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Transcriptional Repression of the Glycoprotein Hormone α Subunit Gene by Androgen May Involve Direct Binding of Androgen Receptor to the Proximal Promoter*

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Testicular androgens suppress the synthesis and secretion of the pituitary gonadotropins, in particular, luteinizing hormone. This suppressive effect includes transcription of both the common α subunit gene and the unique β subunit genes. Herein, we demonstrate that 1500 base pairs (bp) of proximal 5′-flanking region derived from the human α subunit gene and a shorter 315-bp segment of the bovine α subunit gene confer negative regulation by androgen to the gene encoding bacterial chloramphenicol acetyltransferase in transgenic mice. Cotransfection assays with human androgen receptor indicated that the 1500-bp promoter region of the human α subunit gene also confers androgen regulation (transcriptional suppression) to reporter genes in both pituitary and placental cell lines. This raises the possibility of a role for DNA binding in suppression of α subunit transcription by activated androgen receptor. Consistent with this possibility, we have used a gel-mobility shift assay to detect several high affinity binding sites for androgen receptor located in the proximal promoter of the human α subunit gene. The strongest androgen receptor binding site is located at approximately −101 in the proximal 5′-flanking region. This steroid receptor binding site overlaps another binding site that defines one of several contiguous *cis*-acting regulatory elements required for basal transcriptional activity. Thus, binding of activated androgen receptor to this region may block the binding of a requisite *trans*-acting factor and lead to an attenuation in transcription. We conclude that this interaction, which occurs directly at the level of the pituitary, represents one of several physiological avenues through which androgens regulate gonadotropin gene expression.

The gonadotropins luteinizing hormone (LH)[1] and follicle stimulating hormone (FSH) are members of the glycoprotein hormone family and as such are heterodimers composed of an identical α subunit and distinct β subunits (Fiddes and Talmadge, 1984). The common α subunit and each of the β subunits are encoded by unique single copy genes that are expressed in specific cell types (gonadotropes) of the anterior pituitary gland (Fiddes and Talmadge, 1984; Gharib *et al.*, 1990).

Synthesis and secretion of LH and FSH are exquisitely regulated via negative feedback by gonadal estrogen and androgen acting either directly at the pituitary or at the hypothalamus to affect secretion of gonadotropin releasing hormone or GnRH (Brinkley, 1981; Desjardins, 1981; Gharib *et al.*, 1990). Accordingly, castration typically results in an elevation of both pituitary and circulating concentrations of LH and FSH that can be attenuated by replacement with either estrogen or androgen. Negative feedback effects of gonadal steroids include transcription of both the common α and unique β subunit genes (Gharib *et al.*, 1990). Consequently, changes in synthesis and secretion of intact heterodimers result from coordinated and regulated expression of both subunits even though the molecular mechanisms that control expression may be distinct for the different genes.

Due to the lack of pituitary cell lines that synthesize and secrete biologically active LH or FSH, we used transgenic mice to characterize the proximal promoter-regulatory regions of the human and bovine glycoprotein hormone α subunit genes. Previously, we have demonstrated that 1500[2] bp of 5′-flanking region derived from the gene encoding the human α subunit is sufficient to confer both estrogen and GnRH regulation to a heterologous reporter gene (Keri *et al.*, 1991; Hamernik *et al.*, 1992). Moreover, this promoter fragment targets expression of several different reporter genes to the pituitary and, specifically, to gonadotropes within the pituitary (Hamernik *et al.*, 1992). Similar results were obtained

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[1] The abbreviations used are: LH, luteinizing hormone; FSH, follicle stimulating hormone; bp, base pair(s); ER, estrogen receptor; AR, androgen receptor; ARE, androgen response element; GnRH, gonadotropin releasing hormone; GR, glucocorticoid receptor; GRE, glucocorticoid response element; CRE, cAMP response element; CREB, CRE-binding protein; JRE, junctional regulatory element; rAR-DBD, rat AR DNA binding domain; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; MMTV, mouse mammary tumor virus; T, testosterone; E, estradiol-17β; DHT, 5α-dihydrotestosterone.

[2] In most of our previous publications, this fragment has been referred to as a 1500-bp fragment. Preliminary sequence analysis indicates that the correct size is 1635 bp. For convenience and continuity, we will continue to refer to this region as a 1500-bp fragment (estimated by agarose gel electrophoresis).

with a shorter fragment of the promoter from the bovine α subunit gene (315 bp of proximal 5′-flanking region) (Kendall *et al.*, 1991; Hamernik *et al.*, 1992), suggesting that the promoters of both the human and bovine α subunit genes share highly conserved mechanisms regarding gonadotrope-specific expression and regulation by estradiol and GnRH (Keri *et al.*, 1991; Hamernik *et al.*, 1992).

The negative effect of estrogen on transcriptional activity of the human α subunit promoter is unusual as this region lacks a high affinity binding site for estrogen receptor (ER) (Keri *et al.*, 1991). In addition, activated ER failed to suppress expression of a chimeric human αCAT vector in cotransfection studies (Keri *et al.*, 1991). Thus, estrogen appears to regulate expression of the human α subunit promoter through an indirect mechanism that remains undefined but may involve both the hypothalamus (inhibition of GnRH secretion) and pituitary (interference with some component of the GnRH signal transduction pathway).

A significant component of the negative feedback by androgen may be due to inhibition of GnRH synthesis and secretion (Desjardins, 1981; Crowley *et al.*, 1985; Tilbrook *et al.*, 1991). Several lines of evidence, however, prompted us to further explore the potential for a direct effect of androgen on expression of the α subunit gene. First, like ER, androgen receptor (AR) is expressed in the anterior pituitary gland (Naess *et al.*, 1975; Thieulant and Pelletier, 1979; Schanbacher *et al.*, 1984; Bonsall *et al.*, 1985). Second, there have been several reports indicating that androgen can suppress levels of α subunit mRNA in pituitary cells devoid of hypothalamic input (Wierman *et al.*, 1988; Ahlquist *et al.*, 1990; Paul *et al.*, 1990; Wierman and Wang, 1990). Third, Akerblom *et al.* (1988) suggested that glucocorticoid can inhibit expression of the human α subunit gene by a mechanism involving binding of activated glucocorticoid receptor (GR) to several sites that either overlap or are in close proximity to two tandemly repeated cAMP response elements (CRE) residing in the proximal promoter. More recently, Chatterjee *et al.* (1991) suggested that mutually exclusive binding of GR and CREB represents an unlikely mechanism for glucocorticoid repression of α subunit gene expression. Rather, these authors implicate a squelching mechanism whereby GR directly binds CREB or binds to a shared class of transactivating proteins. An essential feature of both reports, however, is a GR-mediated interference of CREB transactivation. In light of these conflicting data and the well established convergence of DNA binding sites for AR and GR (Cato *et al.*, 1987; Quarmby *et al.*, 1990; Tan *et al.*, 1992), we were intrigued with the possibility that the tandem CREs of the human α subunit gene might be physiological targets of AR.

Accordingly, we have extended the use of our transgenic paradigm and incorporated both cotransfection and DNA-binding studies to determine whether androgen suppresses transcription of the human α subunit gene through a direct mechanism. Herein we report a surprising finding. Androgens suppress activity of the human α subunit promoter in transgenic mice. Moreover, the proximal α subunit promoter contains a high affinity binding site for AR. However, rather than co-map with the CREs, as did GR (Akerblom *et al.*, 1988), the AR binding site maps to a different region positioned between the downstream CRE and the CCAAT box and referred to as a junctional regulatory element (JRE) (Andersen *et al.*, 1990; Kennedy *et al.*, 1990). Collectively, our results suggest that direct binding of activated AR to the JRE may account, at least partially, for physiological regulation of expression of the human α subunit promoter by androgen.

EXPERIMENTAL PROCEDURES

Materials—Acetyl coenzyme A, 5α-dihydrotestosterone, and estradiol-17β were obtained from Sigma. Radionuclides were obtained from Du Pont-New England Nuclear. DNA modifying enzymes and restriction enzymes were obtained from either Boehringer Mannheim or GIBCO/BRL. Constant time release pellets containing either testosterone (T), 5α-dihydrotestosterone (DHT), or estradiol-17β (E) were obtained from Innovative Research of America (Toledo, OH). The plasmid pMMTV-CAT was a gift from Dr. Steve Nordeen.

Purified AR and Antibody—Bacterially expressed and purified rat AR DNA binding domain (amino acid residues 460–704), henceforth referred to as rAR-DBD, was prepared as previously described (Quarmby *et al.*, 1990). The antibody (AR-52) used in the gel-mobility shift assays is directed against an epitope (amino acid residues 527–541) that maps to the rat AR DNA binding domain (Quarmby *et al.*, 1990).

Animals—Transgenic mice harbored one of two different CAT chimeric genes. Transcription of the CAT gene was directed by either 1500 bp of 5′-flanking sequence from the human α subunit gene or 315 bp of the bovine counterpart. These two lines of mice (HαCAT or BαCAT) were produced and characterized previously (Bokar *et al.*, 1989). Both lines have been propagated in our laboratory; the animals used in the present experiments were at least 10 generations from founder animals and harbored the transgenes at single integration sites.

Castration and Steroid Replacement Paradigms—Castrations were performed under general anesthesia using a combination of ketamine and acepromazine (Hamernik *et al.*, 1992). At the time of castration, a constant release pellet containing either T (5 mg/pellet), DHT (5 mg/pellet), E (0.25 mg/pellet), or placebo was implanted subcutaneously. At 2 weeks post-castration, animals were euthanized and pituitaries, brains, and livers were harvested for assay of CAT activity (Bokar *et al.*, 1989; Keri *et al.*, 1991). Trunk blood samples were collected for assay of serum hormone concentrations.

Assays—Lysates were prepared from tissues and CAT activity was assayed as previously described (Bokar *et al.*, 1989; Keri *et al.*, 1991). Nonspecific CAT activity or background was determined by including homologous tissues from non-transgenic mice in each assay. Serum concentrations of E and T were measured using Pantex (Santa Monica, CA) radioimmunoassay kits validated in our lab for use with mouse serum (Keri *et al.*, 1991). Serum concentrations of LH and DHT were measured using validated radioimmunoassay procedures (Hamernik *et al.*, 1992).

Plasmids—Plasmids pHα(−1500)CAT, pRSVCAT, and pSV0CAT have been described by Bokar *et al.* (1988, 1989). Estrogen receptor expression vector (pHEO; henceforth referred to as hER) and pSV1vERECAT (henceforth referred to as pVERECAT) have been described by Keri *et al.* (1991). Human AR expression vector (pCMVhAR) consists of the full-length AR cDNA fused to the cytomegalovirus promoter (Quarmby *et al.*, 1990). Plasmid pMMTV-CAT consists of a 2.9-kilobase pair *Bam*HI fragment containing the MMTV long terminal repeat fused to CAT (Darbre *et al.*, 1986).

Cell Lines and Transfections—For transient transfection studies, αT3 cells (Windle *et al.*, 1990) were plated in Dulbecco's modified Eagle's medium with 5% fetal bovine serum, 5% horse serum and antibiotics at a density of 2×10^6 cells/100-mm plate on the day prior to transfection. Culture conditions for BeWo cells have been described previously (Silver *et al.*, 1987). Cells were transfected using the indicated amounts of vectors by calcium-phosphate coprecipitation (Gorman *et al.*, 1982) and were harvested at 3 days post-transfection. On the day after transfection, cells were cultured in complete medium containing either 10^{-6} M DHT, 10^{-6} M E in ethanol, or a similar amount of ethanol without steroid. Cells were replenished with fresh media on the day prior to harvesting. After harvesting, cellular lysates were prepared and assayed for CAT activity as described (Silver *et al.*, 1987; Bokar *et al.*, 1989). Initial transfections were carried out using media free of phenol red; however, later experiments indicated no difference in either the presence or absence of phenol red. Thus, data from both treatment groups were pooled for analyses. Charcoal-stripped serum was used in all transfections. In each cotransfection with pCMVhAR, a positive control for androgen response (pMMTV-CAT) and a negative control (pRSVCAT) for promoter specificity was included.

Gel-mobility Shift Assays—End-labeled DNA fragments were used as probes in gel-mobility shift assays using purified rAR-DBD. The procedural details of the assay are as described by Tan *et al.* (1992). Briefly, rAR-DBD (500 μg) was preincubated at 4 °C for 15 min in

binding buffer (10 mM Tris-HCl, pH 7.5, 25 mM KCl, 10% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol) with 2 μg of poly(dI-dC) and 100 μg of bovine serum albumin. Approximately 10,000 cpm (0.1–0.5 ng) of probe was added to the reaction, and binding was allowed to proceed for 15 min at room temperature. Subsequently, the anti-AR-DBD antibody, AR-52, was added to the appropriate binding reactions and incubations were continued for an additional 15 min at room temperature. Following an additional incubation at 4 °C for 30 min, 2 μl of 0.2% bromphenol blue was added to each sample and samples were subjected to electrophoresis in 4% polyacrylamide gels (0.5 × TBE) at 4 °C for 4–5 h. Gels were dried and exposed to Kodak X-Omat film at −70 °C.

Competition gel-mobility shift assays were performed with four different oligonucleotides corresponding to different regions of the α subunit promoter.

α36: 5′-AAATTGACGTCATGGTAAAAATTGACGTCATGGTAA-3′
(position −146/−111)

FGH: 5′-GTCATGGTAATTACACCAAGTACCCTTCAA-3′
(position −120/−90)

GHI: 5′-TTACACCAAGTACCCTTCAATCATTGGATG-3′
(position −110/−80)

IJK: 5′-TCATTGGATGGAATTTCCTGTTGATCCCAG-3′
(position −90/−60)

A 215-bp fragment of the α subunit promoter corresponding to bases −170 to +45 was also end-labeled by a fill-in reaction with the Klenow fragment of DNA polymerase I; 21 fmol of probe were used in each assay. Competitors were added immediately preceding the addition of probe in amounts corresponding to 12, 50, and 100 times the amount of probe in each reaction. The reactions remained on ice for 10 min. Antibody (AR-52) was added, and the reactions were further incubated at room temperature for 15 min followed by a 5-min incubation on ice before loading onto a 5% polyacrylamide gel.

RESULTS

Castration Leads to a Pituitary-specific Increase in αCAT Expression in Transgenic Mice—Adult male transgenic mice harboring either the Hα(−1500/+45)CAT or Bα(−315/+45)CAT transgenes were castrated. At 2 weeks post-castration, pituitaries, brains (frontal cortex), and livers were harvested and assayed for CAT activity (Bokar *et al.*, 1989; Keri *et al.*, 1991).

Pituitary CAT activity was significantly higher in castrated transgenic mice as compared to intact controls (Fig. 1). Consistent with localization of the transgene to gonadotropes (Kendall *et al.*, 1991; Hamernik *et al.*, 1992), the increase in CAT expression was pituitary-specific. The CAT activity observed in the brain of the HαCAT transgenic mice agrees with our previous studies of this line of mice (Bokar *et al.*, 1989; Keri *et al.*, 1991) and may reflect colocalization of transgene activity with previously observed patterns of extra-pituitary sites of expression of thyroid stimulating hormone and LH (Hojvat *et al.*, 1982; Hostetler *et al.*, 1987).

Based on these results we concluded that 1500 bp of proximal 5′-flanking region from the Hα subunit gene, or 315 bp of the bovine counterpart, is sufficient to confer responsiveness to negative feedback effects by testicular hormones. The predominant gonadal steroids secreted by the mouse testis are T and DHT, with minimal to undetectable levels of E (Desjardins and Chubb, 1983). We therefore tested whether replacement with either androgen or estrogen would act to attenuate the post-castrational rise in pituitary CAT activity.

Androgen and Estrogen Suppress the Post-castrational Rise in Pituitary CAT Activity in Either HαCAT or BαCAT Transgenic Mice—Adult, male transgenic mice were castrated and fitted with subcutaneous, constant release pellets containing either T, DHT, or E. Control animals were castrated and received pellets containing inert carrier. At 2 weeks post-castration, tissues were analyzed for CAT activity as above. Trunk blood was collected and serum was assayed for concentrations of T, DHT, E, and LH.

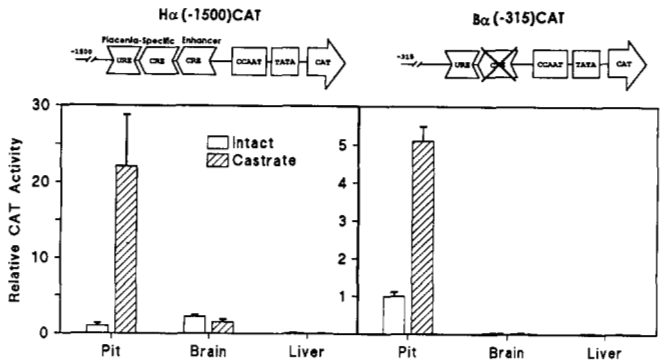

FIG. 1. **Castration results in a pituitary-specific increase in human and bovine αCAT expression in transgenic mice.** Adult male transgenic mice harboring either Hα(−1500/+45)CAT (n = 7) or Bα(−315/+45)CAT (n = 6) were castrated. At 2 weeks post-castration, pituitaries, brains, and livers were harvested from the castrates (*shaded bars*) and intact controls (*open bars*) and assayed for CAT activity. The cartoon at the *top* of the figure depicts the major regulatory regions of the human and bovine α subunit promoters (*URE*, upstream regulatory element; *CRE*, cAMP response element) (Bokar *et al.*, 1989; Silver *et al.*, 1987; Delegeane *et al.*, 1987). Aside from length, a primary difference between the human and bovine α transgenes is the absence of a functional CRE in the bovine α promoter.

The constant release pellets resulted in physiological concentrations of T and DHT (T = 7.8 ± 1.8 ng/ml; DHT = 1.4 ± 0.8 ng/ml) (Desjardins and Chubb, 1983) in serum. Serum concentrations of E averaged 323 ± 62 pg/ml. To further verify that biologically active concentrations of androgen were achieved, we also isolated seminal vesicles from each mouse. Consistent with the physiological concentrations of androgen assayed in serum, the paired weight of seminal vesicles was higher in T-implanted mice (178 ± 13 mg) and DHT-implanted mice (166 ± 7 mg) than either controls (41 ± 2 mg) or E-implanted mice (59 ± 3 mg).

The CAT activity assayed in pituitaries of either the HαCAT or BαCAT transgenic mice receiving T implants was significantly lower than that measured in pituitaries of castrate controls (Fig. 2, *A* and *B*). Thus, T replacement was effective in suppressing the post-castration increase in expression of both the Hα and Bα transgenes. To provide a physiological correlate for pituitary CAT activity, we also measured serum concentrations of LH. Consistent with the attenuation in pituitary CAT activity, treatment with T resulted in severely suppressed serum concentrations of LH. Thus, 1500 or 315 bp of proximal 5′-flanking region derived from either the Hα or Bα subunit gene is sufficient to confer negative regulation by T in transgenic mice.

The suppressive effects of DHT on transgene expression were not as pronounced as T. In light of the relative instability of DHT in blood, one explanation for the reduced suppression by DHT is simply that the dose of DHT was only minimally effective; however, serum concentrations of DHT were in a physiological range and sufficient to maintain seminal vesicle weight. Another explanation is that suppression of α transgene expression in T-implanted mice was a consequence of both a direct effect of androgen and an indirect effect that occurs after either target site or peripheral aromatization to estrogen. This would explain the partial suppression of pituitary CAT activity that occurred when animals were treated with either DHT (a non-aromatizable androgen) or E alone. Nevertheless, replacement with DHT did result in signifi-

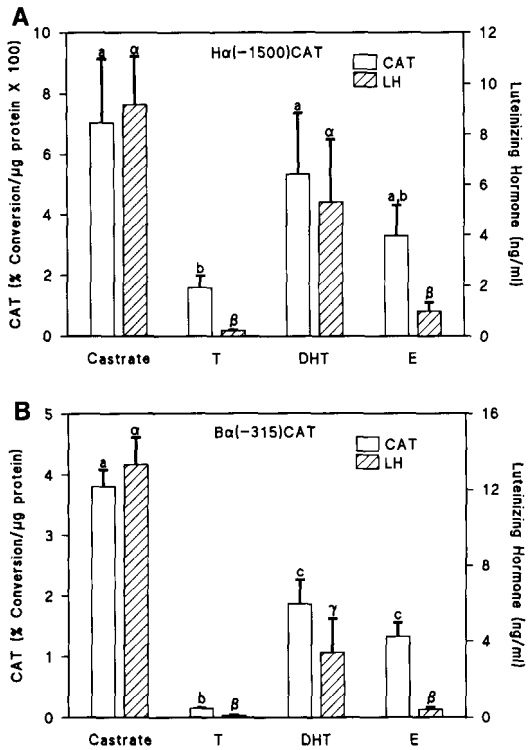

FIG. 2. **Androgen and estrogen suppress the post-castrational rise in pituitary CAT activity and serum concentrations of LH in either the Hα(−1500/+45)CAT (*panel A*) or Bα(−315/+45)CAT (*panel B*) transgenic mice.** Adult male, transgenic mice were castrated and fitted with constant release pellets containing either testosterone (*T*), 5α-dihydrotestosterone (*DHT*), or estradiol-17β (*E*). Control animals were castrated and received pellets containing inert carrier. Each treatment group contained a minimum of 7 animals. At 2 weeks post-castration, pituitaries were harvested and analyzed for CAT activity. Trunk blood was collected, and serum was assayed for LH concentrations. *Vertical bars* with different lettered superscripts represent statistical differences in CAT activity ($p < 0.05$). Similarly, *vertical bars* with different Greek symbols (α, β, γ) represent significantly different levels of serum LH ($p < 0.05$).

cantly suppressed pituitary expression of the BαCAT transgene. Thus, androgenic regulation of the α subunit gene in transgenic mice is demonstrable in two distinct lines of mice harboring α subunit transgenes from different species.

In general, effects of androgen replacement on serum concentrations of LH were similar to those for α transgene expression. That is, T replacement resulted in severely suppressed serum concentrations of LH in both lines of transgenic mice as compared to castrate controls. The effects of DHT were less pronounced and resulted in LH levels that were intermediate between those measured for castrate controls or mice implanted with either T or E.

In contrast to the effects of E in partially suppressing expression of the HαCAT and BαCAT transgenes, serum concentrations of LH in E-treated mice were not different from animals treated with T. As LH is composed of both an α and a β subunit, it may be that expression of LHβ is more sensitive than the α subunit to negative feedback effects of E.

From these experiments, we conclude that sequences contained within the proximal promoter regions of the Hα and Bα subunit genes can confer both androgenic and estrogenic regulation on a bacterial reporter gene in male, transgenic mice.

Co-expression of Human AR Suppresses Hα(−1500)CAT Expression in a Steroid-dependent Manner—Negative transcriptional regulation of the Hα subunit promoter by AR was studied by cotransfecting a human AR expression vector (pCMVhAR) with pHα(−1500)CAT into two cell lines known to express the α subunit gene −αT3 cells, a mouse cell line of gonadotrope lineage (Windle *et al.*, 1990) and BeWo cells, a human choriocarcinoma cell line (Bokar *et al.*, 1989). Data in the cotransfection experiments are presented as relative CAT activity. This was calculated from the activity of each treatment group (±AR, ±DHT) as percent of activity from the control plates. Controls are defined as activity of the test vector pHα(−1500)CAT in cells lacking treatment with both exogenous AR (transfection) and steroid. The co-expression studies were confined to the Hα subunit promoter for two important reasons. First, consistent with its normal pattern of expression, the Bα subunit promoter is inactive in BeWo cells (Bokar *et al.*, 1989). Second, although somewhat surprising in light of the robust expression of Bα(−315)CAT in transgenic mice, expression of this same construct is 10–30-fold lower than Hα(−1500)CAT in αT3 cells (data not shown), thus compromising any functional analysis of negative regulatory elements.

In αT3 cells, there was no effect of DHT at 50 ng of cotransfected AR (Fig. 3). At 0.5, 1.0, and 5.0 μg of AR, treatment of cells with DHT resulted in a significant reduction in expression of pHα(−1500)CAT. In addition to steroid-dependent suppression, cotransfection with 5.0 μg of pCMVhAR also resulted in steroid-independent suppression of CAT activity. It may be that this reflects steroid independent binding of AR to DNA due to high levels of expression of AR. Alternatively, the constitutively active CMV promoter may compete for binding to limiting *trans*-acting factors. In fact, a significant suppression of CAT activity was observed in both BeWo cells and αT3 cells when 10 μg of Hα(−1500)CAT was cotransfected with 5.0 μg of a CMV-growth hormone reporter vector (data not shown). Thus, "steroid-independent" suppression by pCMVhAR probably reflects a combination of high levels of expression of AR and competition for *trans*-acting factors by the CMV promoter. Regardless of the exact mechanism, both steroid-dependent

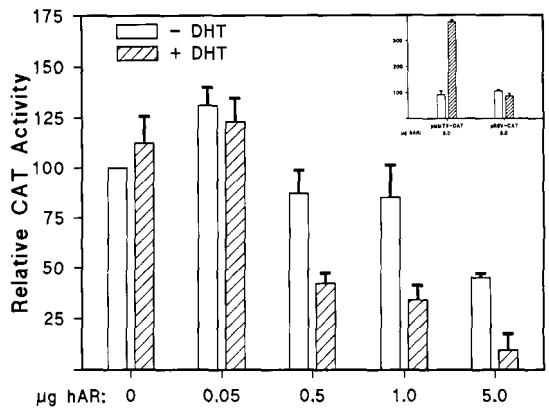

FIG. 3. **Co-expression of human AR in αT3 pituitary cells suppresses Hα(−1500/+45)CAT expression in a steroid-dependent manner.** Ten μg of pHα(−1500/+45)CAT were transiently cotransfected with the indicated amounts of human androgen expression vector (*AR*) into αT3 cells. On the day after transfection, cells were treated with medium containing 10^{-6} M DHT in ethanol or ethanol alone. Cells were harvested approximately 48 h later, and CAT assays were performed. Relative CAT activity is the percentage of CAT activity measured in lysates from cells transfected with 10 μg of pHα(−1500/+45)CAT in the absence of DHT and AR. Values represent the mean ± S.E. of triplicate plates in 4 independent transfections. CAT activities for pMMTV-CAT and pRSV-CAT cotransfected with 5.0 μg of pCMVhAR are expressed as the percentage of CAT activity measured for vector alone (see *inset*).

and steroid-independent suppression of Hα(−1500)CAT activity appear to be promoter-specific, because neither effect was observed when AR was cotransfected with pRSVCAT. A significant, steroid-dependent induction of CAT activity was observed when 5.0 μg of AR was co-expressed with pMMTV-CAT, consistent with the presence of multiple GRE/ARE sequences within the long terminal repeat of the MMTV promoter (Cato *et al.*, 1987; Otten *et al.*, 1988).

The ability of AR to inhibit expression of Hα was also demonstrable in BeWo cells. As in αT3 cells, there was no effect of DHT on expression of Hα(−1500)CAT at 50 ng of cotransfected AR; at 0.5 μg of AR, the inclusion of DHT in the media led to a steroid-dependent reduction in CAT activity (data not shown).

In contrast to AR, we were not able to demonstrate any steroid-dependent suppression of Hα(−1500)CAT expression when αT3 cells were cotransfected with a human ER expression vector (Fig. 4). This further confirms our previous observations in BeWo and MCF-7 (a human breast carcinoma cell line) cells that estrogen, unlike androgen, cannot directly regulate expression of the human α subunit gene (Keri *et al.*, 1991).

Collectively, these data suggest that AR can negatively regulate activity of the Hα subunit promoter in a steroid-dependent, promoter-specific manner. We next asked whether AR is capable of binding directly to sequences located within the proximal 1500 bp of 5′-flanking region of the Hα subunit gene.

AR Binds to an Element Residing within the Promoter-regulatory Region of the Human α Subunit Gene—To investigate binding of AR to the Hα subunit promoter, we prepared a series of restriction fragments spanning 1500 bp of the proximal 5′-flanking region (Fig. 5*A*, *upper panel*). Each fragment was end-labeled and used as a probe in gel-mobility shift assays using bacterially expressed and purified DNA binding domain of the rat AR (rAR-DBD). AR bound to probe

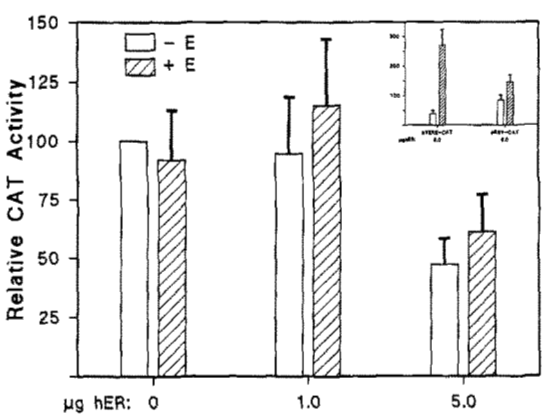

FIG. 4. **Estradiol fails to suppress expression of pHα(1500/+45)CAT transiently cotransfected with human ER expression vector in αT3 pituitary cells.** Ten μg of pHα(−1500/+45)CAT was transiently cotransfected with the indicated amounts of human ER expression vector (*hER*) into αT3 cells. On the day after transfection, cells were treated with medium containing either 10^{-6} M estradiol-17β in ethanol or ethanol alone. Cells were harvested approximately 48 h later, and CAT assays were performed. Relative CAT activity is the percentage of CAT activity measured in lysates from cells transfected with 10 μg of pHα(−1500/+45)CAT in the absence of estradiol and hER. Values represent the mean ± S.E. of triplicate plates in 2 independent transfections. CAT activities for pVERE-CAT and pRSV-CAT cotransfected with 5.0 μg of hER are expressed as the percentage of CAT activity measured for vector alone (see *inset*). The pVERE-CAT plasmid contains the vitellogenin ERE positioned upstream of the SV40 promoter without its enhancer (Keri *et al.*, 1991).

was identified by including a specific antibody to the rAR DNA binding domain (AR-52) (Tan *et al.*, 1992). Inclusion of both rAR-DBD and AR-52 further retards the migration of the probe through the gel if a binding site for AR is present within the probe sequence (illustrated in Fig. 5*A*, *lower panel*).

The results of a gel-mobility assay using eight restriction fragments spanning 1500 bp of Hα 5′-flanking region are shown in Fig. 5*B*. Also included in the assay was a fragment containing a previously defined ARE derived from the androgen-responsive C3 gene (Tan *et al.*, 1992). An antibody-dependent band with retarded migration was readily detected when the C3 ARE fragment was incubated with rAR-DBD and AR-52 (Fig. 5*B*, *lane 3*). Likewise, two fragments from the Hα subunit promoter appeared to bind AR. Fragment 2, located between −1100 and −850, probably represents a weak interaction as judged by the faint band (*lane 18*). In contrast, fragment 4, located between −486 and +45, appears to possess the highest intrinsic capacity to bind AR based on the intensity of the antibody-dependent band (*lane 12*). In fact, when fragment 4 was cleaved with *Sau*I (see Fig. 5*A*), a pronounced "super-shifted" band was evident for fragment 4B (−170/+45; *lane 6*), whereas no binding site was detected for fragment 4A (−486/−170; *lane 9*). Collectively, these data suggest that the binding of AR to fragment 4 can be fully accounted for by sequences located within the proximal 170 bp of Hα 5′-flanking region.

Akerblom *et al.* (1988) suggested that GR is capable of binding to three different regions within 170 bp of proximal 5′-flanking region of the human α subunit gene. At least one of these sites mapped to a region overlapping one of two tandemly repeated 18-bp CRE located between −146 and −110. To determine if AR might also interact with sequences spanning one or both of the CRE, we conducted the gel-mobility shift assay using −170-bp human α fragments with either one or both of the CRE deleted (hα-Δ18 and -Δ22, respectively). Surprisingly, the ability of these fragments to bind AR was not significantly affected despite the absence of the CRE (data not shown). To further explore the requirement for a functional CRE in AR binding, we tested the proximal promoter (−315/+45) of the Bα subunit gene that lacks a functional CRE (Bokar *et al.*, 1989). This region interacted with AR but with an apparent lower affinity than the homologous region from the Hα subunit promoter (data not shown). Significant sequence differences in the suspected AR binding region probably account for the differences in binding affinity noted for the human and bovine α subunit promoters (see Fig. 8). Thus, these data suggest that the interaction of AR with the α subunit promoter does not require sequences contained within tandemly repeated or single CREs.

To better define the sequences required for binding of AR, we further digested the Hα −170/+45 fragment (4B) with *Rsa*I (which cleaves at position −99; see Fig. 5*A*), end-labeled each fragment, and subjected each to gel-mobility shift assay. Neither subfragment exhibited binding to rAR-DBD suggesting that digesting with *Rsa*I effectively disrupted the AR binding site (Fig. 6). Curiously, the *Rsa*I site is contained within a region referred to as the junctional regulatory element (JRE) previously implicated as important for expression of human α subunit gene (Andersen *et al.*, 1990).

If the JRE does represent the critical binding site for AR, then this element (−120/−90) should be capable of displacing binding of AR to its larger parent radiolabeled fragment of the human α promoter, *i.e.* fragment 4B (−170/+45). This possibility was tested directly by oligonucleotide competition with four different oligonucleotides corresponding to sequences near the *Rsa*I site. Competitions were performed with

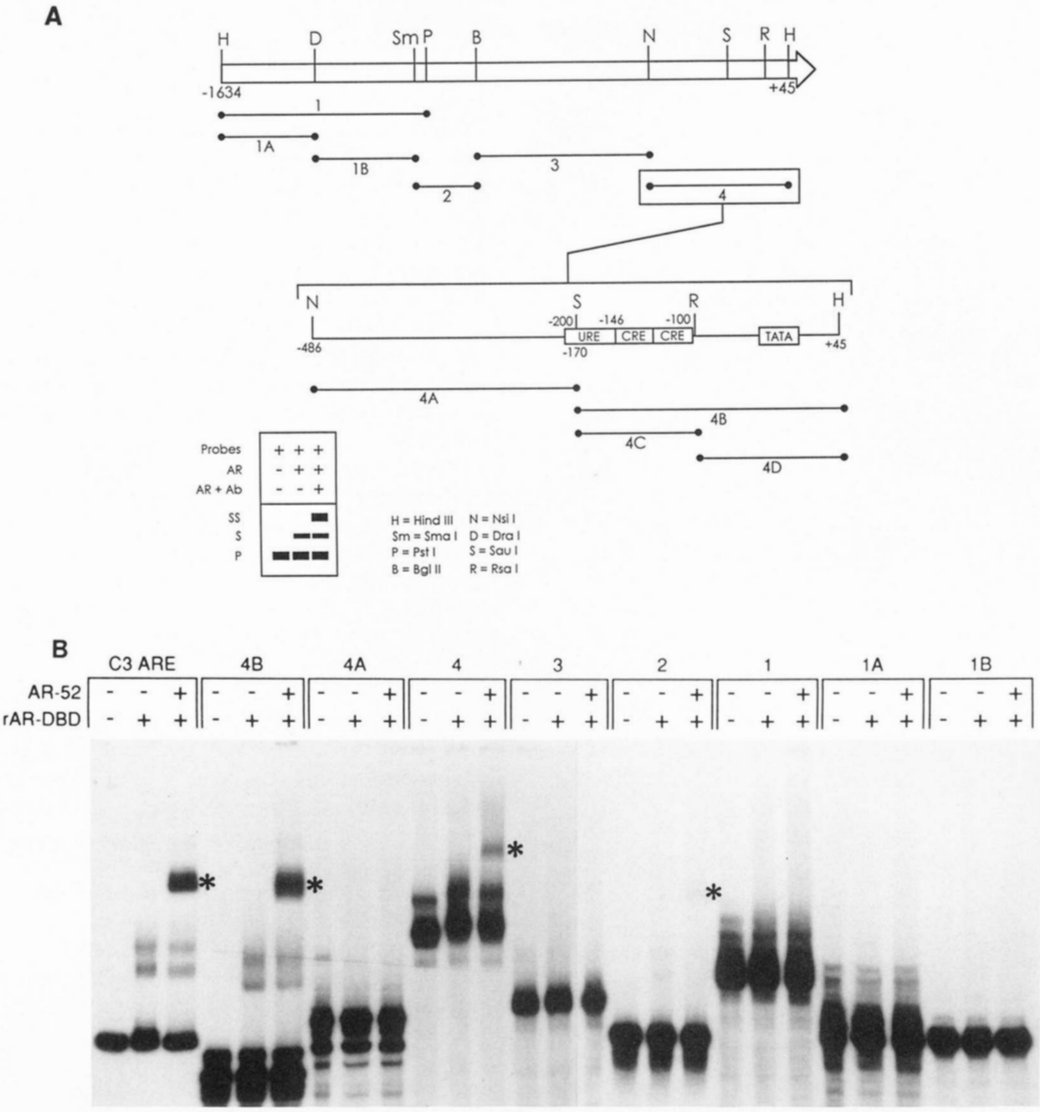

FIG. 5. **A high affinity binding site for AR resides within the proximal 170 bp of the human α subunit gene promoter.** Restriction fragments were prepared from approximately 1500 bp of proximal 5′-flanking region of the human α subunit gene (*panel A*). The *inset* to *panel A* shows an expected antibody-dependent shift (super-shift or *SS*). Also shown is the unshifted probe (*P*) and an antibody-independent shift (*S*). Fragments were end-labeled with ^{32}P and used as probes in a gel-mobility/super shift assay (*panel B*). Each fragment is represented in the mobility shift assay by three lanes; −/− = probe alone; −/+ = probe + rat AR DNA-binding domain (rAR-DBD); +/+ = probe + rAR-DBD + anti-rAR-DBD (AR-52). C3-ARE is an authentic androgen-responsive element derived from the C3 gene (Tan *et al.*, 1992). *Asterisks* indicate specific AR-DNA complexes as identified by their altered migration relative to binding assays carried out in the absence of antibody.

sequences representing either the tandem CRE (α36, −146/−111) (Bokar *et al.*, 1988), the JRE (FGH, −120/−90) (Andersen *et al.*, 1990), the CCAAT box (GHI, −110/−80) (Kennedy *et al.*, 1990), or a nonfunctional element (IJK, −90/−60) (Kennedy *et al.*, 1990). Consistent with AR binding specifically to the JRE region of the human α promoter, only the −120/−90 (FGH) fragment was capable of displacing binding of AR to the larger radioactive probe (fragment 4B), even when the other competitors were used at 100-fold excess (Fig. 7). Collectively, these results indicate that the JRE may represent a physiological target for binding of activated AR and suggest a mechanism for transcriptional attenuation whereby binding of AR to the JRE would prevent binding of another factor required for full transcriptional activity.

DISCUSSION

Hormonal regulation of gonadotropin synthesis and secretion is extremely complex involving, at the minimum, hypothalamic input of GnRH and gonadal input of steroids and regulatory peptides (Brinkley, 1981; Desjardins, 1981; Gharib *et al.*, 1990). The effects of gonadal steroids can occur both at the level of the hypothalamus to affect the synthesis and secretion of GnRH and at the level of the anterior pituitary (Crowley *et al.*, 1985; Gharib *et al.*, 1990). Finally, since gonadotropins are composed of two different subunits encoded by different genes, changes in the synthesis and secretion of the intact heterodimer may reflect coordinate but independent alterations in expression of both the common α subunit gene and the unique β subunit genes.

The dynamics of such a system are difficult to recapitulate *in vitro*. This problem is further exacerbated by the lack of continuous cell lines that synthesize and secrete biologically active LH. To circumvent these problems, we have utilized a transgenic mouse paradigm to study the molecular mechanisms underlying tissue-specific and hormonally regulated expression of the promoter from the gene encoding the com-

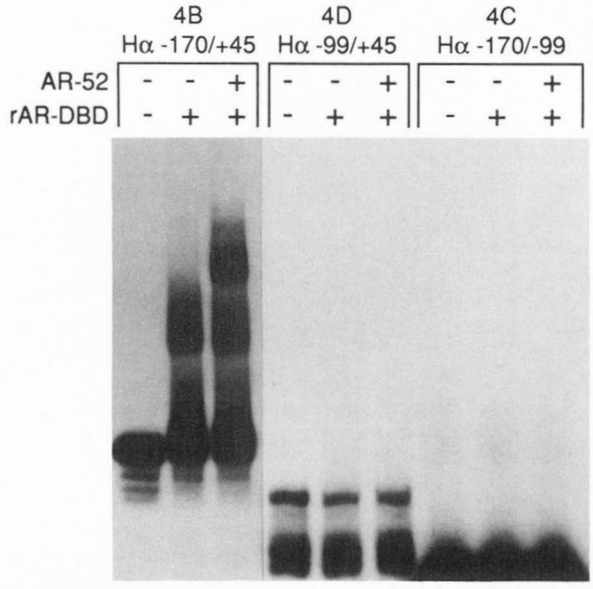

FIG. 6. **Digestion of human α(−170/+45) with *Rsa*I disrupts binding of AR.** Human α −170/+45 (fragment 4B) was isolated and subjected to *Rsa*I digestion resulting in two fragments, −99/+45 (fragment 4D) and −170/−99 (fragment 4C). Each fragment was end-labeled with ^{32}P and used in gel-mobility shift assays with rAR-DBD and AR-52 as described in Fig. 5 and under "Experimental Procedures." Only fragment 4B displayed an antibody-dependent change in migration.

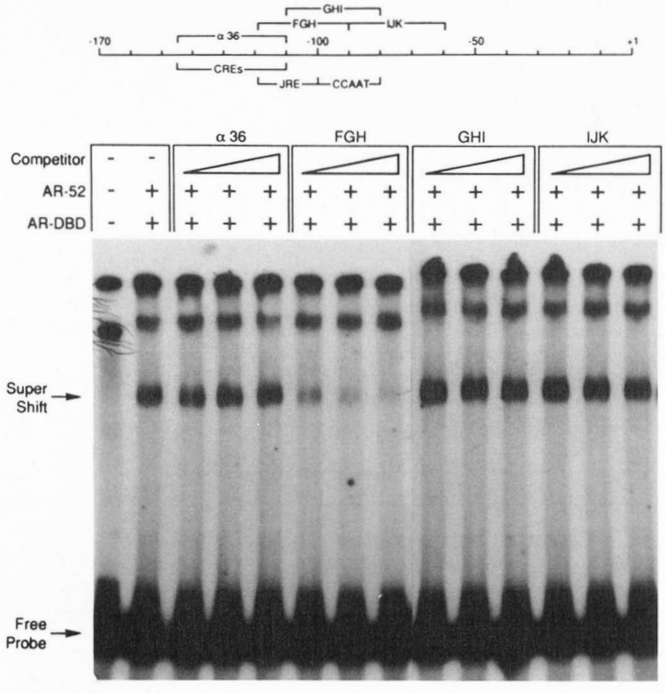

FIG. 7. **Oligonucleotide competition maps the AR binding element to sequences residing between −120/−90 in the human α promoter.** Human α −170/+45 (fragment 4B) was end-labeled with ^{32}P and used in a gel-mobility shift assay with rAR-DBD and AR-52 as described under "Experimental Procedures." Four separate unlabeled oligonucleotides were added to the binding reactions in increasing concentrations as indicated by the *wedge*. The competitor oligonucleotides were added at 12-, 50-, and 100-fold over the concentration of probe. A schematic diagram of the promoter is given at the *top* of the figure depicting previously characterized response elements on the *bottom* and the oligonucleotides used in the competition above.

mon α subunit of the glycoprotein hormones. Using this transgenic mouse model, we demonstrated previously that either 1500 or 315 bp of proximal 5′-flanking region derived from either the human or bovine α subunit genes are sufficient to confer both pituitary-specific and, in the case of the human promoter, placenta-specific expression (Bokar *et al.*, 1989). Furthermore, it appeared that pituitary expression of both transgenes is confined to gonadotropes (Kendall *et al.*, 1991; Hamernik *et al.*, 1992), thus validating the use of hormonal replacement paradigms to study regulated expression of the transgenes.

Recently, we have used castration and steroid replacement experiments to establish that both the human and bovine α subunit transgenes contain sufficient regulatory information to confer responsiveness to estradiol 17-β and GnRH, thus paralleling normal, physiological regulation of the endogenous α subunit gene (Keri *et al.*, 1991; Hamernik *et al.*, 1992). Surprisingly, however, we were not able to demonstrate the presence of a high affinity binding site for ER in the 5′-flanking region of the human α subunit gene, suggesting that negative regulation by estrogen must occur through an indirect mechanism at either the level of the hypothalamus or the anterior pituitary or a combination of the two (Keri *et al.*, 1991).

Our present studies not only expand our analysis of the regulation of the α subunit promoter in transgenic mice to males, where we can demonstrate that both the human and bovine promoters are capable of conferring negative regulation by androgen, they also suggest fundamentally different mechanisms for androgenic and estrogenic regulation of α subunit gene expression. As with the estrogen replacement experiments in females, the pattern of suppression of expression of the α subunit transgenes by androgen mimics the normal physiological role of androgen in suppressing transcription of both the α and β subunit genes of the gonadotropins. Unlike estrogen, however, some component of the negative regulation of α subunit gene expression may be accounted for by a mechanism involving direct binding of activated AR to a negative androgen response element (ARE), which functions as a negative element as a result of its location in the proximal promoter of the α subunit gene. Consistent with the known differences in binding requirements for AR and ER, this putative negative ARE cannot bind ER, nor does it respond to activated ER in either choriocarcinoma cells (Keri *et al.*, 1991) or pituitary cells (Fig. 4).

Evidence for a direct effect of AR on α subunit gene expression stems from three basic experimental paradigms: 1) transgenic mice, 2) transfection analysis, and 3) gel-mobility shift assays. First, we have shown androgen suppression of both Hα- and BαCAT expression in the pituitaries of transgenic mice. Second, co-expression of AR with HαCAT in both BeWo and αT3 cells leads to an androgen-dependent attenuation of HαCAT expression. Third, we have detected a high affinity, sequence-specific binding site for AR located in the proximal promoter of the Hα subunit gene. Finally, the loss of AR binding upon digestion of Hα(−170/+45) with *Rsa*I implicates the region at −101 as the functional AR binding element. As shown in Fig. 8, this region bears homology to a consensus GRE/ARE, and, in fact, purified GR has been shown to protect this region from DNase I digestion (Akerblom *et al.*, 1988). In addition, an analogous region in the bovine α promoter also shares homology to the consensus GRE/ARE, further supporting the notion that this conserved sequence serves as a physiological target for AR.

The molecular mechanism(s) by which the binding of AR to the α subunit promoter attenuates transcription is not yet

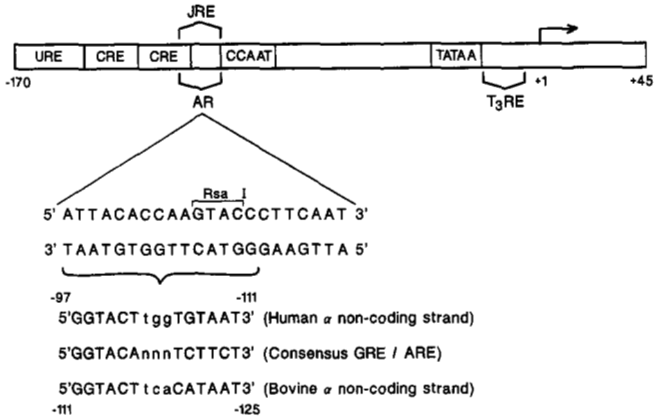

FIG. 8. **Regulatory elements residing within the proximal promoter of the human glycoprotein hormone α subunit gene.** Androgen receptor binds immediately downstream of the tandem CRE in the human α subunit gene promoter. Contained within the AR binding region is a potential GRE (*bracketed region*) identified by Akerblom *et al.* (1988). Previously defined regulatory elements include: upstream regulatory element (*URE*) and CRE (Delegeane *et al.*, 1987; Bokar *et al.*, 1989), JRE (Andersen *et al.*, 1990), CCAAT (Kennedy *et al.*, 1990), and a thyroid hormone response element (*T_3RE*) (Chatterjee *et al.*, 1989).

clear. At least four possible mechanisms have been proposed for transcriptional repression. These include competition, quenching, direct, and squelching (Levine and Manley, 1989). Akerblom *et al.* (1988) suggested that glucocorticoid suppression of human α subunit gene expression could occur by a competition mechanism involving binding of GR to a region overlapping one of two tandemly repeated cAMP response elements in the human α subunit gene promoter. Thus, the binding of GR would presumably block the binding of CREB and so diminish expression of the human α subunit gene. Although we cannot preclude the possibility of spatial interference of CREB binding by AR, it would seem less likely as the tandemly repeated CRE are not necessary for binding of AR. This is indicated by three lines of evidence. First, the ability of AR to bind to Hα(−170/+45) is not compromised with the deletion of either one or both of the tandem CRE (data not shown). Second, AR is capable of binding to the proximal 315 bp of 5′-flanking region of the bovine α subunit gene (data not shown). This region lacks a functional CRE (Bokar *et al.*, 1989). Third, oligonucleotide competition with the tandem CRE was not capable of displacing binding of AR to the human α(−170/+45) region (Fig. 7). Thus, the AR binding site and CREB binding sites do not overlap.

Instead of co-mapping to the CRE, the highest affinity AR binding site appears to reside within the JRE, another regulatory element located just downstream of the tandem CRE (Andersen *et al.*, 1990) (Fig. 8). The JRE region has also been implicated as binding GR (Akerblom *et al.*, 1988). Thus, it appears that the binding of AR may block binding not of CREB, but of another regulatory protein (JRE transcription factor) previously defined as binding to the JRE and important for basal expression of the human α subunit gene (Andersen *et al.*, 1990). Targeted mutation coupled with transfection analysis represents the most direct approach for testing the functional significance of this AR binding site. Unfortunately, block mutants that disrupt AR binding activity also disrupt binding of the JRE transcription factor that in turn is required for transcriptional activity (Kennedy *et al.*, 1990; Andersen *et al.*, 1990). Thus, a direct test of this hypothesis awaits the definition of point mutations that eliminate binding of AR to the α subunit gene while retaining full basal transcriptional activity.

How does androgen attenuate α subunit gene expression *in vivo*? It has long been recognized that testicular androgens can suppress synthesis and secretion of pituitary gonadotropins, in particular, LH (Desjardins, 1981). Nevertheless, there remains considerable question as to whether the predominant site of action of androgen negative feedback occurs at the level of the hypothalamus or at the pituitary. Some have suggested that the negative feedback effects of androgen on LH synthesis and secretion in cattle and sheep occur predominantly at the level of the hypothalamus (Tilbrook *et al.*, 1991). In contrast, in men it appears that androgen effects occurring directly at the level of the pituitary may play an important role in regulation of LH synthesis and secretion (Finkelstein *et al.*, 1991). The transgenic mouse studies presented herein shed some light on the question of site of action of androgen in regard to the α subunit gene. In contrast to our previous work with ER, the presence of a high affinity, sequence-specific binding site for AR, located within the proximal promoter of the human α subunit gene, provides a clear target for AR. In addition, we have shown that activity of the human α subunit promoter can be inhibited in a steroid-dependent manner upon co-expression of AR in both pituitary cells and choriocarcinoma cells. Thus, these data point toward a direct pituitary site of action for androgen in negative regulation of α subunit gene expression. Clearly, a pituitary site of action does not preclude a hypothalamic site of action for androgen as well. In fact, full negative regulation of α subunit gene expression by androgen likely involves a combination of the two. The construction of transgenic mice harboring mutant α subunit promoters that retain full transcriptional activity but do not bind AR should provide a powerful approach for assessing the relative contribution of hypothalamic and pituitary sites of action of androgen in suppressing transcription of the α subunit gene.

REFERENCES

Ahlquist, J. A. O., Franklyn, J. A., Ramsden, D. B., and Sheppard, M. C. (1990) *J. Mol. Endocrinol.* **5,** 1–6

Akerblom, I. E., Slater, E. P., Beato, M., Baxter, J. D., and Mellon, P. L. (1988) *Science* **241,** 350–353

Andersen, B., Kennedy, G. C., and Nilson, J. H. (1990) *J. Biol. Chem.* **265,** 21874–21880

Bokar, J. A., Roesler, W. J., Vandenbark, G. R., Kaetzel, D. M., Hanson, R. W., and Nilson, J. H. (1988) *J. Biol. Chem.* **263,** 19740–19747

Bokar, J. A., Keri, R. A., Farmerie, T. A., Fenstermaker, R. A., Andersen, B., Hamernik, D. L., Yun, J., Wagner, T., and Nilson, J. H. (1989) *Mol. Cell. Biol.* **9,** 5113–5122

Bonsall, R. W., Rees, H. D., and Michael, R. P. (1985) *J. Steroid Biochem.* **23,** 389–396

Brinkley, H. J. (1981) *Biol. Reprod.* **24,** 22–43

Cato, A. C. B., Henderson, D., and Ponta, H. (1987) *EMBO J.* **6,** 363–368

Chatterjee, V., Lee, J. K., Rentoumis, A., and Jameson, J. L. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 9114–9118

Chatterjee, V., Madison, L. D., Mayo, S., and Jameson, J. L. (1991) *Mol. Endocrinol.* **5,** 100–110

Crowley, W. F., Jr., Filicori, M., Spratt, D., and Santoro, N. F. (1985) *Recent Prog. Horm. Res.* **41,** 473–531

Darbre, P., Page, M., and King, R. J. B., (1986) *Mol. Cell. Biol.* **6,** 2847–2854

Delegeane, A. M., Ferland, L. H., and Mellon, P. L. (1987) *Mol. Cell. Biol.* **7,** 3994–4002

Desjardins, C. (1981) *Biol. Reprod.* **24,** 1–21

Desjardins, C., and Chubb, C. (1983) *Am. J. Physiol.* **244,** E575–E580

Fiddes, J. C., and Talmadge, K. (1984) *Recent Prog. Horm. Res.* **40,** 43–74

Finkelstein, J. S., Whitcomb, R. W., O'Dea, L., Longcope, C., Schoenfeld, D. A., and Crowley, W. F., Jr. (1991) *J. Clin. Endocrinol. Metab.* **73,** 609–620

Gharib, S. D., Wierman, M. E., Shupnik, M. A., and Chin, W. W. (1990) *Endocr. Rev.* **11,** 177–199

Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) *Mol. Cell. Biol.* **2,** 1044–1051

Hamernik, D. L., Keri, R. A., Clay, C. M., Clay, J. N., Sherman, G. B., Sawyer, H. R., Jr., Nett, T. M., and Nilson, J. H. (1992) *Mol. Endocrinol.* **6,** 1745–1755

Hojvat, S., Baker, G., Kirsteins, L., and Lawrence, A. M. (1982) *Neuroendocrinology* **34,** 327–332

Hostetler, G., Eaton, A., Carnes, M., Gildner, J., and Brownfield, M. S. (1987) *Neuroendocrinology* **46,** 185–193

Kendall, S. K., Saunders, T. L., Jin, L., Lloyd, R. V., Glode, L. M., Nett, T. M., Keri, R. A., Nilson, J. H., and Camper, S. A. (1991) *Mol. Endocrinol.* **5,** 2025–2036

Kennedy, G. C., Andersen, B., and Nilson, J. H. (1990) *J. Biol. Chem.* **265,** 6279–6285
Keri, R. A., Andersen, B., Kennedy, G. C., Hamernik, D. L., Clay, C. M., Brace, A. D., Nett, T. M., Notides, A. C., and Nilson, J. H. (1991) *Mol. Endocrinol.* **5,** 725–733
Levine, M., and Manley, J. L. (1989) *Cell* **59,** 405–408
Naess, O., Attramadal, A., and Aakvaag, A. (1975) *Endocrinology* **96,** 1–9
Otten, A. D., Sanders, M. M., and McKnight, G. S. (1988) *Mol. Endocrinol.* **2,** 143–147
Paul, J. S., Ortolano, G. A., Haisenleder, D. J., Stewart, J. M., Shupnik, M. A., and Marshall, J. C. (1990) *Mol. Endocrinol.* **4,** 1943–1955
Quarmby, V. E., Kemppainen, J. A., Sar, M., Lubahn, D. B., French, F. S., and Wilson, E. M. (1990) *Mol. Endocrinol.* **4,** 1399–1407
Schanbacher, B. D., Winters, S. J., Rehm, T., and D'Occhio, M. J. (1984) *J. Steroid Biochem.* **20,** 1227–1232
Silver, B. J., Bokar, J. A., Virgin, J. B., Vallen, E. A., Milstead, A., and Nilson, J. H. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 2198–2202
Tan, J., Marschke, K. B., Ho, K. C., Perry, S. T., Wilson, E. M., and French, F. S. (1992) *J. Biol. Chem.* **267,** 4456–4466
Thieulant, M.-L., and Pelletier, J. (1979) *J. Steroid Biochem.* **10,** 677–687
Tilbrook, A. J., de Kretser, D. M., Cummins, J. T., and Clarke, I. J. (1991) *Endocrinology* **129,** 3080–3092
Wierman, M. E., and Wang, C. (1990) *Biol. Reprod.* **42,** 563–571
Wierman, M. E., Gharib, S. D., LaRovere, J. M., Badger, T. M., and Chin, W. W. (1988) *Mol. Endocrinol.* **2,** 492–498
Windle, J. J., Weiner, R. I., and Mellon, P. L. (1990) *Mol. Endocrinol.* **4,** 597–603