrease within 8 h suggests that AR mRNA degradation may be an active process.

Total androgen was measured by RIA in pooled sera from the treatment groups. Serum androgen decreased from 3.8 ng/ml in the intact animal to less than 0.25 ng/ml within 24 h of castration (see Fig. 3E). After treatment with testosterone propionate, serum androgen levels increased rapidly for 2 h to greater than 10-fold higher than in untreated controls and decreased gradually over

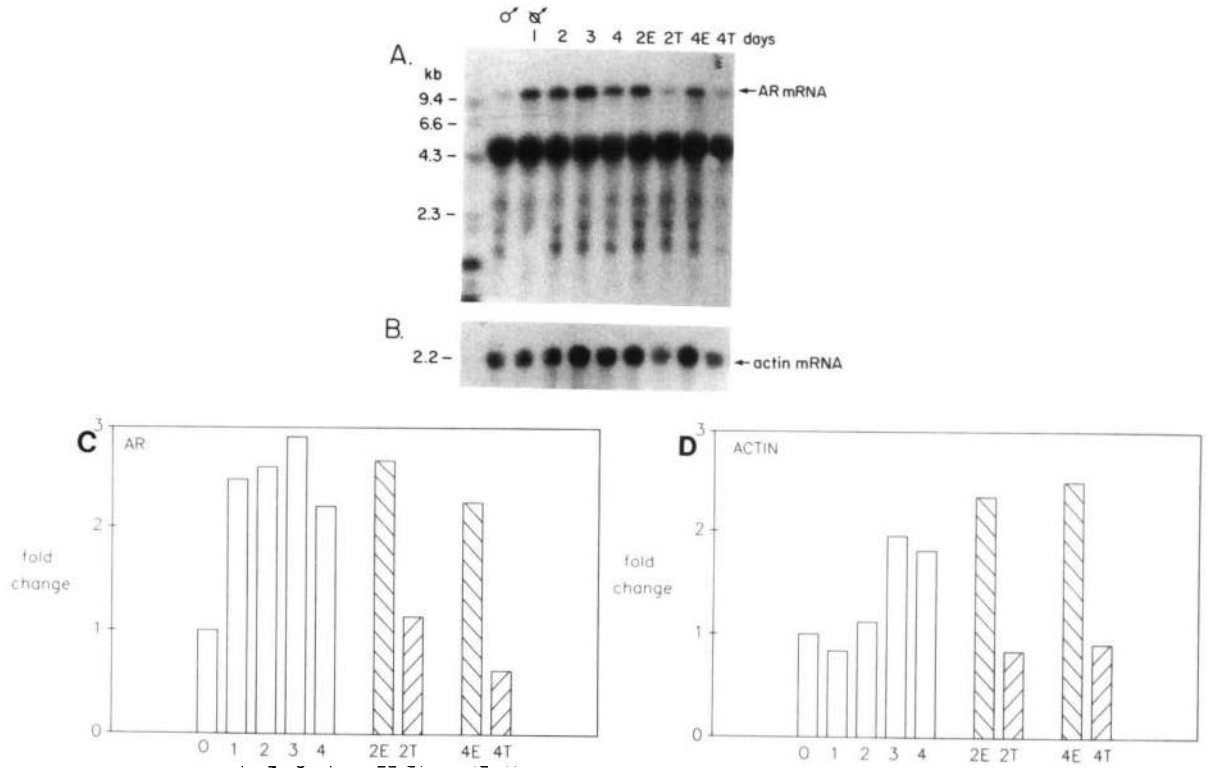

Fig. 2. Northern Blot of Ventral Prostate RNA Various Times after Androgen Withdrawal and Restimulation with Androgen or Estrogen

Ventral prostate RNA was isolated after no treatment (0), (1), (2), (3), and (4) days after castration. At 2 and 4 days after castration, additional rats received an injection of testosterone propionate (2T or 4T) or estradiol (2E or 4E) 24 h before killing. Aliquots of total RNA (20 μg) were analyzed by blot hybridization using an AR cDNA probe (A) as described. The blot was washed and rehybridized to an actin cDNA probe as a control for RNA loading (B). The autoradiographs were quantified for AR (C) and actin (D). Molecular weight markers are described in Fig. 1.

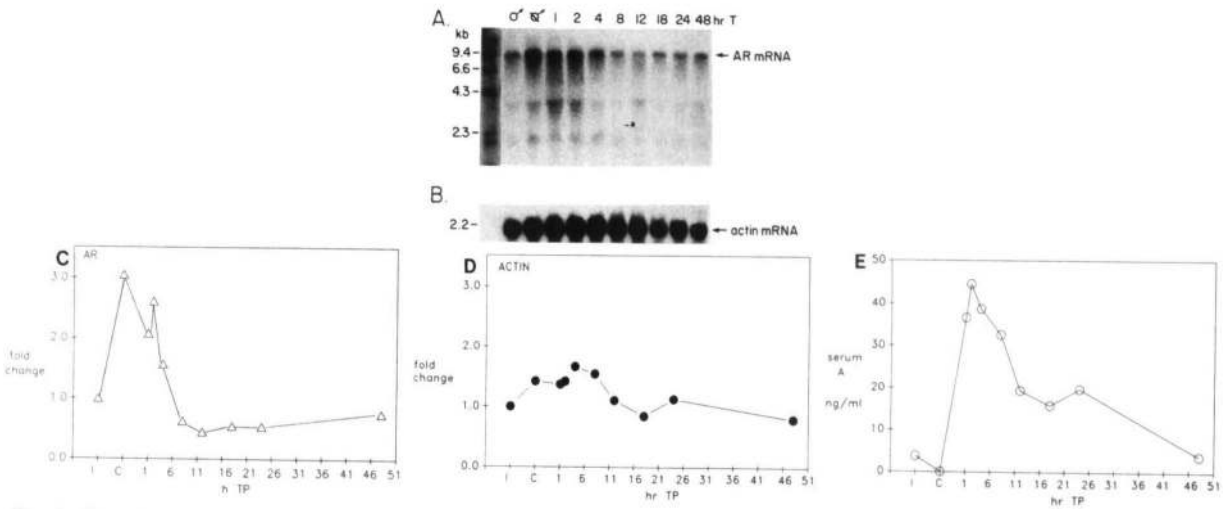

Fig. 3. Time Course of AR mRNA Decline after Androgen Stimulation

Total ventral prostate RNA from untreated intact rats (♂) or rats castrated 2 days earlier (♂) were administered a single injection of testosterone propionate (2 mg, im) and tissues were removed 0, 1, 2, 4, 8, 12, 18, 24, or 48 h later. Aliquots of RNA (20 μg) were analyzed by Northern blot hybridization to an AR cDNA probe as described (A). The blots were washed and rehybridized to actin cDNA as a control for RNA loading (B). Molecular weight markers are described in Fig. 1. Autoradiographs were quantified for AR (C) and actin (D). Pooled serum androgen levels (ng/ml) were measured for each group by RIA (E).

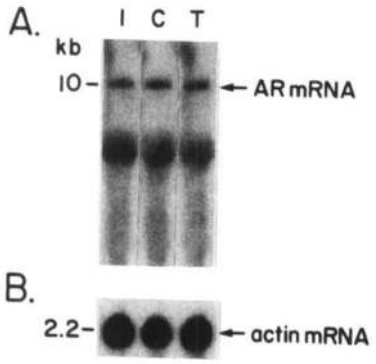

Fig. 4. Regulation of AR mRNA in the *Tfm* rat

RNA was isolated from kidneys of *Tfm* rats that were removed 2 days after castration (C) or 18–24 h after administration of either sesame oil (I) or 10 mg testosterone propionate (T) im. Aliquots of RNA (30 μg) were analyzed by Northern blot hybridization to an AR cDNA probe (A). The blot was washed and rehybridized to actin cDNA (B).

the next 40 h, with a half-time of approximately 7 h (Fig. 3E).

Absence of Down-Regulation in the *Tfm* Rat

Receptor mediated down-regulation of AR mRNA was examined in the androgen insensitive *Tfm* rat (King Holtzman, Stanley Gumbreck *Tfm* rat, Ref. 14). Kidneys from intact *Tfm* and wild type male siblings contained similar levels of 10-kb AR mRNA even though *Tfm* rats had an androgen level (4.4 ng/ml) approximately 3-fold higher than wild type siblings (1.61 ng/ml) (also Refs. 16, 22). Castration of the *Tfm* rat failed to increase AR mRNA in kidney (Fig. 4A) or significantly alter actin mRNA levels (Fig. 4B). Autologous down-regulation of AR mRNA occurs in wild type siblings as described above for the Sprague Dawley rat (data not shown). Thus autologous down-regulation of AR mRNA requires a functional AR which is lacking in the *Tfm* rat.

Autoregulation in the LNCaP Human Prostate Cancer Cell Line

The steady state level of 10-kb AR mRNA was low in the metastatic prostate cancer LNCaP cell line when cells were maintained in media containing 10% fetal calf serum (Fig. 5, A and C) with an androgen concentration of approximately 0.1 nM. AR mRNA increased approximately 10-fold when LNCaP cells were maintained for 2 days in serum-free medium. The addition of testosterone or the antiandrogen, cyproterone acetate, caused AR mRNA levels to decline to, or below, the level observed in the presence of serum. Methyltrienolone, R1881, a synthetic androgen, reduced AR mRNA levels in LNCaP cells but estradiol had no effect. Actin mRNA levels showed little variation among treatment groups (Fig 5B). The low signal intensity of actin in LNCaP cells was in part due to the use of a lower specific activity probe. Thus, the hormonal regulation of AR mRNA in human prostatic LNCaP cells is similar to that observed in rat tissues *in vivo*.

DISCUSSION

Autoregulation of AR mRNA by androgen is a rapid process in prostate and epididymis, which are tissues that regress in the absence of androgen (23, 24), as well as in kidney and brain, which show little morphological change in response to androgen (25, 26). It is likely that down-regulation occurs by decreasing the half-life of AR mRNA and/or its rate of transcription, as suggested with the estrogen (27) and glucocorticoid receptors (28). The absence of androgen regulation of AR mRNA in the *Tfm* rat supports a receptor mediated process. Autologous down-regulation is a prevalent finding among steroid receptor mRNAs (17–20), although vitamin D up-regulates its receptor mRNA (21). The physiological significance of steroid hormone down-regulation of cognate receptor mRNAs is unclear. These hormonal effects may be relatively transient and reflect a homeostasis mechanism modulating the hormonal signal toward steady state.

The most pronounced effect of androgen on AR mRNA was noted in the human prostate cancer cell line, LNCaP, where there was a 10-fold increase in AR mRNA when serum was removed from the incubation medium. In general it has been difficult to document androgen responses in tissue culture cells. LNCaP cells appear therefore to be a useful *in vitro* model system to further study androgen effects in tissue culture. LNCaP cells have been shown to respond to androgen by increasing the secretion of a 42-kilodalton protein (29) and the synthesis of the epidermal growth factor receptor (13, 30).

The presence of a normal steady state level of AR mRNA in the *Tfm* rat differs from the *Tfm* mouse. Our previous study revealed a very low level of AR mRNA in the *Tfm* mouse (2). This difference in AR mRNA levels between the *Tfm* rat and mouse suggests that the mutations associated with androgen insensitivity differ. It is possible that the low amount of *Tfm* mouse AR mRNA results from an alteration in transcriptional regulation. Androgen insensitivity in the *Tfm* rat is due to a single base mutation in the steroid binding domain of the AR (16).

A surprising finding in our study was the agonistic effect of cyproterone acetate in down-regulating AR mRNA since this compound has an antiandrogenic effect when administered to intact rats (31). Recent reports indicate, however, that cyproterone acetate acts as an agonist in LNCaP cells by promoting both an increase in cell number and in the uptake of thymidine (32, 33), in contrast to an earlier report of antagonistic effects of cyproterone acetate in these cells (29). Cyproterone acetate and other antiandrogens, including hydroxyflutamide and RU23908, all of which act as agonists in these cells, likely mediate their effects

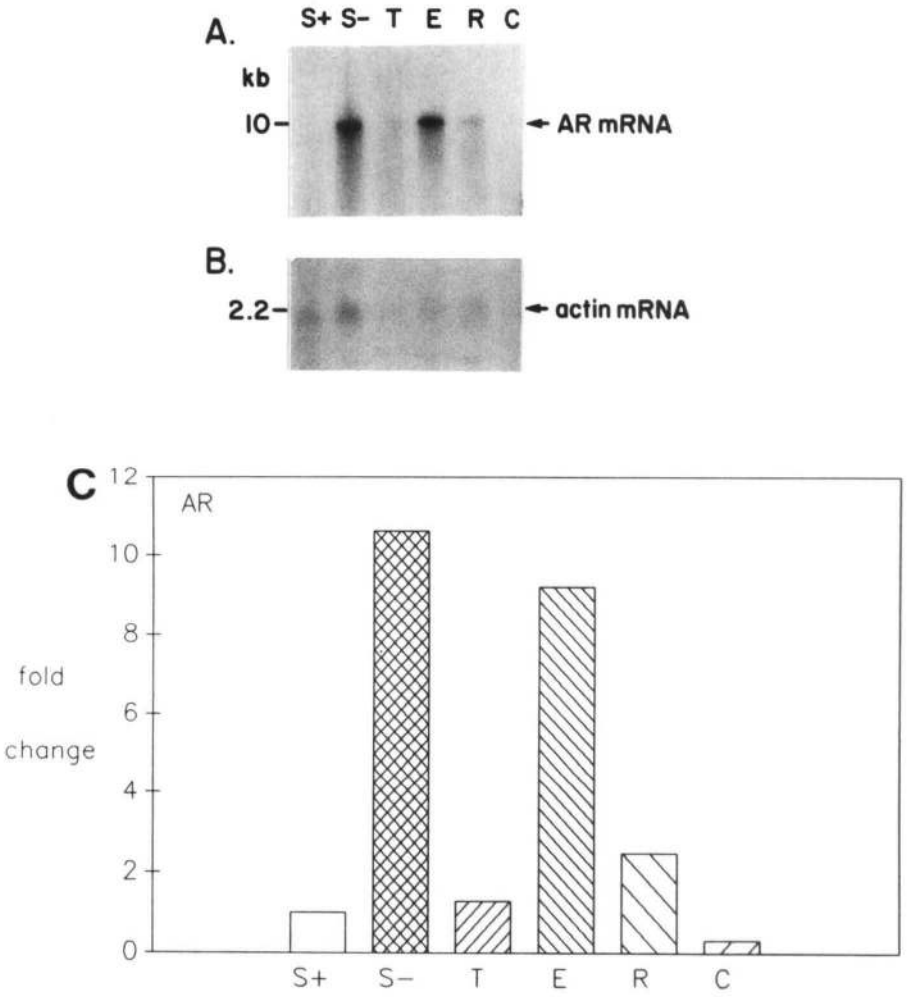

Fig. 5 . Regulation of AR mRNA in LNCaP Cells

LNCaP cells were grown to confluence in RPMI containing 10% fetal calf serum and maintained for 2 days in this medium (S+), in serum free medium (S−), or in serum free medium supplemented with 30 nM testosterone (T), estradiol (E), R1881(R), or cyproterone acetate (C). Total RNA was isolated and aliquots (20 μg) were analyzed by blot hybridization using an AR cDNA (A). The blot was washed and rehybridized to actin cDNA (B). The autoradiographs were quantitated for AR (C). The specific activities of the probes were 2×10^8 dpm/μq DNA for AR and 0.3×10^8 dmp/μq DNA for actin.

through AR since they inhibited the uptake of [^{3}H]-R1881 (32). The explanation for the agonist effects of antiandrogens in LNCaP cells is not known, although the AR in these cells has been reported to have an enhanced affinity for progesterone relative to testosterone (13) suggesting a possible alteration in the steroid binding domain of the LNCaP cell AR. Although cyproterone acetate is also a progestin, it is unlikely that its androgenic agonist effects are mediated through the progesterone receptor since LNCaP cells reportedly lack a progesterone receptor (34). Other antihormones are known to have agonist and antagonist effects. For example the anti-estrogen tamoxifen acts as an estrogen agonist in some systems but as an antagonist in others (35). It will be interesting to test the effects of other antiandrogens and progestins on the regulation of AR mRNA.

MATERIALS AND METHODS

Animals

Adult male Sprague Dawley rats (Zivic Miller, Raleigh, NC) were castrated (8) and some received an injection of testosterone propionate in sesame oil (2 mg, im) or estradiol-17 β in sesame oil (100 μg, sc). Tissues were quick frozen in liquid nitrogen and stored at −80 C. Blood was collected after decapitation and pooled to generate serum samples for RIA. Adult *Tfm* (Stanley Gumbreck) rats (14) and their normal wild type male siblings were provided by Dr. Kathie L. Olsen (State University of New York at Stony Brook). *Tfm* rats had no Mullerian duct derivatives and lacked a normal epididymis and male sex accessory glands; testes were present, but notably smaller than those of age-matched normal siblings. Tissues from *Tfm* and normal male siblings were processed as described above. Animal experiments were conducted in accord with the principles and procedures as outlined by the NIH Guide for the Care and Use of Laboratory Animals.

Cell Culture

The human metastatic prostate cancer LNCaP cell line was obtained from the American Type Culture Collection (Rockville, MD) and grown to confluence at 37 C in a humidified atmosphere of 5% CO_2/95% air in RPMI medium containing phenol red, penicillin, streptomycin, and 10% fetal calf serum (12), equivalent to 0.34 ± 0.02 ng/ml (~1.2 nM) total androgen determined by RIA. In some experiments, cells were grown for 2 days in serum free medium with daily changes or in serum free media with supplements of 30 nM testosterone, methyltrienolone (R1881, New England Nuclear, Dupont, Boston, MA) cyproterone acetate (Schering, Berlin, West Germany) or estradiol-17 β.

RNA Analysis

Total RNA was isolated by homogenizing tissue or lysing cells in guanidine thiocyanate using the procedure of Chirgwin *et al.* (36). Poly(A) RNA was isolated by oligo(dT)cellulose chromatography (37). RNA was denatured in glyoxal and dimethylsulfoxide for 1 h at 50 C (38) and fractionated by electrophoresis on 1.2% agarose gels containing 10 mM sodium phosphate, pH 6.8. RNA was transferred to nylon membranes (0.2 μm Biotrans, ICN, Irvine CA) (39) and cross-linked by brief exposure to UV light (40). ^{32}P-End labeled DNA fragments were used as molecular weight markers. Membranes were hybridized (41) to cDNA probes which were labeled with ^{32}P by nick translation (42) or random priming (43). A 5′-*Eco*RI fragment of rat AR cDNA clone rARep1 or a 0.7-kb *Hin*dIII/*Eco*RI fragment of human AR cDNA clone ARHFL1 was used for AR hybridization (2, 3, 8). A chicken β-actin cDNA (Oncor, Gaithersburg, MD) was used for actin hybridizations (44). For intraspecies hybridizations, filters were washed to high stringency using 0.1 × SSC, 0.1% sodium dodecyl sulfate at 65 C; for cross-species hybridization, filters were washed in the same buffer at 50 C (1 × SSC, 0.15 M NaCl, and 0.015 M sodium citrate). Filters were exposed to Kodak X AR x-ray film at −70 C using intensifying screens. Autoradiographs were scanned with an LKB Ultrascan XL densitometer for quantification of mRNA levels. Filters were stripped according to manufacturer's recommendations before rehybridization.

Serum Androgen

Serum androgen concentrations were determined by RIA (45) using trace ^{125}I-11-α-tyrosylmethylester testosterone (W. Schramm, University of Michigan, Ann Arbor, MI; 12,000 cpm/assay). Sheep antiserum raised against testosterone-11-BSA conjugate was kindly provided by G. Niswender (Colorado State University, Fort Collins, CO). The antiserum exhibits cross-reactivity with dihydrotestosterone (69%), 5 α-androstan-3-β, 17 β-diol (22%) and 5 α-2 androstan-3 α, 17 β-diol (14%). The slopes of the standard curves differed from that of the testosterone standard curve so that the data represent a summation of all cross-reacting androgens and are accordingly termed serum androgen (45).

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