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Androgen Receptor Drives Transcription of Rat PACAP in Gonadotrope Cells

Constance M. Grafer and Lisa M. Halvorson
Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9032
Corresponding author.
Address all correspondence and requests for reprints to: Lisa M. Halvorson, Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75390-9032., E-mail: ude.nretsewhtuoSTU@nosrovlaH.asiL.
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Abstract

Gonadotropin expression is precisely regulated within the hypothalamic-pituitary-gonadal axis through the complex interaction of neuropeptides, gonadal steroids. and both gonadal- and pituitary-derived peptides. In the anterior pituitary gland, the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) modulates gonadotropin biosynthesis and secretion, acting both alone and in conjunction with GnRH. Steroid hormone feedback also influences gonadotropin expression via both direct and indirect mechanisms. Evidence from nonpituitary tissues suggests that PACAP may be a target for gonadal steroid regulation. In the present study, we show that androgen markedly stimulates rat (r) PACAP promoter-reporter activity in the LβT2 mature mouse gonadotrope cell line. 5′-Serial deletion analysis of reporter constructs identifies 2

regions of androgen responsiveness located at (−915 to −818) and (−308 to −242) of the rPACAP promoter. Androgen receptor (AR) binds directly to DNA *cis*-elements in each of these regions in vitro. Site-directed mutagenesis of 3 conserved hormone response element half-sites straddling the (−308 to −242) region dramatically blunts androgen-dependent PACAP promoter activity and prevents AR binding at the mutated promoter element. Chromatin immunoprecipitation demonstrates that endogenous AR binds the homologous region on mouse chromatin in LβT2 cells in both the presence and absence of androgen. These data demonstrate that androgen stimulates PACAP gene expression in the pituitary gonadotrope via direct binding of AR to a specific cluster of evolutionarily conserved hormone response elements in the proximal rPACAP gene promoter. Thus, androgen regulation of pituitary PACAP expression may provide an additional layer of control over gonadotropin expression within the hypothalamic-pituitary-gonadal axis.

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide with wide-ranging, pleiotropic function. A member of the secretin/glucagon/vasoactive intestinal peptide polypeptide family, PACAP was originally isolated from the ovine hypothalamus based on its ability to stimulate cAMP formation in rat pituitary cells (1). Initially defined as a hypothalamic releasing factor, PACAP expression was subsequently found in the gonadotrope subpopulation as well as in the folliculostellate cells of the anterior pituitary gland, suggesting that PACAP may act as an autocrine-paracrine factor in this tissue (2). PACAP and its receptors are expressed in multiple tissues, including the central and peripheral nervous systems, smooth muscle of the lung and intestinal tract, and endocrine organs such as the placenta, adrenal, parathyroid, and endocrine pancreas; however, it is found most abundantly within the hypothalamic-pituitary-gonadal (HPG) axis (3, 4). Reproductive functions include modulation of gonadotropin biosynthesis and secretion within the anterior pituitary gland, both alone and in concert with GnRH, as well as regulation of gonadal development and function (5–21).

Gonadotropin biosynthesis, secretion and, ultimately, normal reproductive function are tightly controlled within the HPG axis by a complex series of interactions between multiple hormones arising from the hypothalamus, the gonads, and the anterior pituitary gland itself. Steroid hormone feedback is a key feature of the HPG axis with gonadal steroids regulating gonadotropin biosynthesis and secretion at the level of the hypothalamus and pituitary either by directly altering gene transcription, by indirectly altering GnRH pulse patterns in the hypothalamus, or by effects on peptides such as activin B and follistatin made by gonadotropes or other cells within the pituitary (22–29).

The importance of gonadal steroid feedback to gonadotrope cells themselves is highlighted by studies using gonadotrope-specific estrogen receptor (ER)α conditional knockout transgenic mice that display infertile or subfertile phenotypes. Results differed depending on the model, but evidence suggested that the fertility defects could be due to an estrogen-dependent dysregulation of LH expression (30, 31). Gonadotrope-specific androgen receptor (AR) conditional knockouts have not been reported; however, androgens are known to regulate FSHβ expression by altering mRNA transcription and stability in rats as well as by directly regulating the mouse FSHβ promoter activity in synergy with activins via interaction with Sma- and Mad-related proteins (32–36). Androgens have also been shown to repress LHβ subunit expression via AR interactions with the transcription factor steroidogenic-factor 1 (37).

Despite its critical role in multiple physiologic systems, relatively little is known about the transcription factors, hormones, or signaling pathways that regulate PACAP gene expression (38). In a previous study, we showed that GnRH stimulates rat (r) PACAP promoter activity and increases endogenous PACAP mRNA in immortalized mouse gonadotrope LβT2 cells. The action of GnRH is mediated via protein kinase A, protein kinase C, and MAPK intracellular signaling pathways acting on cAMP response element and activator protein 1 sites in the proximal PACAP gene promoter (39). A broad goal of our continuing study was to identify additional hormonal factors that

regulate PACAP expression in pituitary gonadotropes. A number of studies in nonpituitary tissues have suggested that PACAP may be regulated by steroid hormones. Estrogens stimulate PACAP expression in the ventromedial nucleus and arcuate nucleus, whereas progesterone increases PACAP and PACAP receptor mRNAs in the rat hypothalamus (40, 41). In PC12 rat pheochromocytoma cells PACAP mRNA is increased by dexamethasone (42). However, to our knowledge, there is no information regarding the effect of androgens on PACAP expression.

In the current study we hypothesized that the PACAP gene is a target for androgen regulation via interaction with the ligand-activated androgen receptor (AR) in gonadotrope cells. Accordingly, using transient transfection, EMSA, and chromatin immunoprecipitation (ChIP), we now present data demonstrating that androgen activates PACAP gene transcription via interaction of AR with a noncanonical grouping of hormone response elements (HREs) in the proximal PACAP promoter.

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Materials and Methods

Cell lines and reagents

LβT2 mouse gonadotrope cells were generously provided by Dr P. L. Mellon (University of California, San Diego, California) and were maintained in high-glucose DMEM (Gibco/Life Technologies, Gaithersburg, Maryland) supplemented with 10% fetal bovine serum (FBS, Gibco) (vol/vol), 1 mM sodium pyruvate, and 1% penicillin/streptomycin (vol/vol) at 37°C in humidified 5% CO_2/95% air. 5α-androstan-17β-ol-3-one (5-dihydrotestosterone, DHT) was purchased from Sigma-Aldrich (St Louis, Missouri) and dissolved in absolute ethanol.

RNA extraction, reverse transcription, and PCR

LβT2 cells (1×10^6) were cultured for 24 hours in phenol red-free DMEM supplemented with 5% charcoal:dextran-stripped FBS (Gemini Bio-Products, West Sacramento, California). Pituitary

and ovary were collected from an 8-week-old female C57BL/6 mouse. Total RNA was prepared using TRIzol according to manufacturer's instructions (Ambion [Austin, Texas]/Life Technologies), and DNase treated using the Turbo DNA-free kit (Ambion/Life Technologies). DNase-treated total RNA (1 μg) was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen [Carlsbad, California]/Life Technologies) primed with random hexamer. A parallel reaction lacking reverse transcriptase was used as an additional negative control. PCRs contained 1× PCR buffer with 1.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphate, 200 nM of each primer, 100 ng cDNA template, and 0.5 U Platinum Taq DNA polymerase (Invitrogen/Life Technologies). Cycling conditions were as follows: 94°C for 2 minutes, then 35 cycles at 94°C × 30 seconds, 55°C × 30 seconds, and 72°C × 60 seconds and a final extension for 2 minutes at 72°C. Primers for mouse AR exon 1 and mouse α-tubulin have been previously described (39, 43) and are detailed in Supplemental Table 1 published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org. PCR products were separated on 1% agarose gel and photographed using a ChemiImager 4400 Imaging System (Alpha Innotech, Santa Clara, California). The identity of the amplified AR bands for LβT2 cells and mouse pituitary were confirmed by purification and sequencing of the PCR product.

Western blot analysis

LβT2 cells (10×10^6) were plated overnight in 100-mm dishes in high-glucose DMEM supplemented with 10% (vol/vol) FBS, 1 mM sodium pyruvate, and 1% (vol/vol) penicillin/ streptomycin, cultured an additional 24 hours in phenol red-free Opti-MEM (Gibco/Life Technologies) supplemented with 5% charcoal:dextran-stripped FBS, and then treated with 100 nM DHT or vehicle in plain phenol red-free Opti-MEM for 6–48 hours, as indicated. Nuclear extracts were prepared using the method described by Andrews and Faller (44). Mouse testis tissue extract was prepared from snap-frozen, pulverized adult C57BL/6 testis in radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitor cocktails. Protein

was quantitated with the BCA Protein Assay Kit (Thermo Scientific Pierce, Rockford, Illinois), separated on a 10% SDS-PAGE gel and transferred onto Immobilon-P membrane (Millipore Corp, Billerica, Massachusetts). After blocking, the membrane was probed with a 1:250 dilution of anti-AR antibody (PG21, Millipore) followed by a 1:20 000 dilution of anti-rabbit HRPO secondary antibody (NA34VS, GE HealthCare, Pittsburgh, Pennsylvania). Bound secondary antibody was visualized using the SuperSignal West Dura chemiluminescent substrate kit (Thermo Scientific Pierce) and exposure to autoradiography film. The membrane was then stripped with Restore Western Blot Stripping Buffer (Thermo Scientific) and reprobed with 1:5000 anti-α-tubulin (DM1A, Sigma-Aldrich) followed by antimouse-HRPO (NA931VS, GE HealthCare) and visualized with SuperSignal West Pico substrate kit (Thermo Scientific Pierce).

EMSA

LβT2 cells were grown in 100-mm culture dishes and treated with 100 nM DHT or vehicle for 48 hours as described for Western blot analysis. Cells were harvested by scraping in cold PBS containing 0.6 mM EDTA, the resulting sample was pelleted, and nuclear proteins were extracted according to the method of Andrews and Faller (44). Recombinant full-length AR was overexpressed in Sf9 insect cells using a human Flag-AR baculovirus construct graciously provided by Dr. Elizabeth M. Wilson at The University of Colorado. Production of recombinant hFlag-AR was carried out at the University of Colorado Cancer Center Protein Production/MoAb/Tissue Culture core facility. Sf9 cell infection, growth conditions, and subsequent generation of whole-cell extracts were carried out as previously described (35). Double-stranded oligonucleotides were end labeled with [γ-^{32}P]ATP and purified over a Quick Spin G-25 Sephadex Column (Roche Applied Science, Indianapolis, Indiana). Oligonucleotide probe sequences are provided in Supplemental Table 1. Sequence for the consensus HRE probe has been previously reported (35). Nuclear protein or hFlag-AR extracts were incubated with 60 000 cpm of probe in DNA-binding buffer containing 10 mM Tris-HCl, pH 7.5, 40 mM KCl, 0.2 mM EDTA, 10 mM DTT, 10% (vol/vol) glycerol, 0.8 mg/ml BSA, and 50 μg/ml

poly[d(I-C)] for 1 hour prior to resolution electrophoresis on a 5% nondenaturing polyacrylamide gel in 0.5× Tris-borate-EDTA buffer. Where appropriate, 2 μL of antisera were added for the final 30 minutes of incubation and are the same as listed for ChIP. The gel was dried and subjected to autoradiography.

Transient transfection of cell lines

LβT2 cells (2×10^5)/well were plated overnight in 24-well plates, rinsed in HBSS, and cultured an additional 24 hours in phenol red-free Opti-MEM with 5% charcoal:dextran-stripped FBS, and then transfected approximately 48 hours after plating with 200 ng/well luciferase reporter vector, 100 ng pSG5-AR, and 2.5 ng/well pGL4.74 expressing *Renilla* luciferase (Promega Corp., Madison, Wisconsin) as an internal control using Fugene 6 (Roche) at a 3:1 (microliters/μg of DNA) ratio in fresh 5% stripped fetal calf serum phenol red-free-Opti-MEM according to manufacturer's instructions. The cells were treated the following day with DHT (100 nM or as indicated) or vehicle for 24 hours. Cell extracts were prepared and luciferase activity was assessed with the Promega Dual-Luciferase Reporter Assay System according to manufacturer's instructions using a Berthold Detection Systems luminometer (Pforzheim, Germany). Relative light units (RLU) for luciferase in each sample were corrected against RLU of a cotransfected *Renilla* luciferase reporter. The fold-change following androgen treatment was then calculated relative to the matching vehicle-treated control. The fold-induction of deletion or mutant promoter-reporter constructs was expressed as the percent activity relative to the fold-induction of the full-length or wild-type control construct, as applicable.

Plasmids

The rPACAP promoter-pGL3 luciferase reporter constructs have been previously described (39). Additional 5′-deletion constructs and site-directed mutations were generated by PCR or using the Stratagene QuikChange II Site-Directed Mutagenesis Kit, respectively (La Jolla, California). Sequences of mutated residues are identical to those reported for EMSA probes in

Supplemental Table 1. The fidelity of all constructs was verified by nucleotide sequencing. The pXP2-FSHβLUC luciferase reporter construct, containing −2000/+698 of the rat FSHβ promoter, was provided by Dr. U. B. Kaiser (Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts). The pSG5-AR human AR expression construct was provided by Dr. D. P. McDonnell (Duke University, Durham, North Carolina).

ChIP assay

LβT2 cells (5×10^6) were plated to 100-mm dishes overnight in high-glucose DMEM with 10% FBS, sodium pyruvate, and antibiotics. Cells were rinsed the following day with HBSS and cultured an additional 48 hours in phenol red-free Opti-MEM with 10% charcoal:dextran-stripped FBS, sodium pyruvate, and antibiotics, treating for the indicated times with either 100 nM DHT or vehicle. Chromatin preparation and immunoprecipitation procedures were adapted from the method of Dahl and Collas (45). At the time of harvest, the cells were rinsed with Dulbecco's PBS containing 20 mM sodium butyrate (NaB), and then fixed in 1% methanol-free formaldehyde (P/N 28906, Thermo Scientific) in PBS/NaB buffer for 8 minutes at room temperature with gentle rotation. Fixation was halted by addition of 125 mM glycine for 5 minutes at room temperature with gentle rotation. Fix solution was removed, and cells were rinsed 1× with PBS/NaB and collected to 1.5-mL microcentrifuge tubes by scraping in PBS/NaB. Suspended cells were washed 1× in PBS/NaB and cell pellets were resuspended in 120 µL lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% sodium dodecyl sulfate, 20 mM NaB, 1 mM phenylmethylsulfonylfluoride, 1× EDTA-Free complete Mini protease inhibitor cocktail (P/N 11836170001, Roche) and 1× PhosSTOP phosphatase inhibitor cocktail (P/N 04906837001, Roche), incubating 5 minutes on ice. Chromatin was sheared by sonication with 6 × 5 minute cycles in an ice-cold Bioruptor bath sonicator (Diagenode, Inc, Denville, New Jersey) to the 100- to 500-bp range, which was confirmed by separation on a 1% agarose gel with ethidium bromide staining. Sheared chromatin was clarified by centrifugation at 15 000 × *g*, 4°C and the supernatant was collected in a new tube. The sample was

diluted to a standard 2 A_{260} Units (a minimum of 10-fold dilution) with RIPA Buffer (1× RIPA (P/N 20–188, Millipore) containing 20 mM NaB, 1 mM phenylmethylsulfonylfluoride, 1× protease inhibitor cocktail, 1× phosphatase inhibitor cocktail) and stored at −80°C. An aliquot of the diluted chromatin for each sample was reserved as an input reference sample.

The diluted chromatin was incubated overnight with rotation at 4°C with Protein A Dynabeads (Life Technologies) conjugated with either PG-21 anti-AR (Millipore), normal rabbit IgG (Santa Cruz Biotechnology, Inc, Santa Cruz, California) or no antibody, then washed 3× in RIPA buffer with protease and phosphatase inhibitors and 1× in Tris-EDTA, pH 8.0, with transfer to a new tube prior to final elution to reduce background. Immunoprecipitations and input samples were incubated overnight at 68°C in elution buffer (20 nM Tris-HCl, pH 7.5; 5 mM EDTA; 50 nM NaCl) containing 1% sodium dodecyl sulfate, and 50 µg/mL Proteinase K, the eluted material collected, and the beads were reeluted for an additional 5 minutes at 68°C, pooling the eluate with the first fraction. DNA was isolated from the eluate by extraction with phenol-chloroform-isoamyl alcohol, reextracted with chloroform-isoamyl alcohol, and then ethanol precipitated at −80°C. The nucleic acid pellet was washed 2× with 70% ice-cold ethanol, dried, and resuspended in Tris-EDTA, pH 8.0.

Immunoprecipitated DNA eluates and 1% of input samples were analyzed by conventional PCR with primers amplifying 300 bp of the mouse PACAP proximal promoter 5′-untranslated region (UTR) that is homologous to rPACAP −410 to −115 (relative to the transcriptional start site) which spans HRE4/5 and HRE6, or genomic mouse PACAP exon 5. Primer sequences are listed in Supplemental Table 1. PCRs were as follows: 4 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 45 seconds at 51°C, and 30 seconds at 72°C, and a final extension for 1 minute at 72°C. PCR product was separated on a 1% agarose gel in the presence of ethidium bromide and photographed with a Fujifilm LAS-3000 (Quansys Biosciences, Logan, Utah).

Statistical analysis

Statistical calculations were performed using the SigmaPlot statistical software package (Systat Software, Inc, Chicago, Illinois). Data were analyzed for normality followed by one-way ANOVA and Tukey's test for post hoc comparisons. Statistical significance was set as indicated in each figure legend with at least a $P < .05$.

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Results

Expression of AR mRNA and protein in gonadotropes

Immortalized LβT2 mouse gonadotrope cells have been used as a model system for the study of steroid regulation of gonadotropin gene expression and secretion, and express many factors characteristic of mature gonadotropes including the gonadotropin subunits, GnRH-receptor, follistatin, inhibin, activin, and activin receptors (22, 35, 43, 46–51). They express AR mRNA but the expression of AR protein has been poorly characterized (35, 43, 47). Although early studies reported AR immunoreactivity comparable to that of mouse and rat pituitary extracts, more recent studies have reported difficulty detecting endogenous AR, concluding that the overall level of endogenous AR in LβT2 cells is low (35, 36, 46). Similarly, unstable expression of ER, progesterone receptor (PR), and glucocorticoid receptor (GR) has been reported in LβT2 cells despite having readily measurable levels of mRNA (35, 52). In light of this, we wished to confirm that LβT2 cells, in our hands, retained the ability to express AR and respond to androgen stimulation in order to support use of this cell line as a model system to study androgen regulation of PACAP expression.

AR expression is regulated at multiple levels with posttranscriptional regulation occurring independent of, and oftentimes opposite to, changes in mRNA levels (53, 54). To confirm that our LβT2 cells were expressing AR, we first used RT-PCR to detect AR mRNA. As shown in Figure 1A, AR transcripts were readily detected in mouse ovary, anterior pituitary, and untreated LβT2 cells. AR mRNA levels were not

affected by exposure to androgen (data not shown). This result is consistent with previous findings for LβT2 cells (47).

A variety of studies have demonstrated that androgen treatment leads to an overall increase of AR within the nucleus due to a decrease in the rate of AR degradation and conformational changes that promote dimerization, translocation into the nucleus, and high-affinity DNA binding (53, 55–58). Therefore, we reasoned that AR protein expression in LβT2 cell nuclear extracts would be highest following androgen treatment. We prepared nuclear extracts from cells cultured for 6, 12, 24, or 48 hours with 100 nM DHT vs vehicle control. After repeatedly failing to detect AR with conventional substrates, we were ultimately successful in detecting AR by Western blot, which depended on the use of highly sensitive, extended duration chemiluminescent substrates, an indication that the overall level of AR in LβT2 cells is extremely low. Western blot analysis (Figure 1B) revealed that AR (110 kDa) was faintly detectable in nuclear extracts of non-androgen-treated cells at 6 hours, gradually declining and becoming undetectable at later time points. In contrast, AR was detected at 6 hours in nuclear extracts from DHT-treated cells and increased in abundance over the 48-hour treatment period, indicating that LβT2 cells do, indeed, retain the ability to respond to exogenous androgen treatment, at least in terms of increasing nuclear AR.

We next wished to determine whether the AR could bind a hormone response *cis*-element on DNA. Nuclear extracts of DHT-treated vs untreated LβT2 cells were tested using EMSA (Figure 1C). Multiple binding complexes were formed when LβT2 nuclear extracts were incubated with a radiolabeled probe containing a consensus HRE (described in Reference 35 and Supplemental Table 1). Coincubation with an AR-specific antibody produced an AR-specific supershift in the nuclear extract of DHT-treated but not control-treated cells (compare Figure 1C, lanes 9 and 12). As a positive control, whole-cell extract from Sf9 insect cells containing a baculovirus expressed human Flag-tagged AR (Flag-AR) was found to bind robustly to the same consensus HRE (Figure 1C, lanes 3–5). As with Western blot, extended exposure times were necessary to detect

the LβT2 AR-specific signal on EMSA, also suggesting that AR levels in LβT2 cells are quite low.

Androgen stimulates PACAP transcription in gonadotropes

Gonadally derived androgens are well-described modulators of gonadotropin biosynthesis through feedback mechanisms (59). We hypothesized that androgen may also regulate the synthesis of PACAP within gonadotropes. LβT2 cells were transiently cotransfected with the rPACAP promoter (−1916 to +906 relative to the transcription start site) fused to the pGL3 luciferase reporter with or without an AR expression construct, and treated with varying doses of DHT. DHT-specific promoter stimulation was observed when the PACAP promoter reporter construct was cotransfected in the presence of the AR expression construct (Figure 2). Androgen stimulation of promoter activity was dose dependent and maximal stimulation (nearly 17-fold) was attained following 1 nM (10^{-9} M) DHT treatment. We did not observe any androgen-specific promoter activity in the presence of the endogenous LβT2 AR alone. A similar phenomenon was observed for the DHT response of the positive control FSHβ reporter construct included in our experiment, as has also been reported in other studies using LβT2 cells (35, 48). As such, we elected to perform all subsequent transient transfection assays in the presence of the cotransfected AR expression plasmid.

The androgen response maps to 2 regions of the rPACAP gene promoter

We next wished to determine which specific nucleotide sequence(s) within the −1916/+906 PACAP gene promoter confer androgen responsiveness using the same transient transfection approach. Transfection of serial 5′-deletions of the rPACAP gene promoter fused to the pGL3 luciferase reporter (constructs previously described in Reference 39) demonstrated a significant loss of androgen responsiveness between base pairs −915 and −402 (48% loss) with a further dramatic loss of promoter activity to the level of the empty reporter vector following deletion to position −77 (87% loss) (Figure 3A).

In order to more finely map the regions of androgen responsiveness, an additional series of 5′-truncations were generated within regions −915 to −402 (Figure 3B) and −402 to −77 (Figure 3C). Our results identified 2 androgen-responsive regions located between bases −915 and −818 and between −308 and −242 of the rPACAP promoter.

Androgen responsiveness coincides with consensus HREs identified by in silico analysis

The AR is a member of the Class I steroid receptor family which also includes the PR and the GR. Ligand-activated AR typically binds DNA as a homodimer to classical steroid HREs represented by the sequence 5′-TGTTCT-3′, arranged as inverted repeats separated by a 3-nucleotide spacer (60). In silico analysis of the rPACAP promoter sequence was used to identify candidate hormone response *cis*-elements falling within the areas of androgen responsiveness identified by transient transfection analysis (61). No canonical bipartite androgen-response elements were found; however, multiple regions with similarity to consensus steroid HRE half-sites were identified within, or closely flanking, the areas of interest identified by the 5′-deletion analysis as indicated by the schematic representation of the rPACAP promoter in Figure 4.

Two steroid HRE half-sites, designated HRE1 and HRE2, were identified within the −915 to −818 region and a third, designated HRE3, was found to directly flank the junction that was arbitrarily created at base −818 during the creation of our 5′-truncation constructs. Similarly, sequence analysis revealed a grouping of steroid hormone response half-sites associated with the −308 to −242 region. Two overlapping half-sites were designated as HRE4/5 that straddle the junction created with our truncation at base −242. Because the 2 overlapping elements, HRE4/5, formed a contiguous sequence, they were treated as a single regulatory element for the purpose of downstream analyses. The element, HRE6, lies just 14 bp beyond the −242 junction. Although HRE6 lies beyond the boundary of the identified androgen-responsive region, clustering of multiple HRE half-sites is commonly found in steroid-responsive genes

and has been found to synergistically promote cooperative binding of steroid receptors (62); therefore we elected to include HRE6 in further studies. The candidate HREs share a high level of conservation when compared with analogous regions of the mouse and human PACAP promoters.

HREs located in or near the −915 to −818 region of the PACAP promoter bind AR but show minimal functional contribution to the overall androgen response

Transient transfection experiments were performed in LβT2 cells to analyze the importance of the candidate HRE1, HRE2, and HRE3 *cis*-elements in region −915 to −818 of the rPACAP promoter. These elements were mutated singly or in combination in the context of the long −1916/+906 rPACAP promoter reporter construct and analyzed for effects on the androgen response by luciferase assay (Figure 5A). Mutation of the HREs alone demonstrated a modest, but not statistically significant, blunting of the androgen response. Mutation of all 3 elements together showed a marginally significant reduction (18%) in androgen response (*P* = .048 vs wild-type).

EMSA was used to assess the ability of AR to bind the wild-type and mutated HRE DNA sequences. To maximize the AR signal, we elected to perform the gel-shift assays using baculovirus-expressed recombinant AR. As shown in Figure 5B, a wild-type oligonucleotide probe spanning −861 to −828 of the rPACAP promoter, containing HRE1 and HRE2, binds AR from baculovirus-infected whole-cell extracts (Figure 5B, lane 2) creating a high-molecular weight protein-nucleotide complex. No similar binding was observed in mock-infected cell extracts (Figure 5B, lane 1). Addition of anti-AR antibody (Figure 5B, lane 4) but not control IgG (Figure 5B, lane 3) resulted in a supershift of the complexes as well as an enhancement of binding intensity. Enhancement of steroid receptor-DNA binding intensity following addition of receptor-specific antibody has been previously reported for both AR and PR EMSA (63, 64) and may represent antibody-associated stabilization of the receptor-DNA binding complex. Note that the AR antibody plus labeled

oligonucleotide probe alone, in the absence of protein extract, shows no complex formation (Figure 1C, lane 2).

Oligo probes carrying the same mutations in HRE1 and HRE2 or the double mutant as used in the aforementioned transfection analysis were also tested for AR binding by EMSA (Figure 5B). Mutation of HRE1 nearly eliminated AR binding (Figure 5B, lanes 5 and 6) even in the presence of anti-AR antibody whereas mutation of HRE2 had little effect (Figure 5B, lanes 7 and 8). Mutation of HRE1+2 together (Figure 5B, lanes 9 and 10) was similar to the mutation of HRE1 alone. These findings are consistent with our transfection data wherein the greater loss of AR binding paralleled the loss of ability to stimulate promoter transcriptional activity.

AR binding at HRE3 was tested with another oligonucleotide probe spanning −825 to −791 (Figure 5C). An AR-specific supershift was observed for high-molecular weight protein-nucleotide complexes in extracts containing overexpressed AR but not in mock-infected cell extracts (Figure 5C, lane 5 vs lane 2). Mutation of HRE3 eliminated AR binding (Figure 5C, lanes 7 and 8) on this oligonucleotide. This result was surprising because the identical mutation had no effect on androgen responsiveness in transient transfection (Figure 5A). The triple mutation of HRE1+2+3 was not tested on EMSA due to the extreme length of the probe that would be necessary. Therefore, although AR seems capable of binding the HRE3 promoter element, HRE3 alone is inadequate to confer androgen responsiveness. We cannot rule out the possibility of a cooperative effect with HRE1 and HRE2 but the transfection data suggest that HRE3 does not confer any significant additional effect over that of HRE1+2 alone.

HREs located in or near the −308 to −242 region of the PACAP promoter strongly bind AR and are critical to the overall androgen response

As shown in Figure 6, transfection of mutated promoter-reporter constructs and EMSA were again used to evaluate the ability of AR to bind and promote transcription via candidate HREs

identified by our in silico analysis of the rPACAP promoter, this time focusing on the −308 to −242 region. Three HRE half-sites fell within this region of interest: HRE4/5, which was subsequently treated as a single unit, and HRE6. The HREs were mutated singly or in combination in the context of the long −1916/+906 rPACAP promoter reporter construct and transiently transfected into LβT2 cells, which were then treated with androgen and evaluated for promoter activity by luciferase assay. We observed a profound loss of androgen responsiveness upon mutation of either HRE4/5 or HRE6 (55% and 45% reduction, respectively) (Figure 6A). The combined effect of mutating HRE4/5 and HRE6 together resulted in a 73% loss of androgen response. Finally, we asked what the comparative contribution was from the upstream androgen-responsive region HRE1+2 in relation to that of HRE4/5 and HRE6. Assay of the combined mutants indicates that the contribution of HRE1+2 appears to be minor and additive in the context of the downstream HRE sites, given that the mutation of HRE1+2 resulted only in a small and statistically insignificant additional loss of androgen responsiveness when combined with either the HRE4/5 or HRE6 mutations.

EMSA with overlapping oligonucleotide probes was used to examine AR binding at the HRE4/5 and HRE6 promoter elements (Figure 6B). Labeled wild-type probes were tested in binding reactions with whole-cell extracts from either mock-infected or baculovirus-AR-infected insect cells. rPACAP (−308/−279), containing no putative HREs and included as a negative control, did not bind AR (Figure 6B, lanes 1–3). The probe containing HRE4/5 alone (−278/239) did bind AR (Figure 6B, lane 5), which was supershifted in the presence of anti-AR antibody (Figure 6B, lane 6). The probe containing HRE6 alone (−238/−214) surprisingly did not bind AR (Figure 6B, lanes 11 and 12), but the probe containing both half-sites, HRE4/5+6 (−255/−216), bound AR with much greater intensity and produced larger molecular weight binding complexes than HRE4/5 alone, suggesting that the presence of the HRE6 half-site is essential for promoting high-affinity AR binding in this region.

AR binding at HRE4/5+6 was further dissected by examining the AR binding characteristics of the wild-type and mutant rPACAP (−255/−216) oligonucleotide probes (Figure 6C). The mutated residues are identical to those present in the mutant luciferase reporter constructs used in Figure 6A. Mutation at HRE4/5 (Figure 6A, lane 6) eliminated the ultrahigh molecular weight binding complexes seen with the wild-type probe. Remaining binding complexes were completely supershifted with anti-AR antibody (Figure 6A, lane 7), confirming that AR was still bound to the probe even in the presence of the HRE4/5 mutation. Interestingly, mutation of HRE6 (Figure 6A, lane 8) eliminated most the lower molecular weight binding complexes but some ultrahigh molecular weight complexes still persisted. These complexes were also supershifted with anti-AR antibody (Figure 6A, lane 9), confirming that AR was a component of these binding complexes as well. The combined HRE4/5+6 mutant virtually eliminated all protein-DNA complex formation even in the presence of supershifting antibody, indicating that both HRE4/5 and HRE6 participate in AR binding at this locus. The results of our EMSA analysis agree well with the functional data obtained for HRE4/5 and HRE6 in our transfection experiments, confirming the importance of AR binding at HRE4/5+6 for androgen responsiveness of the PACAP promoter.

AR binds the endogenous proximal mouse PACAP promoter in LβT2 gonadotrope cells

ChIP analysis was performed to evaluate the ability of AR to bind to the endogenous mouse PACAP proximal gene promoter. Nontransfected LβT2 cells were cultured in the presence or absence of 100 nM DHT and fixed at 1 hour or 48 hours after treatment. PCR primers were used to amplify a region of the mouse PACAP proximal promoter 5′-UTR, which is analogous to the rat HRE4/5 and HRE6 regions that confer primary androgen responsiveness. We found that endogenous AR bound to the proximal PACAP promoter in LβT2 cells and that this binding was maintained following DHT treatment (Figure 7). Although there appeared to be a trend toward increased AR binding following DHT treatment at the 48-hour time point, measurements of band intensity normalized against input values

over a series of 5 experiments did not indicate a statistically significant difference between control and DHT-treated samples. As a control for specificity, primers amplifying PACAP exon 5, located 5.3 kb downstream, were also used in PCR analysis. No bands could be amplified from the precipitated DNA in this region even though a robust signal was obtained from the input chromatin control samples.

Go to:

Discussion

As would be predicted by their central role within the HPG axis, the expression of gonadotropin genes is tightly regulated at both the biosynthetic and secretory levels through the complex interaction of neuropeptides (GnRH and PACAP), gonadal steroids (estrogens, progestins, and androgens), and gonadal- and pituitary-derived peptides (activin, inhibin, and follistatin). PACAP modulates gonadotropin biosynthesis and secretion, acting both alone and in concert with GnRH. Although less potent than GnRH, PACAP has been demonstrated to increase secretion of LH, FSH, and free α-subunit by perifused primary pituitary cells (5–7). The stimulatory effects of PACAP on LH secretion have been confirmed in vivo (8). PACAP also has been shown to regulate α-, LHβ, FSHβ, and GnRH-receptor gene promoter activity and mRNA levels (9–15). Furthermore, PACAP stimulates gonadotrope and folliculostellate cell production of follistatin, a binding protein that blocks activin stimulation of FSH biosynthesis (65).

There is strong evidence that androgen feedback directly acts on gonadotrope cells to regulate gonadotropin gene expression, but it has not been known whether androgen regulates pituitary expression of the neuropeptide PACAP (33, 35, 36, 66). We have used transient transfection of gonadotrope LβT2 cells with various rPACAP-luciferase reporter constructs to identify functional androgen-response regions of the PACAP promoter and to evaluate the role of specific candidate HREs. Multiple studies have used this cell line to examine androgen repression (37, 46, 67) and activation (35, 36, 48) of gonadotropin gene

expression through direct and indirect mechanisms. Because androgen stimulation in the presence of the endogenous LβT2 AR is relatively weak, several of these studies have amplified the steroid hormone response by cotransfecting AR expression constructs, a method we have adopted in the current study. The same strategy has been employed to study ER, PR, and GR in LβT2 cells (35, 36, 52, 68, 69). In the studies reported here, the rPACAP promoter was robustly activated by androgen in the context of the immortalized LβT2 gonadotrope, with activation that is dependent on the presence of AR.

To our knowledge, our study is the first to directly examine androgen regulation of PACAP gene expression; however, our findings are consistent with the few prior studies that have examined PACAP expression in males, wherein androgen levels were altered through effects of castration with or without testosterone replacement. Castration of 2-month-old rats dramatically reduced PACAP peptide in all brain areas, including the pituitary, within the first week after castration (70), suggesting that androgen or other gonadal factors positively regulated PACAP expression. Interestingly, the levels of PACAP in the castrated animals gradually recovered, and pituitary PACAP concentrations were significantly higher than initial levels within 4 months after castration. Based on prior findings by our laboratory that GnRH induces PACAP expression (39), we could hypothesize that this increase resulted from the rise in hypothalamic GnRH levels that follow castration. It must be noted that castration produces complex and wide-ranging effects within the HPG axis. For example, gain or loss of hormones or other gonadal factors such as inhibin affects GnRH production in the hypothalamus and directly alters gonadotropin expression in the pituitary.

Androgen responsiveness mapped to 2 distinct regions on the rPACAP promoter: (−915 to −818) and (−308 to −242). Of the 6 putative HREs identified by in silico sequence analysis within these regions, site-directed mutagenesis and EMSA studies indicate that the combination of HRE4/5 and HRE6 is critical for mediating DHT-dependent PACAP promoter activity, whereas the cluster of HRE1+2+3 perhaps plays an ancillary role. This

finding is consistent with the fact that HRE4/5 and HRE6 and flanking sequences show a higher degree of cross-species sequence conservation in comparison with the HRE1+2+3 region, suggesting that the HRE4/5 and HRE6 carry a greater functional value within the organism.

The blunting of androgen response following mutagenesis of HRE1, 2, or 3, or combinations thereof, never achieved the degree of loss seen with truncation of the −915 to −818 promoter fragment. It is possible that our site-directed mutagenesis failed to destroy core AR-binding residues; however, our EMSA analysis indicates that AR does not bind oligonucleotides carrying the mutated HRE1+2 or mutated HRE3. In addition, sequence analysis of the mutated promoter regions fails to detect any remaining sequence elements with homology to known HREs. Therefore, the possibility remains that there may be other unidentified *cis*-elements in this region important for mediating the androgen response, perhaps through interaction of AR with DNA-bound transcription factors, as reported for inhibition of LHβ gene expression (37).

Although HRE4/5 and HRE6 contain half-sites that conform well to consensus HRE sequences, they are too far apart to be considered a canonical androgen response element (ARE) represented by the sequence 5′-TGTTCT-3′, arranged as inverted repeats separated by a 3-nucleotide spacer (60). Nevertheless, site-directed mutagenesis of HRE4/5+6 resulted in a dramatic loss of androgen-dependent PACAP promoter activity and a loss of AR binding at the mutated promoter element, indicating that the combined elements constitute a functional androgen response element. ChIP-on-chip approaches have recently been used in an attempt to define functional AREs on human chromosomes and found that the majority did not conform to the sequence of a canonical ARE and had variable length spacers (71, 72). HRE4/5 is comprised of two HREs overlapping by 1 residue and bears similarity to an ARE direct repeat. It is separated from HRE6 by a 12-residue spacer and HRE5+6 is similar to a head-to-head ARE. A later study concluded that the architecture of a few reported noncanonical ARE sequences was incorrect, proposing instead that the AREs actually conformed

to a general rule of 2 TGTTCT-like motifs separated by exactly 3 nucleotide spacers (73). Whether this finding would hold true for all noncanonical AREs identified by ChIP-on-chip is unclear.

The promoters of steroid-responsive genes frequently contain clusters of HREs that act in a synergistic fashion to promote cooperative binding of steroid hormone receptors (62, 74). AR binding at rPACAP HRE4/5+6 fits this model very well. In fact, AR binding on the −255/−216 probe containing HRE4/5+6 resulted in the formation of what appear to be multimeric ultrahigh molecular weight protein-DNA complexes even in the absence of supershifting anti-AR antibody. This higher molecular weight complex might be the result of cooperative binding of multiple AR dimers. Alternatively, it could represent the incorporation of additional factors into a macromolecular binding complex.

The promoter sequence surrounding, and including, the HRE4/5+6 element is almost completely conserved in rat, mouse, and human. As we have shown by ChIP, endogenous AR binds this region on mouse chromatin, suggesting that this locus is important for androgen responsiveness of the PACAP gene in other mammalian species as well.

This study provides the first direct evidence that androgen stimulates PACAP gene expression in the context of the pituitary gonadotrope. The mechanism of activation occurs, in part, through direct binding of AR to a specific cluster of evolutionarily conserved HREs located within the proximal rPACAP gene promoter. Regulation of steroid-mediated transcription in the LβT2 cell line appears to be much more complex than initially imagined. Although we have established in our study that HRE4/5+6 is important for driving androgen-mediated PACAP transcription in the context of the promoter-reporter construct, to date, we have been unable to show that androgen alone induces endogenous PACAP mRNA in LβT2 cells, even in the context of AR overexpression (data not shown). In the context of the endogenous gene, an additional, as yet unknown, series of events may need to occur to initiate this process.

We must note that it is entirely possible that there are additional positive and negative regulatory regions of the endogenous PACAP promoter that fall outside the region we studied, and that may influence the overall responsiveness to androgen in the context of the whole cell. In fact, PACAP testis-specific exons have been identified 13.5 kb upstream of the translational start sites in rat (16) and 10.9 kb upstream in humans (17), which result in the expression of a unique testis-specific PACAP isoform. Interestingly, in a parallel study, we have observed that cotreatment of LβT2 cells with DHT significantly augments the induction of endogenous PACAP mRNA by GnRH and also synergistically increases the transcriptional response of the PACAP-luciferase-reporter construct (Grafer, C. M., and L.M. Halvorson, unpublished data). The mechanism for these observations has not yet been elucidated.

Androgen regulation of pituitary PACAP expression could provide an additional layer of control over gonadotropin expression within the HPG axis. Androgens are only one group within a larger family of steroid hormones known to act on the gonadotrope. We note that androgens have been shown to suppress LHβ and α-subunit expression, but have also been repeatedly shown to up-regulate FSHβ transcriptional activity and mRNA levels (32). Thus, androgens may increase FSHβ gene expression via direct transactivation as well as via increases in intrapituitary PACAP levels. Interestingly, it has been reported that PACAP is preferentially stimulated by slow GnRH pulse frequencies that also preferentially stimulate FSHβ relative to LHβ gene expression (75). Thus, PACAP may contribute to differential gonadotropin gene expression when present in a more physiologic hormonal context. Further studies are also underway to determine whether PACAP gene expression is modulated by other gonadal steroids such as estrogens and progestins.

Go to:

Supplementary Material

Supplemental Data:

Go to:

Acknowledgments

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Disclosure Summary: The authors have nothing to disclose.

Go to:

Footnotes

Abbreviations:

AR
androgen receptor
ARE
androgen response element
ChIP
chromatin immunoprecipitation
DHT
dihydrotestosterone
FBS
fetal bovine serum
GR
glucocorticoid receptor

HPG
: **hypothalamic-pituitary-gonadal**

HRE
: **hormone response element**

PACAP
: **pituitary adenylate cyclase-activating polypeptide**

PR
: **progesterone receptor**

RIPA
: **radioimmunoprecipitation assay**

RLU
: **relative light unit**

UTR
: **untranslated region.**

Go to:

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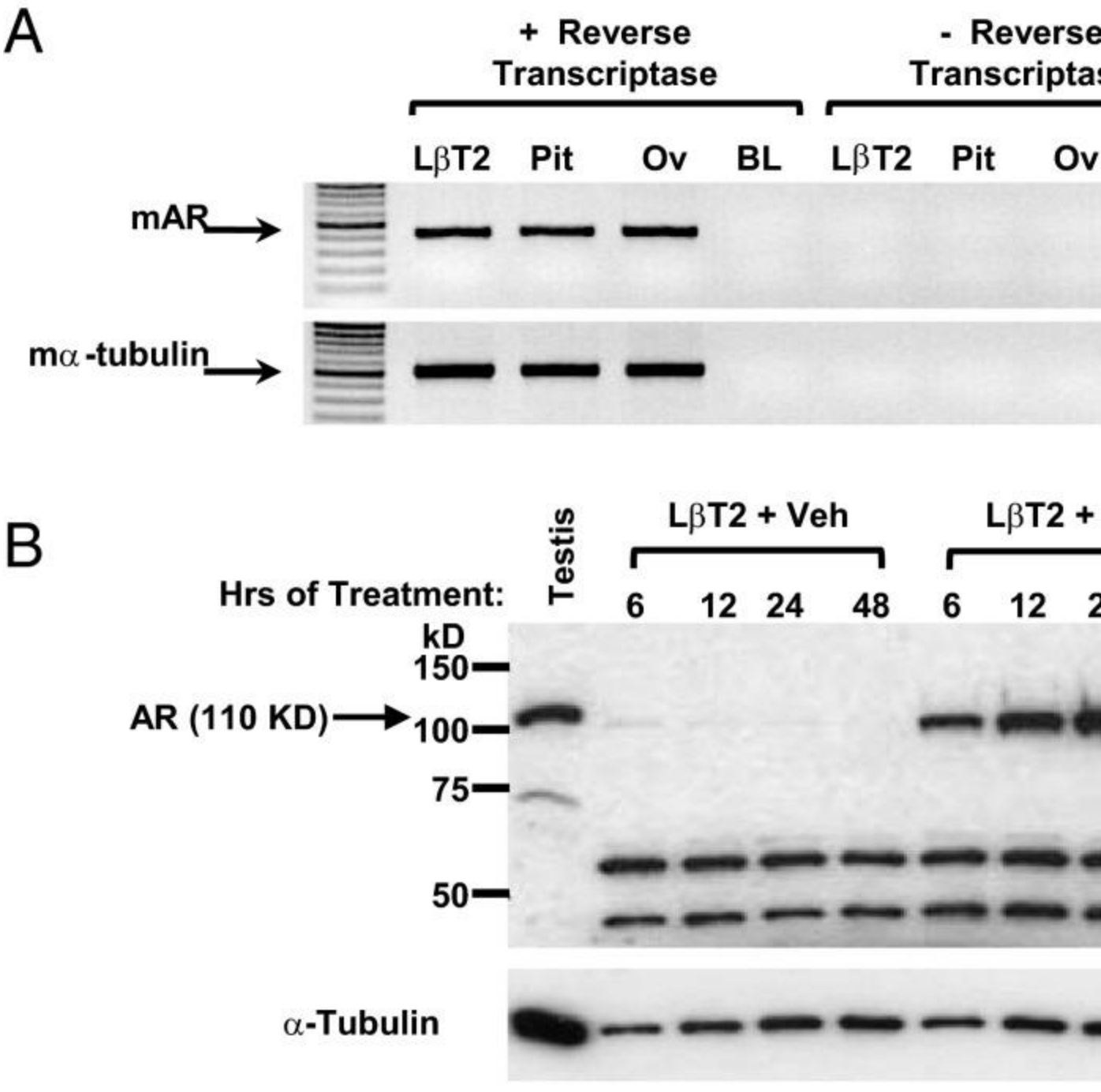
Go to:

Figures and Tables

Figure 1.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75
50
AR (110 KD)
α-Tubulin
C
Flag-AR
LβT2 Extract
DHT:
Ab:
Ig
AR
--
Ig
AR
--
Ig
AR
--
Ig
AR
SS
AR-DNA

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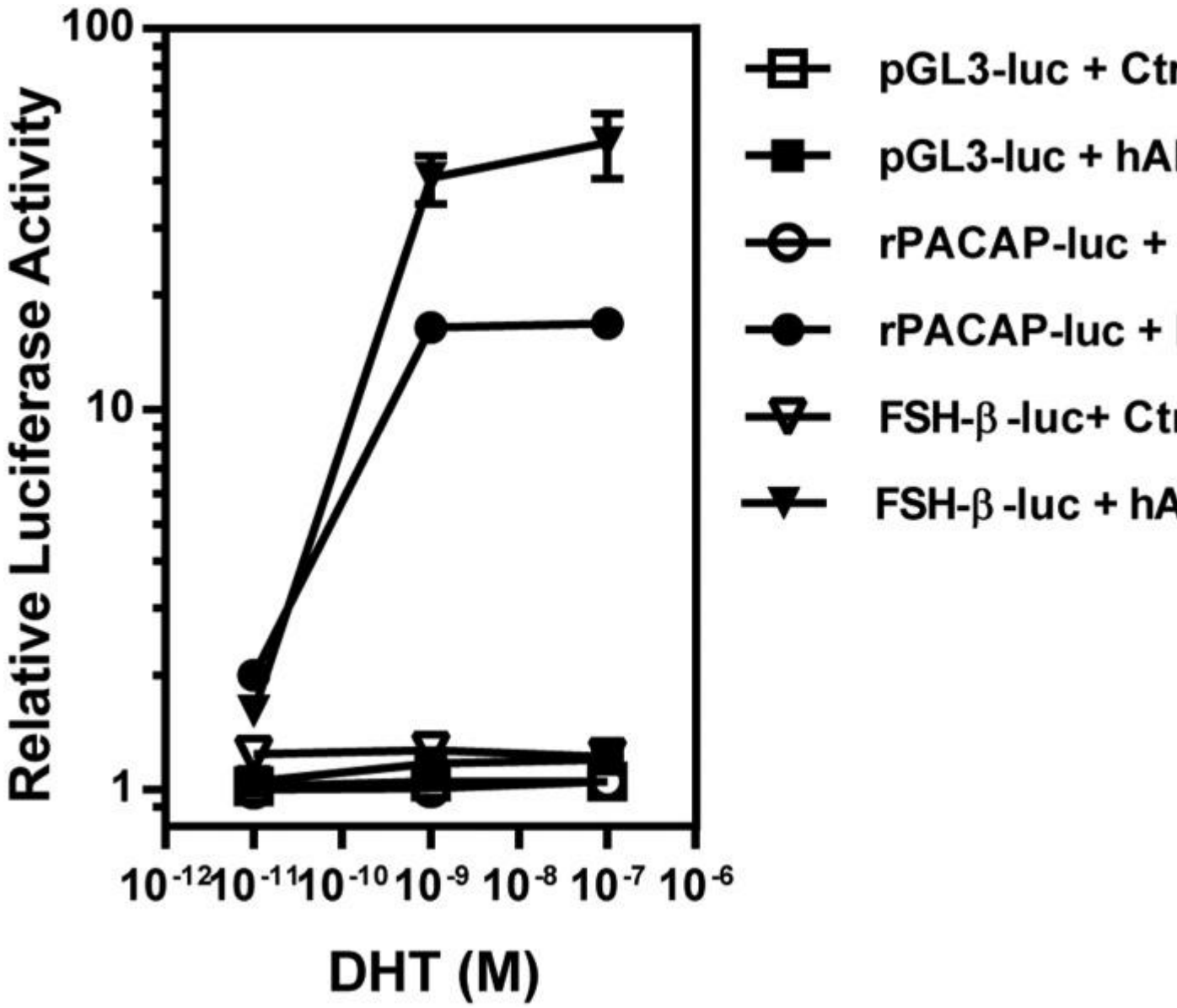
AR mRNA Is Expressed in the Mouse LβT2 Gonadotrope Cell Line and Nuclear AR Protein Increases following DHT Treatment. A, Total RNA was reverse transcribed in the presence (+) or absence (−) of reverse transcriptase and analyzed by PCR with intron spanning AR-specific or mouse α-tubulin-specific primers. Ab, Antibody; Pit, pituitary; Ov, Ovary; BL, Blank. B, Mouse LβT2 cells were treated for indicated times with 100 nM DHT or vehicle. LβT2 nuclear extracts or whole mouse testis extract were analyzed for AR or α-tubulin expression by Western blot. C, Nuclear extracts from LβT2 cells treated with 100 nM DHT or vehicle for 48 hours were incubated with a ^{32}P-labeled oligonucleotide probe containing a consensus steroid HRE in the presence or absence of anti-AR antibody and tested for complex formation by EMSA. Whole-cell extract containing overexpressed Flag-AR from baculovirus-infected Sf9 insect cells was included as a positive control. Arrows indicate protein-DNA complexes of interest; SS, Supershifted complexes.

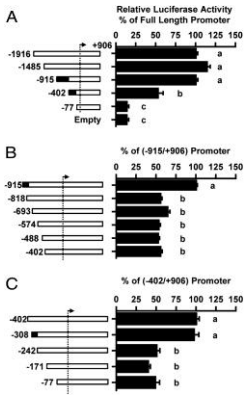
Figure 2.



Androgen Stimulates rPACAP Expression in Mouse Gonadotrope LβT2 Cells in a Dose-Dependent Manner. Dose-dependent stimulation of PACAP promoter activity in LβT2 cells by DHT (24-h treatment) following transient transfection of (−1916 to +906) rPACAP-luciferase reporter with or without cotransfected AR expression vector. FSHβ-luciferase and empty pGL3-luciferase reporters were included as positive and negative controls, respectively. RLU values for luciferase were corrected against RLU values of a cotransfected *Renilla* luciferase reporter and expressed as fold-change relative to

vehicle control. All experiments were performed in triplicate with data expressed as the mean ± SEM.

Figure 3.



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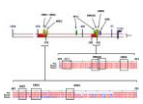
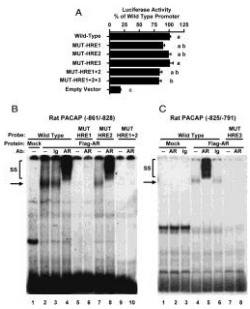
Androgen Response Maps to 2 Regions of the rPACAP Gene Promoter. Gonadotrope LβT2 cells were transiently cotransfected with various lengths of the rPACAP gene promoter fused to a luciferase reporter and the pSG5-hAR expression vector, followed by treatment with 100 nM DHT for 24 hours. A, Serial 5′-truncations were generated for the rPACAP promoter across region −1916 to +906 relative to the transcriptional start site. B, Detailed serial 5′-truncation analysis from position −915 to −402. C, Detailed serial 5′-truncation analysis from position −402 to −77. RLU values for luciferase were corrected against RLU values of a cotransfected *Renilla* luciferase reporter, calculated as fold-change relative to vehicle-treated control. All experiments were performed at least 3 times in triplicate with data expressed as the mean ± SEM. The fold-induction by androgen of each deletion construct was expressed as the % activity relative to the fold-induction of the full-length construct set at 100% for each experiment. Significantly different (*P* < .001) levels of induction among truncations are indicated by differing letters.

Figure 4.



Clusters of Candidate HREs Colocalize with Androgen-Responsive Regions (indicated by red bars) of the rPACAP Promoter. rPACAP promoter sequence elements, arbitrarily designated HRE1 through HRE6 (yellow rods), with homology to reported mammalian steroid HREs were identified using the TESS (Transcription Element Search System) and are shown here in relation to previously identified gonadotrope transcription factor binding elements (activator protein 1 [AP-1], green rods; cAMP response element [CRE], blue rod) (39). Comparison of homologous promoter regions containing putative HREs from rat, mouse, and human indicates a high degree of conservation within most candidate elements. Conserved bases are shown in red; nonconserved bases are shown in blue.

Figure 5.



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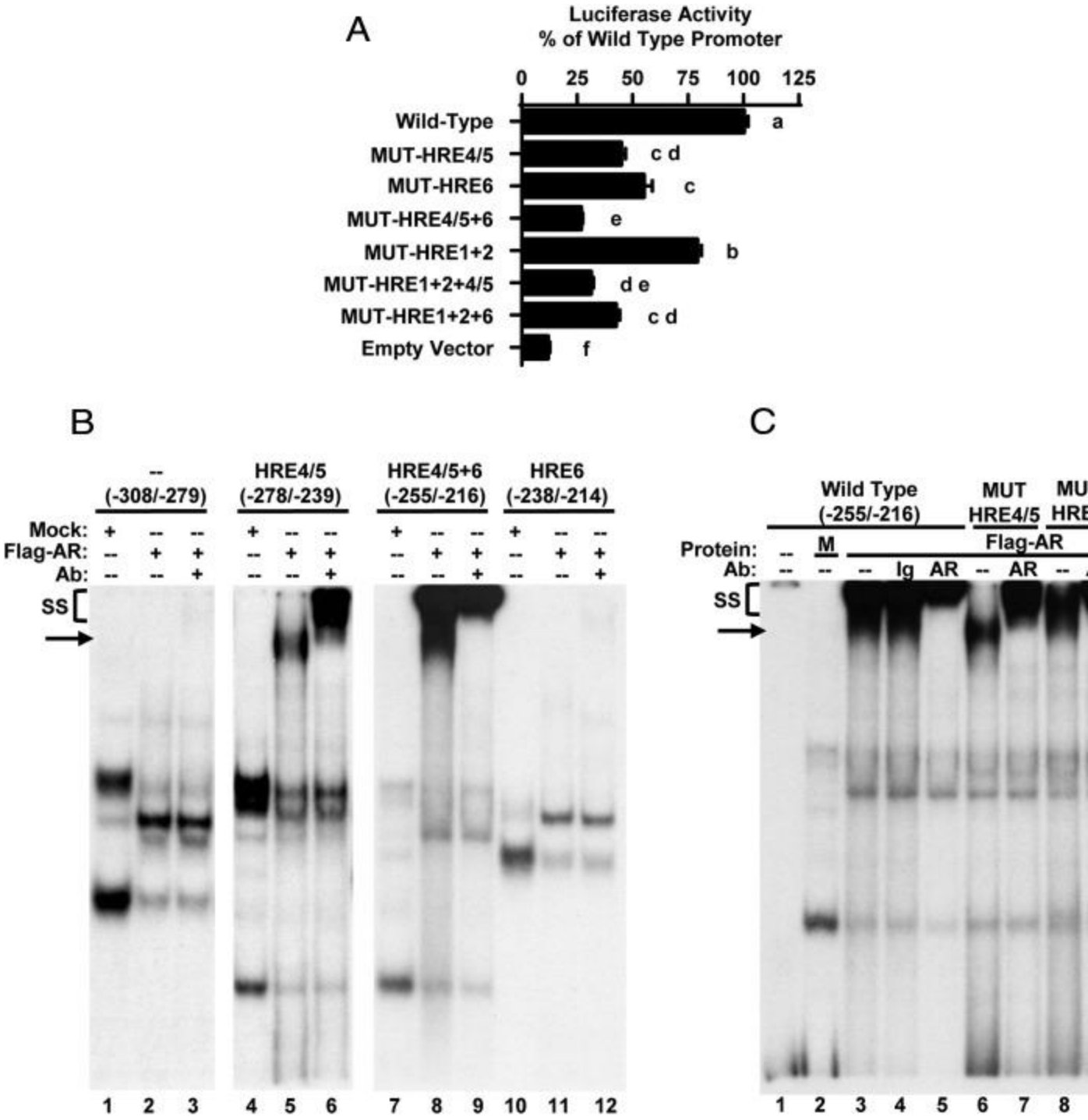
AR Binds Putative HREs of the rPACAP Promoter within the (−915 to −818) Region Identified by Truncation Analysis but These HREs Contribute Minimally to the Overall Functional Androgen Response. A, Effect of single and combinatorial site-directed mutations in the putative HRE1, -2. and -3 elements. Mutations were created in the context of the full-length (−1916 to +906) rPACAP-luc reporter and transiently cotransfected into LβT2 cells with the AR expression construct. All experiments were performed a minimum of 3 times in triplicate. The fold-induction by androgen of each mutant construct was expressed as the % activity relative to the fold-induction of the wild-type construct set at 100% and shown as the mean ± SEM. Significantly different (*P* < .05) levels of induction between constructs are indicated by differing letters. B, Analysis of

putative AR-binding elements HRE1 and HRE2 in the rPACAP promoter by EMSA. Baculovirus-infected Sf9 whole-cell extracts containing overexpressed Flag-AR were incubated with wild-type or mutant ^{32}P-labeled oligonucleotide probes spanning region (−861 to −828) in the presence or absence of supershifting anti-AR antibody. Complex formation on the wild-type probe was also tested with mock infected Sf9 cell extracts and preimmune rabbit IgG antibody (Ig) as an additional negative control. C, Analysis of putative AR-binding element HRE3 in the rPACAP promoter region by EMSA. AR-containing Sf9 cell extracts were incubated with wild-type or mutant ^{32}P-labeled oligonucleotide probes spanning region (−825 to −791). AR-containing complexes are indicated by an arrow; Ab, antibody; MUT, mutant construct; SS, supershifted complexes. Please refer to Supplemental Table 1 for details on wild-type and mutant oligonucleotides.

Figure 6.



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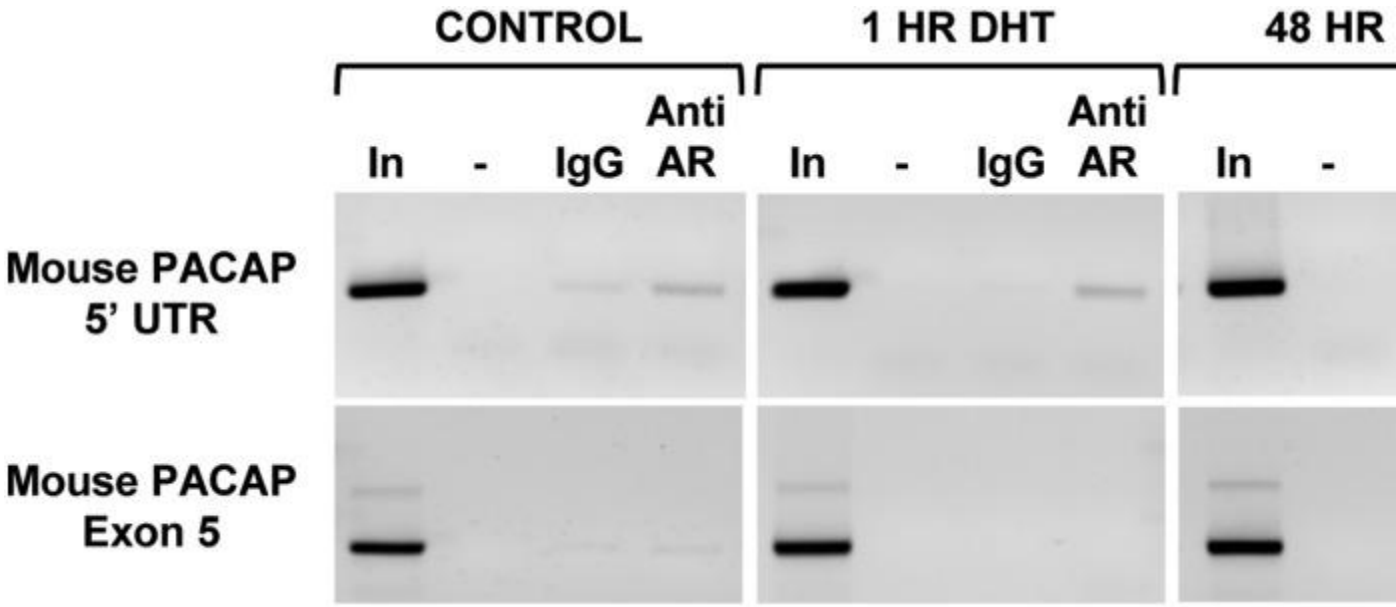
AREs in the Proximal rPACAP Promoter Region (−308 to −214) Confer the Greatest Degree of Androgen Responsiveness and Display High-Affinity AR Oligonucleotide Binding. A, Effect of single and combinatorial site-directed mutations in the putative HRE4/5 and HRE6 elements within region (−308 to −214) relative

to the transcription start site. Additional combinatorial mutations including the proximal elements plus the upstream HRE1+2 were also tested. Mutations were created in the context of the full-length (−1916 to +906) rPACAP-luc reporter and transiently cotransfected into LβT2 cells with AR expression construct. All experiments were performed a minimum of 3 times in triplicate. The fold-induction by androgen of each mutant construct was expressed as the % activity relative to the fold-induction of the wild-type construct set at 100% and shown as the mean ± SEM. Significantly different (*P* < .05) levels of induction between constructs are indicated by differing letters. B and C, EMSA analysis of AR binding on the rPACAP promoter region spanning −308 to −214 containing the putative AR-binding elements HRE4/5 and HRE6. Wild-type overlapping probes (panel B) were tested to dissect AR binding properties at discrete HREs, and the importance of mutated bases in each HRE was then confirmed using mutant probes (panel C). Baculovirus-infected Sf9 whole-cell extracts containing overexpressed Flag-AR were incubated with the indicated wild-type or mutant ^{32}P-labeled oligonucleotide probes in the presence or absence of supershifting anti-AR antibody. Complex formation was also tested, as indicated, with mock infected Sf9 cell extracts (M, Mock) or preimmune rabbit IgG antibody (Ig) as additional negative controls. AR-containing complexes indicated by arrow; MUT, mutant construct; SS, supershifted complexes. Please refer to Supplemental Table 1 for details on oligonucleotides.

Figure 7.



AR Binds to the Proximal PACAP Promoter in LβT2 Cells. ChIP analysis was performed using LβT2 cells treated with 100 nM DHT for 1 or 48 hours prior to cross-linking with formaldehyde. Sheared chromatin samples were immunoprecipitated with AR-specific rabbit antibody or with normal rabbit IgG or no antibody (−) as negative controls. Eluted DNA or input samples (In) were analyzed for 35 cycles by PCR using primers amplifying the mouse PACAP proximal promoter 5′-UTR spanning the *cis*-elements analogous to rat HRE4/5 and HRE6, or primers amplifying mouse genomic PACAP exon 5 as a negative control. Data shown are representative of 5 independent experiments with similar results. See Supplemental Table 1 for primer sequences.
