UNIVERSITY OF CALIFORNIA, SAN DIEGO

Repression of Gonadotropin-Releasing Hormone Gene Expression by Androgen Receptor

A thesis submitted in partial satisfaction of the

requirements for the Degree Master of Science

in

Biology

by

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University of California, San Diego

2010

This work is dedicated to
my professors who have inspired my obsession for science and
to my family and friends for their love and support.

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LIST OF ABBREVIATIONS

AR	androgen receptor
ARE	androgen responsive element
β-gal	β-galactosidase
bp	base pair
C/EBP	CCAAT/enhancer binding protein
ChIP	Chromatin Immunoprecipitation
d.s.	double stranded
DBD	DNA binding domain
DHT	5α-dihydrotestosterone
E2	estradiol
EMSA	electrophoretic mobility shift assay
EREs	estrogen responsive elements
F	forward
fp	free probe
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
GnRHe	gonadotropin releasing hormone distal enhancer
GnRHp	gonadotropin releasing hormone proximal promoter
GnRHue	gonadotropin releasing hormone upstream enhancer
GR	glucocorticoid receptor
IgG	immunoglobulin

kb	kilobases
LBD	ligand binding domain
LH	luteinizing hormone
n.s.	non-specific
nGREs	negative glucocorticoid responsive elements
Nkx2.1	NK2 homeobox 1
NTD	n terminal domain
Oct-1	octamer binding transcription factor 1
Oct1BS-a	octamer binding transcription factor 1 binding site
Oct1BS-b	octamer binding transcription factor 1 binding site
P4	progesterone
Pbx1b	pre-b cell leukemia transcription factor
PCOS	polycystic ovarian syndrome
PCR	polymerase chain reaction
PKC	protein kinase C
PR	progesterone receptor
PREs	progesterone responsive elements
R	reverse
R1881	methyltrienolone
rAR	rat androgen receptor
rGnRH	rat gonadotropin releasing hormone
RLU	relative luciferase activity

RSVe	rous sarcoma virus enhancer
RSVp	rous sarcoma virus promoter
s.s.	single stranded
TK	thymidine kinase
TPA	12-O-tetradecanoyl phorbol-13-acetate
TTF-1	thyroid transcription factor 1
WT	wild type

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ABSTRACT OF THE THESIS

Repression of Gonadotropin-Releasing Hormone

Gene Expression by Androgen Receptor

by

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Master of Science in Biology

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Professor Pamela L. Mellon, Chair

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Gonadotropin-releasing hormone (GnRH) is the central regulator of the hypothalamic-pituitary-gonadal axis. Steroid hormones synthesized and released by the gonads, such as androgens, negatively feedback to the hypothalamus to regulate GnRH synthesis and secretion, and precise regulation is vital for proper reproductive function. *In vitro* experiments, utilizing the GnRH immortalized neuronal cell line, GT1-7, were performed to elucidate the mechanisms by which androgen receptor (AR) disrupts GnRH transcription. We show that methyltrienolone (R1881), an AR agonist, represses GnRH expression through the proximal promoter (GnRHp) as well as the distal enhancer (GnRHe) and causes increased AR interaction with those regions. Classical gene regulation by AR involves binding to androgen responsive elements (AREs). However,

GnRHp and GnRHe do not contain putative AREs. Thus, repression may be occurring via AR interaction with DNA-bound transcription factors or with co-factors. We find that multiple regions, including the -91/-86 region in GnRHp, are required for AR-mediated repression rather than a single transcription factor-binding site. On the other hand, repression of GnRHe maps to the -1800/-1766 region and may require nucleotides -1789/-1787. AR and thyroid transcription factor-1 (TTF-1 or Nkx2.1) may be part of unknown protein complexes binding to the -1795/-1790 and -1792/-1784 regions of GnRHe, respectively. Interestingly, we also find that R1881 suppresses the activity of Rous sarcoma virus promoter (RSVp) when three copies of the Nkx2.1 consensus binding sequence are positioned directly upstream of RSVp. In conclusion, AR activation by R1881 represses GnRH expression by utilizing multiple sites throughout GnRHp, the -1800/-1766 region of GnRHe, and possibly involves Nkx2.1.

I

Introduction

Hypothalamic-Pituitary-Gonadal Axis

Proper communication between the hypothalamus, anterior pituitary, and gonads is critical for mammalian reproductive function, such as sexual development, puberty, menstrual cycle, pregnancy, and menopause. The hypothalamic decapeptide gonadotropin-releasing hormone (GnRH) is the ultimate regulator of the hypothalamic-pituitary-gonadal axis. GnRH-expressing neurons in the hypothalamus release GnRH peptide in a pulsatile manner into the portal capillary bed in the median eminence. GnRH from the median eminence reaches the anterior pituitary to mediate production and secretion of gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH, in turn, stimulate the gonads to regulate folliculogenesis, spermatogenesis, and biosynthesis of steroid hormones such as androgen, estrogen, and progesterone. Steroid hormones are secreted into the blood stream and exert both positive and negative feedback effects on the hypothalamus and anterior pituitary to alter expression and secretion of GnRH and gonadotropins, respectively.

GnRH Peptide

Two types of GnRH decapeptides have been identified. GnRH-I, present in the preoptic area and anterior hypothalamus, is the most prevalent and well studied. It is composed of 10 amino acids (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2). The amino acid sequence of GnRH-I is identical across mammalian species, except in guinea pig [2]. GnRH-II contains a different amino acid sequence and is primarily expressed in non-reproductive areas [3]. The experiments presented in this thesis investigated GnRH-I, hereafter referred to as GnRH.

GT1-7 Cell Line

GnRH neurons are scarce in number and scattered through the hypothalamus, making it difficult to study GnRH gene regulation *in vivo*. Our laboratory has developed the GT1-7 model cell line to study GnRH gene expression. It was developed through targeted tumorigenesis of the GnRH neuron by expressing the SV40 T-antigen oncogene from the 5′ flanking region of rat GnRH (rGnRH) gene. The GT1-7 cell line expresses GnRH mRNA and displays pulsatile secretion of GnRH [4-6]. Many studies use this cell model because it mimics *in vivo* GnRH synthesis and secretion responses [7, 8]. Thus, many studies have been successful in elucidating the molecular mechanisms of GnRH gene expression using the GT1-7 cell line as a model system.

GnRH Gene Regulation

The 5′ flanking region of the GnRH gene is highly homologous between human, rat, and mouse [9]. The rat GnRH (rGnRH) gene consists of three well-characterized regulatory regions, the proximal promoter (GnRHp), distal enhancer (GnRHe), and the upstream enhancer (GnRHue) [10]. GnRHp includes base pairs (bp) from -173 to +112, GnRHe includes the region between -1863 and -1571 bp, and GnRHue is located at -2980 to -2631 bp, relative to the transcriptional start site. Many transcription factors have been found to bind to GnRHp, GnRHe, and GnRHue (Figure 1).

The proximal promoter has approximately 80% nucleotide homology among human, rat, and mouse. Octamer-binding transcription factor-1 (Oct-1), a member of the homeodomain protein family, has been shown to be important for GnRH transcription by binding to functional elements in GnRHp, GnRHe, and GnRHue. Oct-1 is involved in tissue-specific expression by interacting with gene-specific transcription factors and cofactors. Oct-1 binds at AT rich sites. GnRHp contains two octamer-like motifs at -47/-40 bp and -110/-88 bp, which share a 6-nucleotide sequence (ATGCAAAT) with the consensus site [11]. GnRHe contains binding sites at -1785/-1771 (Oct1BS-a) and -1702/-1695 (Oct1BS-b) [12]. Oct-1 bound to Oct1BS-a is more crucial to the activity of GnRHe because mutation of its binding site reduces transcriptional activity by 95% [13]. GnRHue contains nine binding sites for Oct-1, which are essential for GnRH expression [10]. Pre-B-cell leukemia transcription factor 1b (Pbx1b) is a co-factor for Oct-1 [14]. Pbx1b, in turn, binds to its cofactor, PBX/knotted 1 homeobox1 (Prep1). The Pbx/Prep1

heterodimer binds at -100 and -75 in GnRHp and -1749 and -1603 in GnRHe and can act as a co-activator for Oct-1 [14].

Androgen Receptor

Androgen receptor (AR) has two natural ligands, testosterone and its metabolite, 5α-dihydrotestosterone, critical for male-specific puberty, reproductive function, and behavior. Although less is known about its normal effect in females, Shiina et al., observed premature ovarian failure in female AR null mice, suggesting an important role in female reproduction [15]. Excess androgens have been correlated with polycystic ovarian syndrome (PCOS) and are the cause of defects seen in female hyperandrogenemia. Regarding sexual differentiation, it is well known that androgens play a role in organizing brain circuits, and sufficient AR levels are vital during critical stages of development. For instance, neonatal castration of male rats causes a decline in AR levels in the hypothalamus [16]. Mutation of the AR gene causes androgen insensitivity syndrome, leading to a wide variety of reproductive defects [17, 18]. Altogether, proper AR function and levels are vital for reproductive function in both males and females.

AR is a member of the ligand-activated nuclear hormone receptor super-family and upon ligand binding, acts as a transcription factor. The protein consists of four separate and functional regions, the DNA-binding domain (DBD), ligand-binding domain (LBD), a hinge domain, and a NH_2-terminal transactivation domain (NTD). The DBD and LBD are highly conserved across species while the NTD is more species-specific.

The DBD and LBD are very similar in sequence and length compared to other nuclear hormone receptor family members and the NTD shows the greatest variability. All regions of AR interact with a variety of transcription factors, co-factors, and other gene-specific proteins.

Androgenic ligands binding to the LBD of AR in the cytoplasm cause a conformational change and a release of chaperone proteins. This altered conformation results in nuclear translocation and phosphorylation. Nuclear AR binds as a homodimer to androgen-response elements (AREs) in the regulatory regions of target genes. A zinc finger motif in the DBD mediates DNA interaction with AREs, and a second zinc finger motif stabilizes DNA binding and mediates dimerization. AREs consist of palindromic sequences, and the consensus ARE is composed of two anti-parallel 5'-AGAACA-3' motifs, separated by 3 bp [19]. Once bound to DNA, AR interacts with co-activators and co-regulators to control transcription.

Conversely, AR can also indirectly influence gene expression, presumably in genes containing no AREs. Possible mechanisms include competing away or regulating expression and activity of transcription factors and co-factors. Studies of the rat dehydroepiandrosterone sulfotransferase gene promoter show that AR may exert negative effects through transcriptional interference between Oct-1 and C/EBP rather than a direct DNA-AR interaction [20]. Also, AR has been shown to repress muscle atrophy F-box expression by interacting with Oct-1 within an untranslated region [21]. Thus, AR tethering to DNA-bound transcription factors is an indirect mechanism of AR-mediated gene repression.

GnRH Regulation by Steroids

Whether androgens affect GnRH neurons directly or indirectly remain controversial because AR has not been shown to be present in GnRH neurons *in vivo*, although it may be present in low amounts [22, 23]. However, it has been shown that GT1-7 cells contain AR mRNA and express both functional AR and the AR co-activator, ARA70 [24]. Studies done by Belsham, et al., also show that treating GT1-7 cells with 5α-dihydrotestosterone results in approximately 55% reduction of GnRH mRNA levels. Their studies also show the presence of membrane AR. However, activation of membrane AR in GT1-7 cells did not have an effect on GnRH gene expression, suggesting different roles between membrane and cytoplasmic AR [25].

Other steroid hormones also have been shown to repress GnRH expression in GT1-7 cells, including estrogen, progesterone, and glucocorticoids. The mechanisms of estrogen (E_2) and progesterone (P_4) are mediated by their receptors binding to estrogen responsive elements (EREs) and progesterone responsive elements (PREs), respectively, in the GnRH gene. The effect of P_4 has been mapped between -171 and -73 bp in the rGnRH proximal promoter, and progesterone receptor (PR) binds to several sites within that region [26]. Glucocorticoids also repress GnRH promoter activity through glucocorticoid receptor (GR) in GT1-7 cells via binding sites in both GnRHp and GnRHe [27]. Chandran, et al., also showed that GR does not bind directly to negative glucocorticoid-responsive elements (nGREs), but interacts with DNA-bound Oct-1, providing another example of indirect steroid hormone-mediated gene repression [28].

Summary

A potential cause of improper GnRH expression and secretion is disruption of gonadal androgen feedback to the hypothalamus, the mechanisms of which are not fully understood. Alterations in androgen levels lead to reproductive defects in both males and females, including hypogonadotropic hypogonadism, anovulation, and infertility. Androgens have been shown to down-regulate GnRH mRNA levels through an AR-dependent mechanism in the GT1-7 cell line. However, the specific molecular mechanisms by which androgens regulate GnRH expression are not known. Transient transfections were performed with luciferase reporters containing GnRHp, GnRHe, and GnRHue alone or in combination. We found that R1881, an AR agonist, repressed GnRH expression through GnRHp and GnRHe, but not through GnRHue. Repression was stronger when both GnRHp and GnRHe were present. Chromatin immunoprecipitation assays showed increased interaction between AR and GnRHp and GnRHe after treatment with R1881. This could be caused by AR binding to DNA directly, or indirectly through interaction with other transcription factors. However, GnRHp and GnRHe do not contain putative AREs, suggesting repression is independent of DNA binding by AR. Further transient transfection assays utilizing luciferase reporters with GnRHp scanning mutations and serial deletions indicated that multiple sites, including the -91/-86 region, were required for androgen-mediated repression. A reporter containing four copies of the -1800/-1766 GnRHe sequence was repressed by R1881. Electrophoretic mobility shift assay (EMSA) experiments showed AR and Nkx2.1 may be a part of protein complexes

binding to regions -1795/-1790 and -1792/-1784, respectively. Collectively, our findings provide a better understanding of the mechanism of AR-mediated GnRH repression.

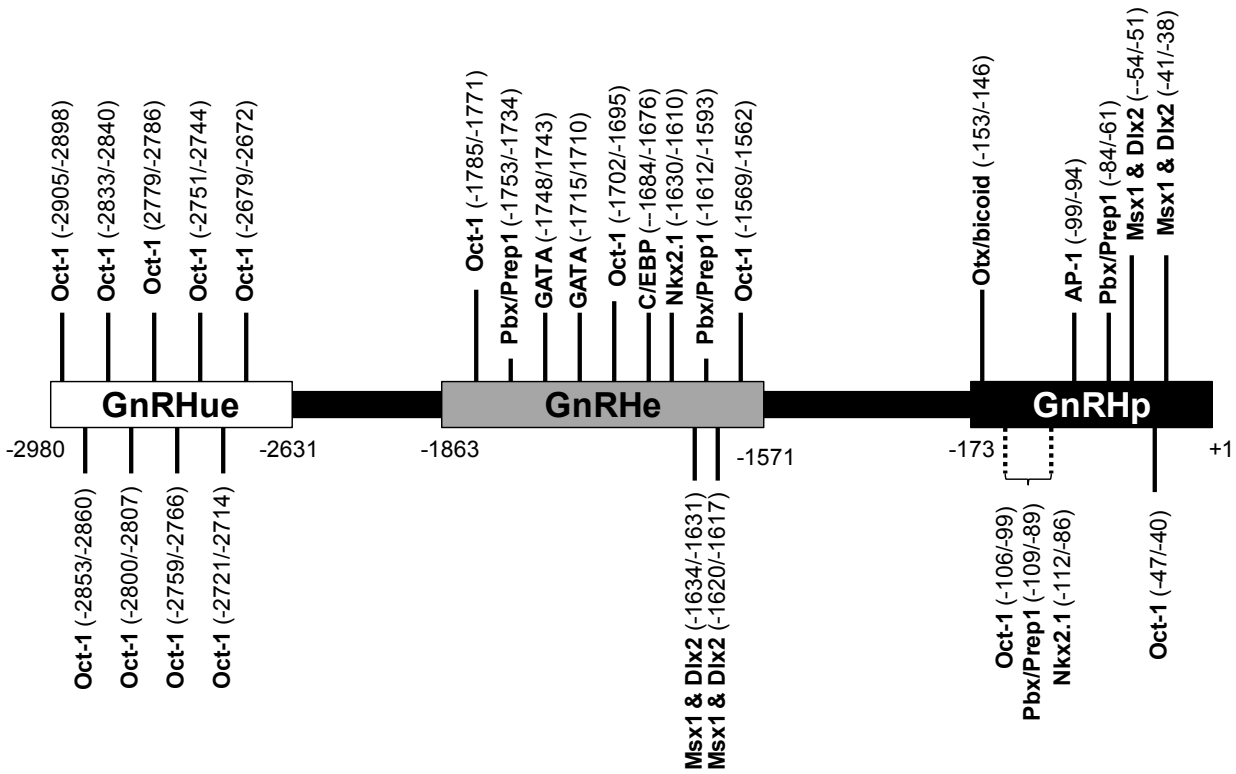

Figure 1: Diagrammatic representation of transcription factors that bind to the GnRH proximal promoter (GnRHp), distal enhancer (GnRHe), and upstream enhancer (GnRHue). Transcription factors are written as their common gene symbol and exact nucleotide binding sites are represented in parentheses, relative to the transcriptional start site. Layout adapted from Lee et al. [1]

II

Methods and Materials

Cell Culture and Transient Transfections for Luciferase Reporter Assays

GT1-7 cells were cultured in DMEM (Cellgro, Mediatech, Inc., Herndon, VA) containing 10% (v/v) μM fetal calf serum (Gemini Bio-Products), and 1% (v/v) penicillin/streptomycin (Invitrogen) in a humidified 5% CO_2 incubator at 37^o C. 150,000 cells were seeded into each well in 24-well plates and incubated overnight at 37^o C before being transiently transfected using FuGENE reagent (Roche Applied Science, Indianapolis, IN). Cells were transfected with 400 ng of expression plasmid, 400 ng of luciferase reporter plasmid and 100 ng of the internal control thymidine kinase (TK) -109 bp promoter on β-galactosidase and incubated for at least 24 hours to ensure protein expression. Cells were treated with 0.1% (v/v) ethanol (vehicle) or 100 nM R1881 (PerkinElmer, Inc., Boston, MA). After 24 hours, cells were harvested and assayed for luciferase (luc) and β-galactosidase (β-gal) as described below.

Luciferase and β-galactosidase Assays

Cells were washed once in 1x phosphate buffered saline (PBS) then resuspended in 50 μl of lysis solution (100 mM potassium phosphate pH 7.8, 0.2% (v/v) Triton X-100). Cells were placed on a shaker (VWR OS-500) for approximately 5 to 15 minutes at speed 4-5. 10 or 20 μl of the cell sample was placed in each well in a 96-well plate for the

ß-gal assay and luc assay, respectively. Luc and β-gal activity were measured using a Veritas Microplate Luminometer (Turner Biosystems). Luc activity was determined by injecting 100 µl per well of a buffer containing 100 mM Tris-HCl pH 7.8, 15 mM $MgSO_4$, 10 mM ATP and 65 µM of luciferin. ß-gal assays were performed using the Galacto-Light Plus Kit (Tropix, Bedford, MA) according to the manufacturer's protocol. Luc values were divided by β-gal values to control for transfection efficiency. All experiments were performed in quadruplicate and repeated a minimum of three times.

Plasmid Constructs

All reporter plasmid constructs were in the pGL3 luciferase backbone. All reporter plasmids not described in the following sections were provided by our lab. The The AR expression vector contained rat cDNA and were in the pSG5 vector [29]. Human Oct-1 and Prep1 were in the pcDNA-1.1 vector [14] and Nkx2.1 was in the pcDNA-1.3 vector [30].

Heterologous Promoters and 3x Multimer Plasmids

Heterologous promoters (-127/-86 GnRHp, -101/-45 GnRHp, and -173/-86 GnRHp) and 3x multimers containing the consensus binding sites for Oct-1, Pbx, and Nkx2.1 were made by designing complimentary single stranded (s.s.) oligonucleotides (Table 1). The oligonucleotides were synthesized by Integrated DNA Technologies (San Diego, CA). Each end corresponded to the cut sites for XmaI and SexAI.

Oligonucleotides were diluted in double distilled water. Annealed (double-stranded, d.s.) oligonucleotides were prepared by boiling 10 pmol of each s.s. oligonucleotide in 50 mM NaCl for 10 minutes and allowed to cool overnight in room temperature. The pGL3 RSVe/RSVp luc backbone was digested between RSVe and RSVp with XmaI and SexAI (New England BioLabs) and then purified using the PCR Purification Kit (Qiagen Sciences, Maryland), according to the manufacturer's protocol. The d.s. oligonucleotides were ligated into the pGL3 RSVe/RSVp luc backbone using the Rapid DNA Ligation Kit (Roche Molecular Biochemicals, Indianapolis, IN), according to the manufacturer's protocol. The vector to insert ratio was 1:5. Two µl of the ligation was transformed into One Shot ® TOP10 Competent Cells (Invitrogen, Carlsbad, CA), following the manufacturer's protocol. Bacteria were plated on luria broth (LB)-ampicillin plates at 25-100 µl per plate and incubated overnight at 37° C. Colonies were separately cultured in 5 mL of LB broth, containing ampicillin, overnight. DNA was extracted using the Qiagen Miniprep Kit and analyzed by KpnI (Sigma-Aldrich) digest. Digested products were run on a 2% gel and positive clones were sequenced by Moore's UCSD Cancer Center. Sequencing was analyzed using the BL2SEQ program by San Diego Supercomputer Center's Biology Workbench (http://workbench.sdsc.edu/).

Mutagenesis

Plasmids listed in Table 2 and 3 were made by site directed mutagenesis using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), according to the manufacturer's protocol. Primers were designed using QuikChange® Primer Design

Program. PCR conditions were as follows: 95°C for 30 seconds, 95° C for 30 seconds, 55°C for 1 minute, and 68° C for 5.5 minutes for 18 cycles. Bacteria were plated on LB-ampicillin plates at 250 µl per plate overnight at 37°. Colonies were cultured and analyzed for correct insert as described previously.

4x (-1800/-1766) Multimer Mutations

Each fragment in the [4x (-1800/-1766) mut Oct] and [4x (-1800/-1766) mut -1787/-1789] reporters was 5′-CAC AGT CCT C**AG** **T**CT GAG ATT GTA CAG TAG GGC A-3′ and 5′-CAC AGT CCT CCT GCT GAG ATT **G**TA CA**G** TAG GGC A-3′, respectively (bolded and underlined nucleotides correspond to substituted nucleotides). Reporters containing these fragments were provided by our lab, but were fused to RSVp. The wild type (WT) reporter was fused to TKp. Thus, the mutated fragments were digested on either end by XmaI and SacI (New England BioLabs). DNA was purified using the QIAquick Gel Extraction Kit (Qiagen Sciences, Maryland) according to the manufacturer's protocol. The fragments were then ligated into the pGL3-TKp backbone using the Rapid DNA Ligation Kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol. TOP 10 bacterial cells were transformed with 2 µl of the ligation, then plated, and cultured as previously described. Clones were analyzed by digestion with XmaI and SacI, and positive clones were confirmed by sequencing (Moore's UCSD Cancer Center).

Nuclear Extracts

GT1-7 cell nuclear extracts were made by first placing the cells in a hypotonic buffer (20 mM Tris pH 7.4, 10 mM NaCl, 1 mM MgCl2, 1 mM PMSF, protease inhibitor cocktail [Sigma-Aldrich], 10 mM NaF, 0.5 mM EDTA, 0.1 mM EGTA) and then passed 3 times through a 25G needle. Nuclear material was spun at 4000 rpm for 4 minutes in 4°C. The pellet was resuspended in hypertonic buffer (20 mM Hepes pH 7.8, 20% glycerol, 420 mM KCL, 1.5 mM MgCl2, 1 mM PMSF, protease inhibitor cocktail from Sigma-Aldrich, 10 mM NaF, 0.5 mM EDTA, and 0.1 mM EGTA.) After 20 minute incubation, samples were spun down at 14,000 rpm for 10 minutes in 4°C and supernatant was aliquoted and stored at -80 °C. Protein concentration was determined by using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) according to the manufacturer's protocol.

Electrophoretic mobility shift assays (EMSA)

EMSA oligonucleotides (Table 1) were synthesized by Integrated DNA Technologies and annealed using the same method described in the *Heterologous Promoters and 3x Multimer Plasmids* section. One pmol of d.s. oligonucleotide was end-labeled with 0.34 millicuries [^{32}P] using T4 Polynucleotide Kinase (New England Biolabs, Inc., Beverly, MA), then incubated for 1 hour at 37°. After the reaction was stopped with 30 µl Tris-EDTA (TE) buffer, the probe was purified using Micro Bio-Spin6 Columns #732-6221 (Bio-rad laboratories, Inc., Hercules, CA). 20 µl binding reactions contained 4 µg of GT1-7 nuclear protein, 100 mM HEPES pH 7.8, 500 mM

KCL, 50 mM $MgCl_2$, 1% NP-40, 1mM DTT, 0.004 µg BSA, PICS, 0.25 ug poly dIdC, and d.s. oligonucleotide competitor or antibody. Reactions were incubated for 5 minutes before addition of 8 fmol of [^{32}P]-labeled oligonucleotide. After 20 minute incubation at RT, samples were electrophoresed on an 8% polyacrylamide gel in 0.25x TBE (Tris-Borate-EDTA). Gels were run at 250 V for 2 hours then dried, under vacuum, and exposed to film at room temperature for 14 days or at -80 °C for 48 hours.

Competition and supershift assays contained 500-fold excess of unlabeled d.s. oligonucleotide or 0.4 ug antibody. Mouse AR and Nkx2.1 antibodies were purchased from Santa Cruz Biotechnologies (sc-815) and Neomarkers (clone 8G7G3/1), respectively. Rabbit IgG was purchased from Santa Cruz Biotechnologies (sc-2027). Competitor probes used were (a) ARE: (5′-GTC TGG TAC AGG GTG TTC TTT TTG-3′), (b) C/EBP: (5′-CTA GGG CTT GGG AAA GAA CTA CTT GAG AC-3′), and (c) Nkx2.1: (5′-CCG GGC ACT GCC CAG TCA AGT GTT CTT GAC ACT GCC CAG TCA AGT GTT CTT GAC ACT GCC CAG TCA AGT GTT CTT GAA-3′), and (d) Nkx2.1 core mutation (5′-CAC TGC CCA GTC **GCC** TGT TCT TGA-3′).

Statistical Analysis

Statistical analysis was performed using JMP version 8 (SAS Institute, Cary, NC). All relative luciferase units (RLU) were optimally transformed by the method of Box and Cox. Raw data were analyzed by ANOVA, followed by Student's t-tests within groups. A P value of less than 0.05 was the requirement for declaring significance.

Table 1: Primer sequences used to create heterologous GnRHp and 3x multimer reporters. Bolded and underlined nucleotides represent substituted nucleotides.

Plasmid	Primer Sequence (5'->3')
-127/-86 Fragment	For: CCGGGCTGTGAAAGTTTTAGCTAAGATTTTAATGACCAAGTTTAAGA
	Rev: CCAGGTCTTAAACTTGGTCATTAAAATCTTAGCTAAAACTTTCACAGC
-127/-86 Fragment mOct-1 B.S.	For: CCGGGCTGTGAAAGTTTTAGCTAAG**CGGCCGC**TGACCAAGTTTAAGA
	Rev: CCAGGTCTTAAACTTGGTCA**GCGGCCG**CTTAGCTAAAACTTTCACAGC
-173/-86 Fragment Forward	For: TTTCCCGGGGGAATTCAACATGTCTGGCTTTT
	Rev: TTTACCTGGTCTTAAACTTGGTCATTAAAATCTTAGC
-173/-86 Fragment Reverse	For: TTTACCTGGTGGAATTCAACATGTCTGGGTTTT
	Rev: TTTCCCGGGCTTAAACTTGGTCATTAAAATCTTAGC
-101/-45 Fragment Forward	For: TTTCCCGGGTAATGACCAAGTTTAAGAAAATGCAAC
	Rev: TTTACCTGGTAATGTAATTGGAACACCTGCTG
-101/-45 Fragment Reverse	For: TTTACCTGGTTAATGACCAAGTTTAAGAAAATGCAAC
	Rev: TTTCCCGGGAATGTAATTGGAACACCTGCTG
3x Oct-1 B.S.	For: CCGGGTGTCGAATGCAAATCACTAGAATGTCGAATGCAAATCACTA…
	GAATGTCGAATGCAAATCACTAGAAA
	Rev: CCTGGTTTCTAGTGATTTGCATTCGACATTCTAGTGATTTGCATTCGACATTCT…
	AGTGATTTGCATTCGACACCATTCGACATTCTAGTGATTTGCATTCGACAC
3x Pbx B.S.	For: CCGGGATCAATCAAATCAATCAAATCAATCAAA
	Rev: CCTGGTTTGATTGATTTGATTGATTTGATTGATTC
3x Nkx2.1 B.S.	For: CCGGGCACTGCCCAGTCAAGTGTTCTTGACACTGCCCAGTCAAGTGTTCTTGA…
	CACTGCCCAGTCAAGTGTTCTTGAA
	Rev: CCTGGTTCAAGAACACTTGACTGGGCAGTGTCAAGAACACTTGACTGGGC…
	AGTGTCAAGAACACTTGACTGGGCAGTGC

Table 2: Primer sequences used to create GnRHp truncations, -126 GnRHp scanning mutants, and triple mutant reporters.

Plasmid Name	Primer Sequence (5'->3')	
-112 Truncation	For:	GACTCTAGAGGATCGATCCCGCTAAGATTTTAATGACCAA
	Rev:	TTGGTCATTAAAATCTTAGCGGGATCGATCCTCTAGAGTC
-101 Truncation	For:	GGACTCTAGAGGATCGATCCCATGACCAAGTTTAAGAAAATG
	Rev:	GCATTTTCTTAAACTTGGTCATGGGATCGATCCTCTAGAGTCC
M1 (-127/-121)	For:	CTTGGACTCTAGAGGATCGATCCCCGGCCGAAGTTTTAGCTAAGATTTTAATGACC
	Rev:	GGTCATTAAAATCTTAGCTAAAACTTCGGCCGGGGATCGATCCTCTAGAGTCCAAG
M2 (-121/-116)	For:	TTGGACTCTAGAGGATCGATCCCCTGTGACGGCCGTAGCTAAGATTTTAATGACCAAGTTTAAG
	Rev:	CTTAAACTTGGTCATTAAAATCTTAGCTACGGCCGTCACAGGGGATCGATCCTCTAGAGTCCAA
M3 (-115/-110)	For:	GACTCTAGAGGATCGATCCCCTGTGAAAGTTTCGGCCGAGATTTTAATGACCAAGTTTAAG
	Rev:	CTTAAACTTGGTCATTAAAATCTTAGCTACGGCCGTCACAGGGGATCGATCCTCTAGAGTCCAA
M4 (-109/-104)	For:	GAGGATCGATCCCCTGTGAAAGTTTTAGCTACGGCCGTAATGACCAAGTTTAAGAAAATGCAACAGAT
	Rev:	ATCTGTTGCATTTTCTTAAACTTGGTCATTACGGCCGTAGCTAAAACTTTCACAGGGGATCGATCCTC
M5 (-103/-98)	For:	AGAGGATCGATCCCCTGTGAAAGTTTTAGCTAAGATTTCGGCCGCCAAGTTTAAGAAAATGCAACAGATAGAC
	Rev:	GTCTATCTGTTGCATTTTCTTAAACTTGGCGGCCGAAATCTTAGCTAAAACTTTCACAGGGGATCGATCCTCT
M6 (-97/-92)	For:	CTGTGAAAGTTTTAGCTAAGATTTTAATGACGGCCGTTAAGAAAATGCAACAGATAGACCAGCAGGT
	Rev:	ACCTGCTGGTCTATCTGTTGCATTTTCTTAACGGCCGTCATTAAAATCTTAGCTAAAACTTTCACAG
M7 (-92/-86)	For:	GTGAAAGTTTTAGCTAAGATTTTAATGACCAAGTCGGCCGAAATGCAACAGATAGACCAGCAGGTGTTCCAATT
	Rev:	AATTGGAACACCTGCTGGTCTATCTGTTGCATTTCGGCCGACTTGGTCATTAAAATCTTAGCTAAAACTTTCAC
Triple Mutation	For:	CTTAGAATGGTGGCTTCAGCTGTGAAAGGGTTAGCTAAGAGGTTAATGACCAAGTTTAAGAAAATGCAAC
	Rev:	GTTGCATTTTCTTAAACTTGGTCATTAACCTCTTAGCTAACCCTTTCACAGCTGAAGCCACCATTCTAAG
	For:	GTTAGCTAAGAGGTTAATGACCAAGGGTAAGAAAATGCAACAGATAGACCAG
	Rev:	CTGGTCTATCTGTTGCATTTTCTTACCCTTGGTCATTAACCTCTTAGCTAAC

Table 3: Primer sequences used to create GnRHe truncations. Templates used for PCR reactions are indicated below.

Plasmid	Primer Sequence (5'->3')	Template
-1636 Truncation	For: CCGAGCTCTTACGCGTCGACTCACAATTTTCTAAATTCAAA	-1800/-1571 GnRHe
	Rev: TTTGAATTTAGAAAATTGTGAGTCGACGCGTAAGAGCTCGG	
-1701 Truncation	For: ATTTCTCTATCGATAGGTACCAGCTCAAAATGATAGCAGTGATG	-1800/-1690 GnRHe
	Rev: CATCACTGCTATCATTTTGAGCTGGTACCTATCGATAGAGAAAT	
-1715 Truncation	For: TTTCTCTATCGATAGGTACCCAGTGATGACTTTGTAACCTTC	-1800/-1690 GnRHe
	Rev: GAAGGTTACAAAGTCATCACTGGGTACCTATCGATAGAGAAA	
-1751 Truncation	For: TTCTCTATCGATAGGTACCGTGAACAACCTCTGTCTTG	-1800/-1730 GnRHe
	Rev: CAAGACAGAGGTTGTTCACGGTACCTATCGATAGAGAA	

III

Results

The GnRH Proximal Promoter and Distal Enhancer are Sufficient for AR-Mediated GnRH Repression

Treatment of GT1-7 cells with 5α-dihydrotestosterone (DHT) results in approximately 55% reduction in GnRH mRNA levels and repression is blocked by hydroxyflutamide, an AR antagonist [31]. Thus, androgen acts directly through AR to repress GnRH gene expression. To further confirm AR-mediated transcriptional repression, GT1-7 cells were transiently transfected with a luciferase reporter driven by a -5 kb GnRH promoter, rat AR expression plasmid, and the thymidine kinase promoter driving β-galactosidase expression (TK-βgal) as a control for transfection efficiency (Figure 2). 24 hours of treatment with 100 nM methyltrienolone (R1881), an AR agonist, led to a significant reduction of luciferase activity. All further transient transfection experiments included AR expression plasmid, unless otherwise noted and contained the TK-βgal reporter as an internal control.

The GnRHp, GnRHe, and GnRHue have been identified as important sites for GnRH gene transcription and are highly homologous between human, rat, and mouse. To determine if these regions are involved in AR-mediated repression, cells were transiently transfected with luciferase reporters either under the control of the GnRHp, GnRHe, or GnRHue alone and also in combination, with the RSVp or enhancer (RSVe), as indicated (Figure 3). 24-hour treatment with 100 nM R1881 led to significant suppression of

GnRHp and GnRHe luciferase reporter activity but had no effect on the GnRHue reporter. Under the same treatment conditions, R1881 reduced reporter activity even further when acting on both the proximal promoter and enhancer. Therefore, experimental results suggest that GnRHp and GnRHe are independently sufficient for AR-mediated suppression and have an additive effect together.

To determine if AR interacts with the GnRHp and GnRHe *in vivo*, chromatin immunoprecipitation (ChIP) was performed on GT1-7 cells transfected with AR expression plasmid. Cells were treated with R1881 for 2 hours and nuclei isolated. An antibody specific for AR was used to immunoprecipitate chromatin associated with AR, and the resulting isolated DNA was used in a polymerase chain reaction (PCR) with primers specific for GnRHp or GnRHe. PCR products were visualized via gel electrophoresis. Levels of chromatin associated with AR were higher in R1881 treated cells compared to vehicle in both regions analyzed (Figure 4). Thus, AR interaction with the GnRH proximal promoter and enhancer increased upon R1881 treatment.

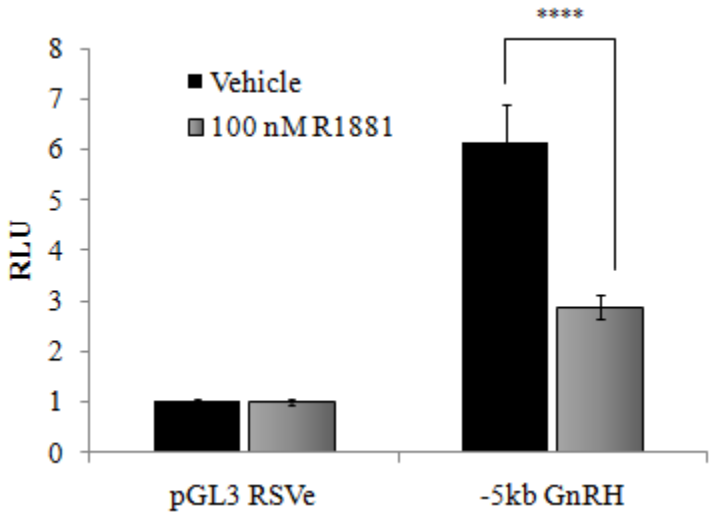

Figure 2: -5 kb GnRH-pGL3-luciferase reporter activity is significantly reduced by R1881. GT1-7 cells were transiently transfected with luciferase reporter containing a -5 kb rat GnRH promoter (-5 kb GnRH) with rat AR expression plasmid. TK-βgal was also transfected into the cells as an internal control. Cells were treated with vehicle (ethanol) or 100 nM R1881 for 24 hours. Cells were assayed for luciferase and β-galactosidase (β-gal) activity explained in *Materials and Methods*. Relative luciferase units (RLU) are luciferase units divided by β-gal units, relative to vehicle or R1881-treated pGL3 RSVe. pGL3 RSVe served as a negative control. The -5 kb GnRH reporter was significantly repressed, indicated by (****), and was determined by Student's t test ($P<0.0001$). Data represent the mean, ± SEM of three experiments, each done in quadruplicate.

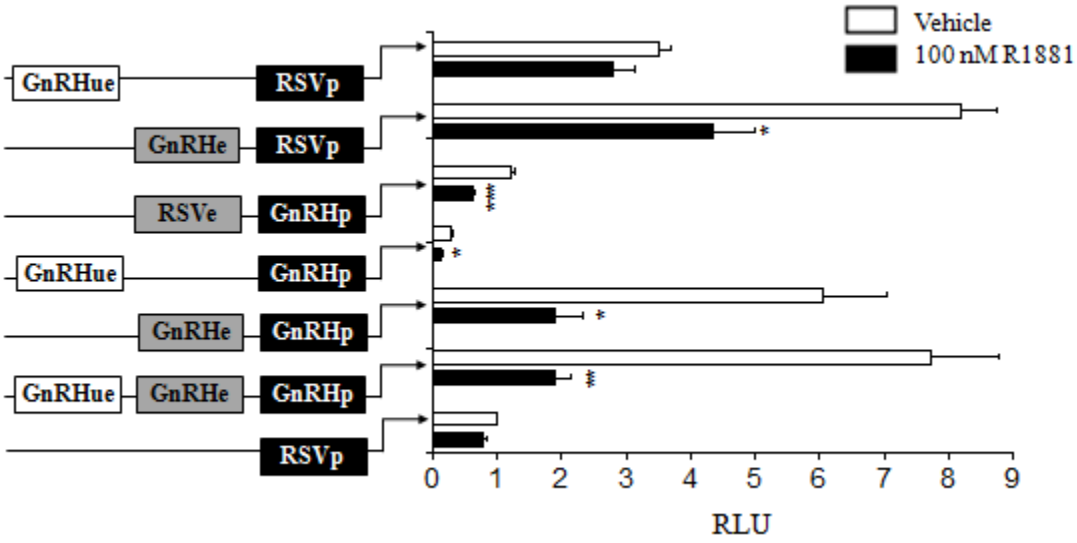

Figure 3: Transient transfections indicate that the GnRH proximal promoter and enhancer are sufficient for repression by AR.

GT1-7 cells were transiently transfected with luciferase reporters containing the proximal promoter (GnRHp), enhancer (GnRHe), or upstream enhancer (GnRHue) alone or in combination, then treated with 100 nM R1881 for 24 hours. Cells were assayed for luciferase and β-gal activity explained in *Materials and Methods*. R1881 repressed activity of all reporters containing GnRHe and/or GnRHp, but not GnRHue alone. Rous sarcoma virus enhancer (RSVe) and promoter (RSVp) were used to enhance expression when no GnRH promoter or enhancer is present. Relative luciferase units are luciferase units divided by β-gal units, relative to vehicle-treated RSVp. Data represent the mean, ± SEM of three experiments, each done in quadruplicate. The *stars* represent statistical significance of R1881 versus vehicle by 1-way ANOVA, t test. (*P<0.05, **P<0.01, ***P<0.001) Data courtesy of Melissa Brayman, Ph.D.

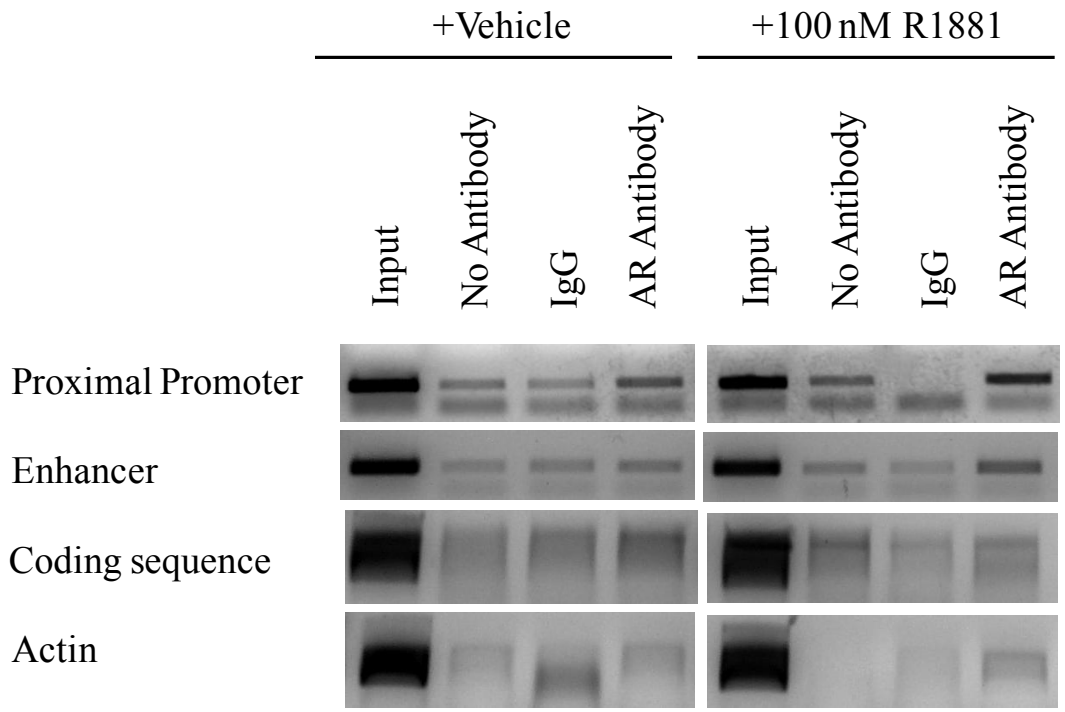

Figure 4: Chromatin immunoprecipitation assay indicates increased AR interaction with the proximal promoter and enhancer after R1881 treatment.

ChIP was performed on GT1-7 cells transiently transfected with rat AR expression plasmid and treated with 100 nM R1881 for 2 hours. An AR antibody was used to immunoprecipitate chromatin interacting with AR. No antibody and an IgG antibody were utilized as controls. PCR primers amplified sequences of the GnRHp and GnRHe. Primers amplifying coding sequences and actin served as controls. Amount of DNA was qualitatively determined by gel electrophoresis. More proximal promoter and enhancer DNA was amplified upon treatment with R1881. Data courtesy of Melissa Brayman, Ph.D.

The -173/-86 and -101/-45 Regions of the GnRH Proximal Promoter are Sufficient for AR-Mediated GnRH Repression

The GnRHp is known to bind to specific transcription factors, including Oct-1 [13], Pbx/Prep1 [14], Nkx2.1 [32], Dlx and Msx [33], and CCAAT/enhancer binding protein-β (C/EBP) [34], that are important for GnRH expression, GnRH neuron development, or both (Figure 5). To characterize the regions of GnRHp necessary for repression by AR, truncation analysis was performed by cloning reporter plasmids with serial deletions from the 5′ end of the proximal promoter, beginning with -126 (relative to the start site of transcription). GT1-7 cells were transiently transfected with the truncation reporter plasmids as indicated, then treated with 100 nM R1881 for 24 hours (Figure 6). Truncation of GnRHp to -112 and -101 did not reverse repression by R1881 treatment, but suppression was lost when truncated to -86. Thus, elements between -101 and -86 bp are likely involved in AR-mediated repression.

Since repression was lost when the proximal promoter was truncated to -86 bp, we tested whether regions upstream are sufficient for AR-mediated repression. Two heterologous promoter reporter constructs were used containing the -127/-86 region and another containing a mutation in the Oct-1 binding site (Figure 7A). Additional heterologous promoters were made containing the -101/-45 region, and another containing base pairs between -173 and -86 (Figure 8A). Since the core GnRH promoter was not included in the reporters, RSVp was added. GT1-7 cells were transiently transfected with the indicated heterologous reporter, then treated with 100 nM R1881 for 24 hours. Treatment with R1881 did not change reporter activity of the -126/-86 region

on a heterologous reporter (Figure 7B). Treatment with R1881 resulted in a suppression of activity of the -101/-45 and -173/-86 regions on the heterologous promoters (Figure 8B). However, the magnitude of repression was much smaller compared to full-length GnRHp. Additional constructs were made with the -101/-45 and -173/-86 fragments in reverse orientation placed between RSVe and RSVp. The reverse orientations also resulted in significant repression. No regulation of RSVp by AR was observed. Thus, the -101/-45 and -173/-86 regions are sufficient for AR-mediated repression, but do not result in the same magnitude of repression compared to the entire proximal promoter.

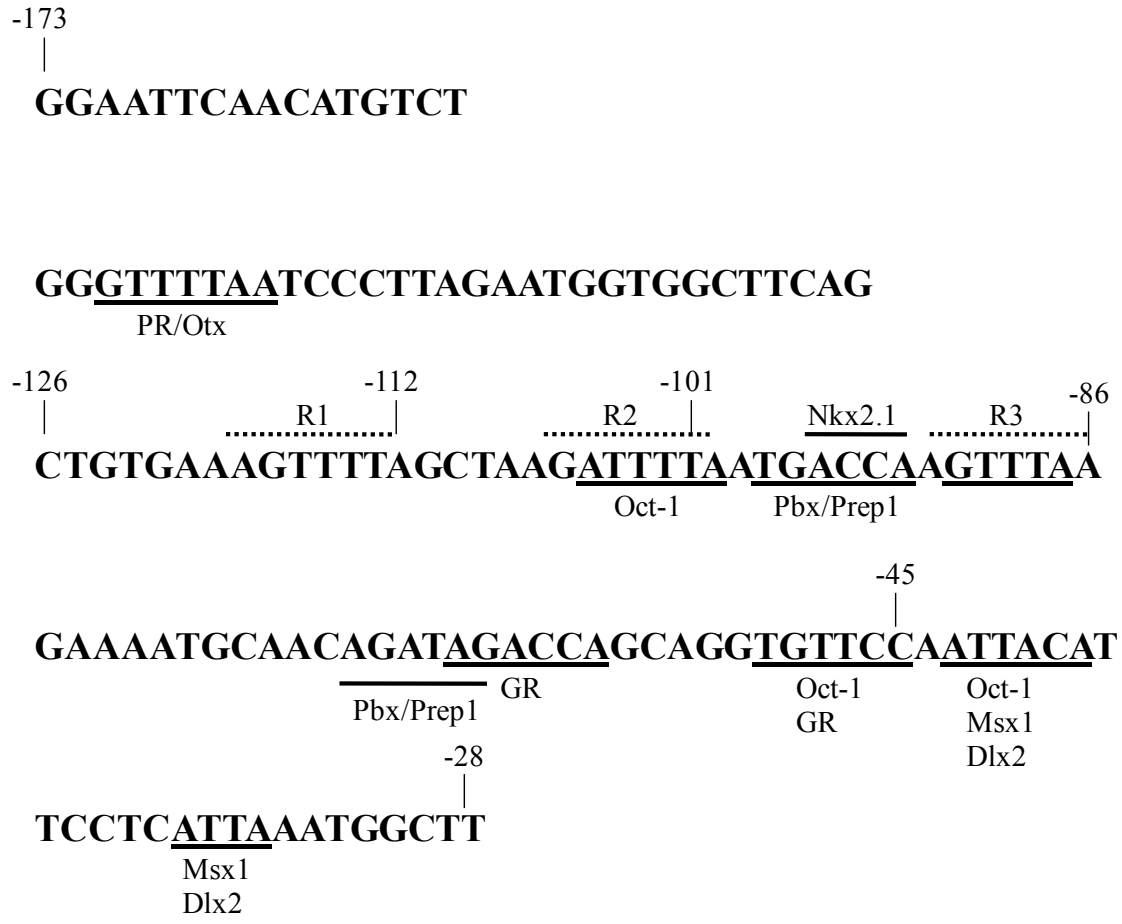

Figure 5: *Cis* elements of the rat GnRH proximal promoter and representation of known transcription factor binding sites.

Lined sequences represent known binding sites for indicated proteins, which are written as their gene symbol. Dotted lines above sequences indicate 3 repeats (R1-R3). R2 and R3 are 1 nucleotide different relative to R1.

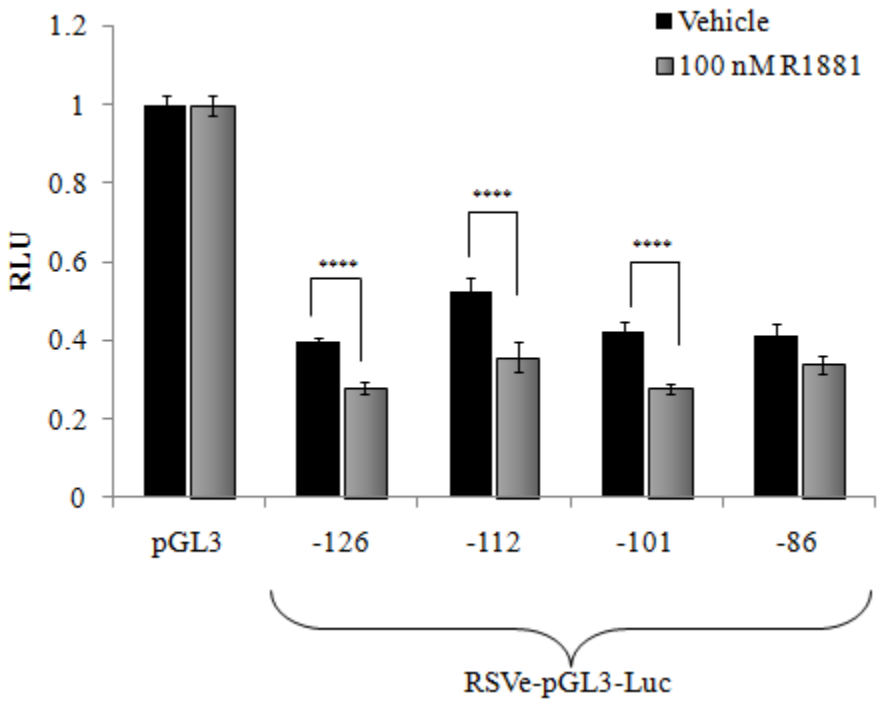

Figure 6: 5′ Truncations of the proximal promoter indicate elements between -101 and -86 bp are involved in AR-mediated repression.

GT1-7 cells were transiently transfected with reporter constructs containing the indicated truncation of GnRHp. Cells were treated with either vehicle (ethanol) or 100 nM R1881 for 24 hours. Cells were assayed for luciferase and β-gal activity explained in *Materials and Methods*. The pGL3 backbone vector served as a negative control. RSVe was included in the deletion reporters to enhance expression. Truncation from -101 to -86 caused a significant loss of repression. Relative luciferase units (RLU) are luciferase units divided by β-gal units, normalized to vehicle or R1881 treated pGL3. Data represent the mean, ± SEM of five experiments, each done in quadruplicate. (****) indicates a statistical significance between the indicated vehicle and R1881-treated reporter as determined by Student's t test with $P<0.0001$.

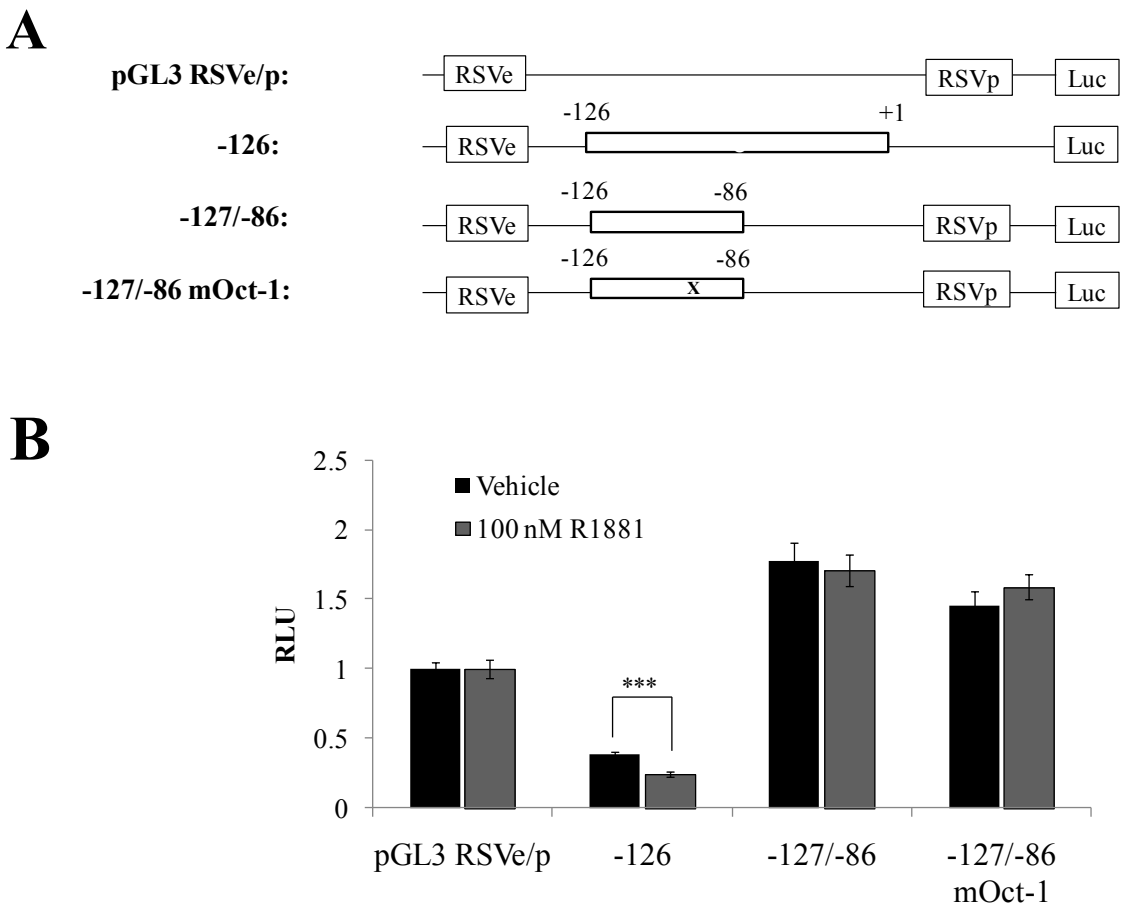

Figure 7: The -127/-86 region of the GnRHp is not sufficient for AR-mediated repression. **A.** Representation of the reporters used in transient transfections. The backbone vector, pGL3 RSVe/p served as a negative control. The -126 pGL3-RSVp-luc (-126) reporter served as a positive control. A reporter construct was made containing the fragment -127/-86 of the proximal promoter, placed between RSVe and RSVp, in the pGL3 backbone. A second -127/-86 heterologous reporter contained a mutation in the Oct-1 binding site, represented by (x). RSVe was included in all reporters to enhance expression.**B.** GT1-7 cells were transfected with the indicated reporter then treated with vehicle (ethanol) or 100 nM R1881 for 24 hours. Cells were assayed for luciferase and β-gal activity explained in *Materials and Methods*. Relative luciferase units (RLU) are luciferase units divided by β-gal units, relative to vehicle or R1881-treated pGL3 RSVe. The -127/-86 reporter was not repressed by R1881. Data represent the mean, ± SEM of three experiments, each done in quadruplicate. (****) indicate statistical difference between the vehicle and R1881 treated -126 reporter as determined by Student's t test with $P<0.001$.

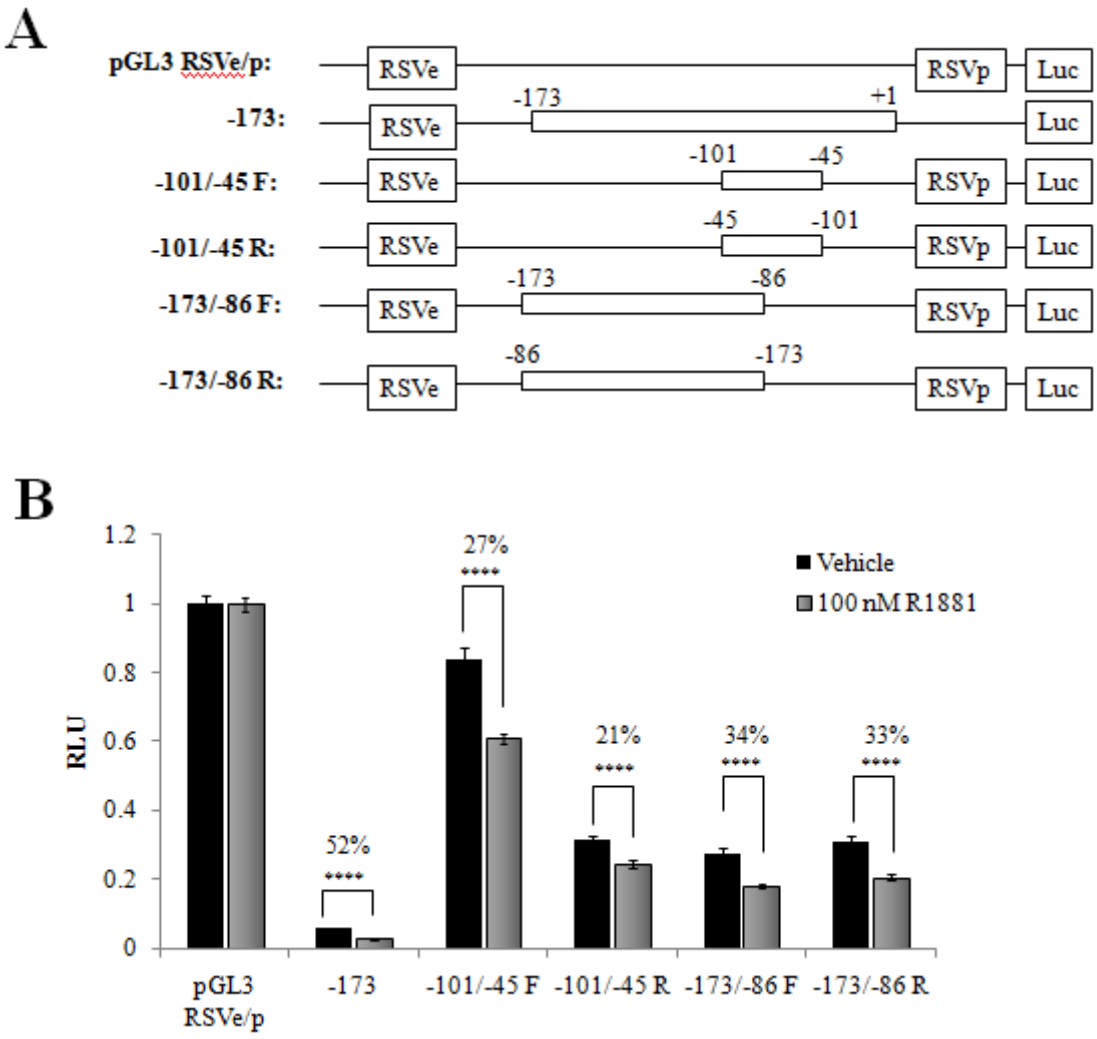

Figure 8: The -173/-86 and -101/-45 regions of the GnRH proximal promoter are sufficient for AR-mediated repression.

A. Representation of the reporters used in transient transfections. The backbone vector, pGL3 RSVe/p, served as a negative control. The -173 pGL3 RSVe/p (-173) served as a positive control. Two reporter constructs were made containing fragments -101/-45 and -173/-86 of the proximal promoter, placed between RSVe and RSVp, in the pGL3 backbone. Each heterologous promoter was made in a forward (F) and reverse (R) orientation. RSVe was included in all reporters to enhance expression. **B.** GT1-7 cells were transfected with the indicated reporter then treated with vehicle (ethanol) or 100 nM R1881 for 24 hours. Relative luciferase units (RLU) are luciferase units divided by β-gal units, normalized to vehicle or R1881 treated pGL3 RSVe/p. Percentages above bars represent approximate magnitude of repression. All heterologous promoters were repressed by R1881, but to a smaller extent compared to -173. Data represent the mean, ± SEM of three experiments, each done in quadruplicate. (****) indicate statistical significance between the indicated vehicle and R1881 treated reporter as determined by Student's t test with $P<0.0001$.

Cis **Elements Between -91/-86 are Necessary for AR-Mediated GnRH Repression**

We utilized site-directed mutagenesis to create 6-bp scanning mutations within the -126/-86 region in the context of the -126-promoter truncation to determine which *cis* elements are involved (Figure 9A). GT1-7 cells were transiently transfected with the indicated mutated reporter and incubated with 100 nM R1881 for 24 hours (Figure 9B). Mutations between -126 and -101 still exhibited significant reduction of reporter activity by R1881. However, mutations at -91/-86 resulted in a relief of repression. Mutations within -103 and -98 resulted in only 23% repression. Thus, another reporter was made containing mutations in both -103/-98 and -91/-86 to determine if the two mutations together would have an additive effect on de-repression. However, 24-hour treatment with R1881 did not result in a greater relief of repression (Figure 10).

Interestingly, there are three AAGTTTA repeats in the -126/-86 region. To determine if they are involved in AR-mediated repression, a reporter was made containing a mutation in each repeat in the context of the full 173 bp promoter (Figure 11A). Even when all three repeats were mutated, the reporter was significantly repressed by R1881, indicating that the repeats do not play a role in repression of P by AR (Figure 11B). Collectively, these results suggest *cis* elements within -91/-86 are likely involved in AR-mediated repression.

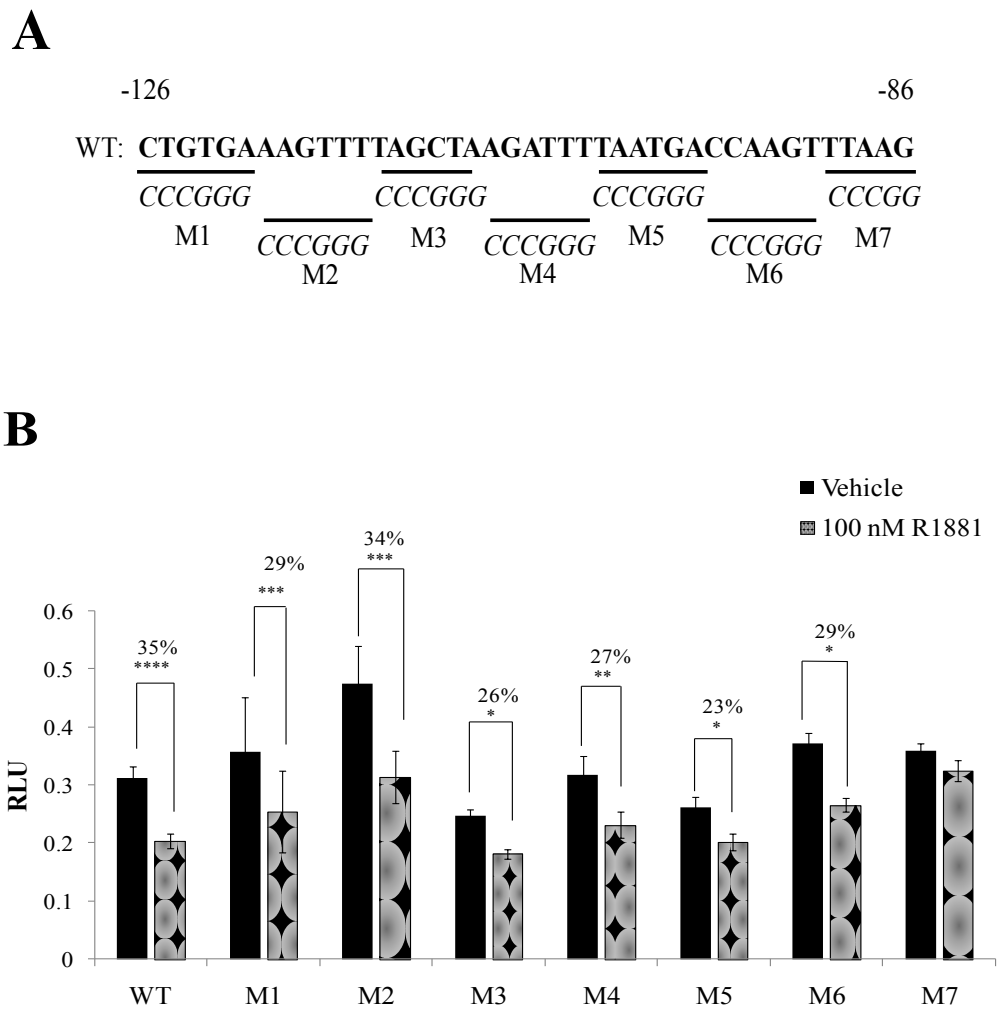

Figure 9: Transient transfections with scanning mutants across the -126/-86 region in the -126 promoter show that the -91/-86 region is necessary.

A. Six bp scanning mutations across the -126/-86 region were made via site directed mutagenesis in the -126 bp GnRHp-RSVe-pGL3 reporter. Bolded *cis* elements represent the wild type (WT) -126/-86 sequence. Underlined WT *cis* elements indicate reporter mutations (M1-M7) and substituted base pairs are italicized. Primers used for mutagenesis are listed in table ?..**B.** Mutant reporters were transiently transfected into GT1-7 cells, then treated with vehicle (ethanol) or 100 nM R1881 for 24 hours. pGL3 served as a negative control and WT served as a positive control. Relative luciferase units (RLU) are luciferase units divided by β-gal units, relative to vehicle or R1881 treated pGL3. Percentages above bars represent approximate magnitude of reduction. Repression was lost when the -91/-86 (M7) region was mutated. Data represent the mean, ± SEM of three experiments, each done in quadruplicate. Statistical significance between the indicated vehicle and R1881 treated reporter, indicated by (*), were determined by Student's t test. (P****<0.0001, P***<0.001, P**<0.01, P*<0.05)

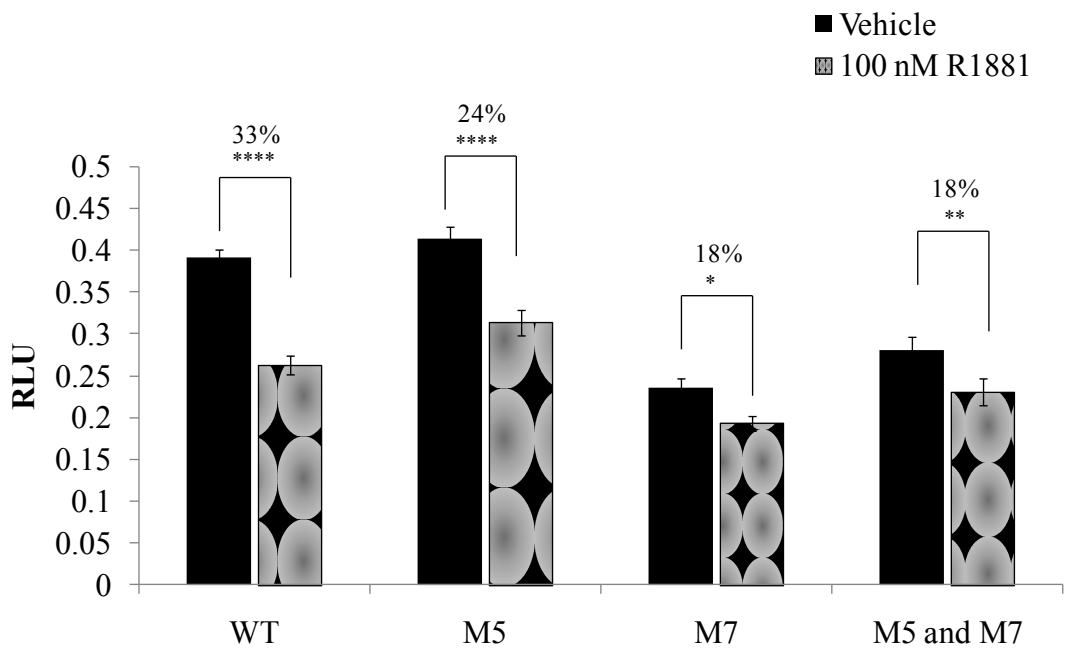

Figure 10: Mutation of both -91/-86 and -103/-98 does not show an additive effect in repression relief.

A reporter was made containing mutations in both -103/-98 (M5) and -91/-86 (M7) in the context of the -126 GnRHp and transiently transfected into GT1-7 cells. WT, M5, and M7 sequences are shown in Figure 9A. Cells were treated with vehicle (ethanol) or 100 nM R1881 for 24 hours then assayed for luciferase and β-galactosidase (β-gal) activity explained in *Materials and Methods*. Relative luciferase units (RLU) are luciferase units divided by β-gal units, normalized to vehicle or R1881 treated pGL3. pGL3 (not shown) and WT reporters served as a negative and positive control, respectively. Percentages above bars indicate approximate magnitude of repression. Mutations in both M5 and M7 resulted in the same magnitude of repression relative to M7 alone. Data represent the mean, ± SEM of seven experiments, each done in quadruplicate. Statistical difference between the indicated vehicle and R1881 treated reporter, represented by (*), were determined by Student's t test. (P****<0.0001, P**<0.01, P*<0.05)

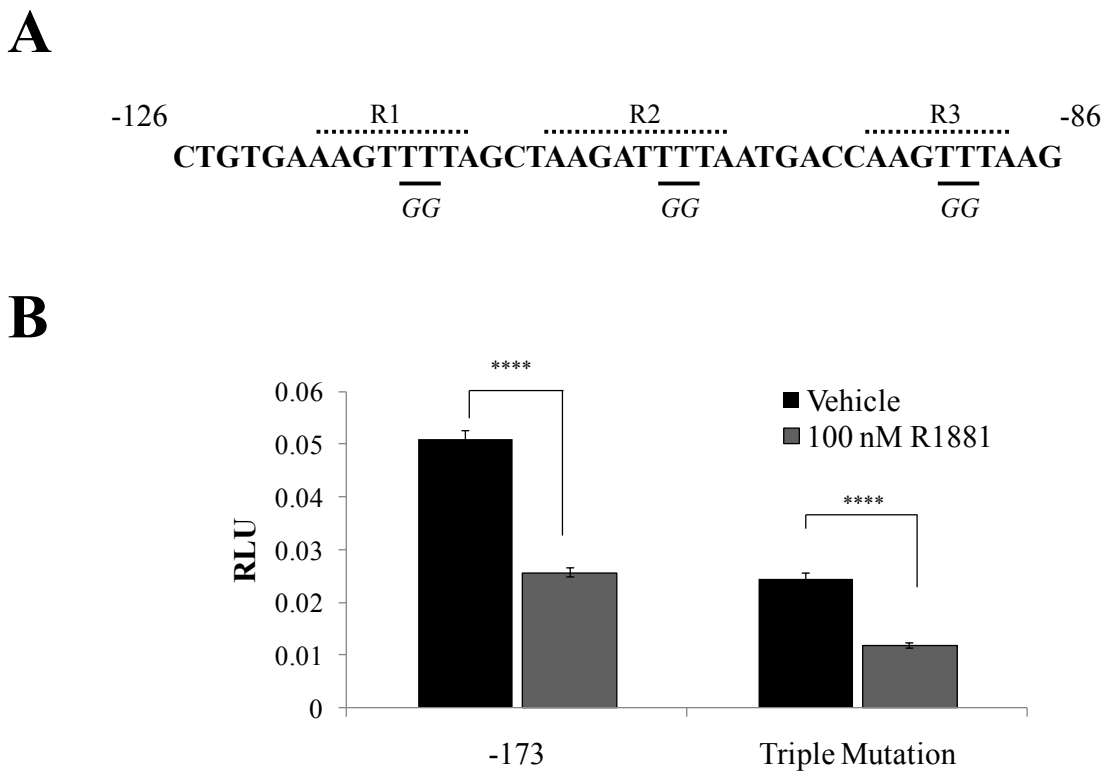

Figure 11: Three AAGTTTA repeats in the -126/-86 region are not required for AR-mediated repression.

A. Site-directed mutagenesis was utilized to make mutations in the -173 GnRHp-RSVe luciferase reporter (-173). The mutations were in each of the three repeats (R1-R3) in the -126/-86 region and italicized *cis* elements represent substituted nucleotides. **B.** The triple mutated reporter was transiently transfected into GT1-7 cells and treated with vehicle (ethanol) or 100 nM R1881 for 24 hours. Cells were assayed for luciferase and β-galactosidase (β-gal) activity explained in *Materials and Methods*. Relative luciferase units (RLU) are luciferase units divided by β-gal units, relative to vehicle or R1881 treated pGL3-RSVe. pGL3-RSVe served as a negative control (not shown). The triple mutated reporter was negatively regulated by treatment with 100 nM R1881. Data represent the mean, ± SEM of four experiments, each done in quadruplicate. (****) represents statistical difference between the corresponding vehicle and R1881 treated reporter as determined by Student's t test with $P<0.0001$.

The -1800/-1780 Region in the GnRH Enhancer is Involved in AR-mediated GnRH Repression and Interacts with AR

Earlier transient transfection experiments showed a significant reduction in GnRHe activity upon R1881 treatment (Figure 3). Furthermore, ChIP results confirmed increased interaction with this region after R1881 treatment (Figure 4). The GnRH enhancer region contains many known transcription factor-binding sites (Figure 12).

Glucocorticoid receptor, another member of the steroid hormone family of nuclear receptors, tethers to DNA-bound Oct-1 to repress GnRH expression [28]. To determine if AR functions through the same mechanism, reporters containing mutations of the Oct-1 binding sites in the GnRHe were utilized in transient transfections (Figure 13). Reporters contained mutations in the binding sites for Oct-1a (-1785 to-1771) and Oct-1b (-1702 to -1695) alone and in combination. 24-hour treatment with R1881 resulted in repression of all three mutated reporters, suggesting that neither Oct-1 binding site is required.

To characterize specific regions or other possible transcription factor binding sites involved in AR-mediated repression, truncation analysis was carried out with serial deletion of the -1800/-1571 enhancer region from the 3′ end (Figure 14). Truncation to -1636 bp resulted in a dramatic decrease in basal activity, but repression by R1881 was still present. Significant repression continued as the enhancer was truncated to -1730 bp, but became significant again when truncated to 1750 bp. Repression was lost again when truncated to -1780 bp, suggesting the -1800/-1780 region contains sites that are involved in AR-mediated GnRH repression.

The GnRHe was further investigated by transiently transfecting cells with a reporter containing GnRHe with base-pair mutations at -1790 and -1791, which is upstream of the Oct-1a binding site (Figure 15). This 2-base pair mutation resulted in a loss of 12-O-tetradecanoyl phorbol-13-acetate (TPA)-mediated GnRH repression [35]. Basal expression from this reporter was much lower than from the wild type enhancer, but AR repression was still present, indicating the 2 base pairs at -1790 and -1791 may be important for both basal activity, but not for repression. Therefore, other elements within the -1800/-1780 region are necessary for AR-mediated GnRH repression.

Figure 12: Sequence of the distal enhancer and representation of known transcription factor binding sites.

Lined sequences represent known binding sites for proteins. Dotted lines indicate the beginning of a binding site that is continued onto the next line. Proteins are written as their gene symbol.

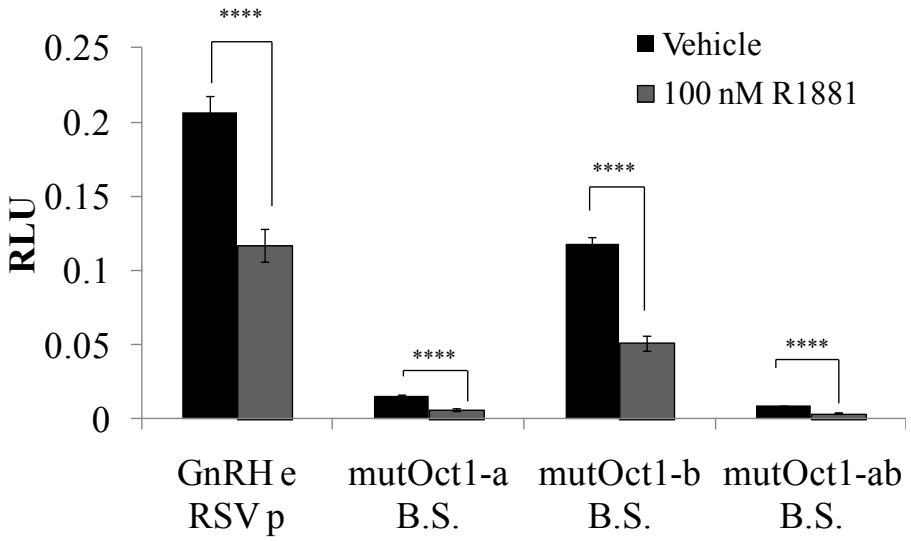

Figure 13: Oct-1 a and b binding sites in the GnRH enhancer are not required for AR repression.

The GnRHe in the pGL3 RSVp luciferase backbone was mutated in either or both of the two Oct-1 binding sites (B.S.), Oct-1a and Oct-1b. Indicated reporters were transiently transfected into GT1-7 cells and were treated with 100 nM R1881 for 24 hours. Cells were assayed for luciferase and β-gal activity explained in *Materials and Methods*. GnRHe RSVp served as a positive control. pGL3 RSVp served as a negative control (not shown). Relative luciferase units (RLU) are luciferase units divided by β-gal units, relative to vehicle or R1881 treated pGL3 RSVp. R1881 repressed all reporters, regardless of mutation. Data represent the mean, ± SEM of three experiments, each done in quadruplicate. (****) represents statistical difference between the corresponding vehicle and R1881 treated reporter as determined by Student's t test with $P<0.0001$.

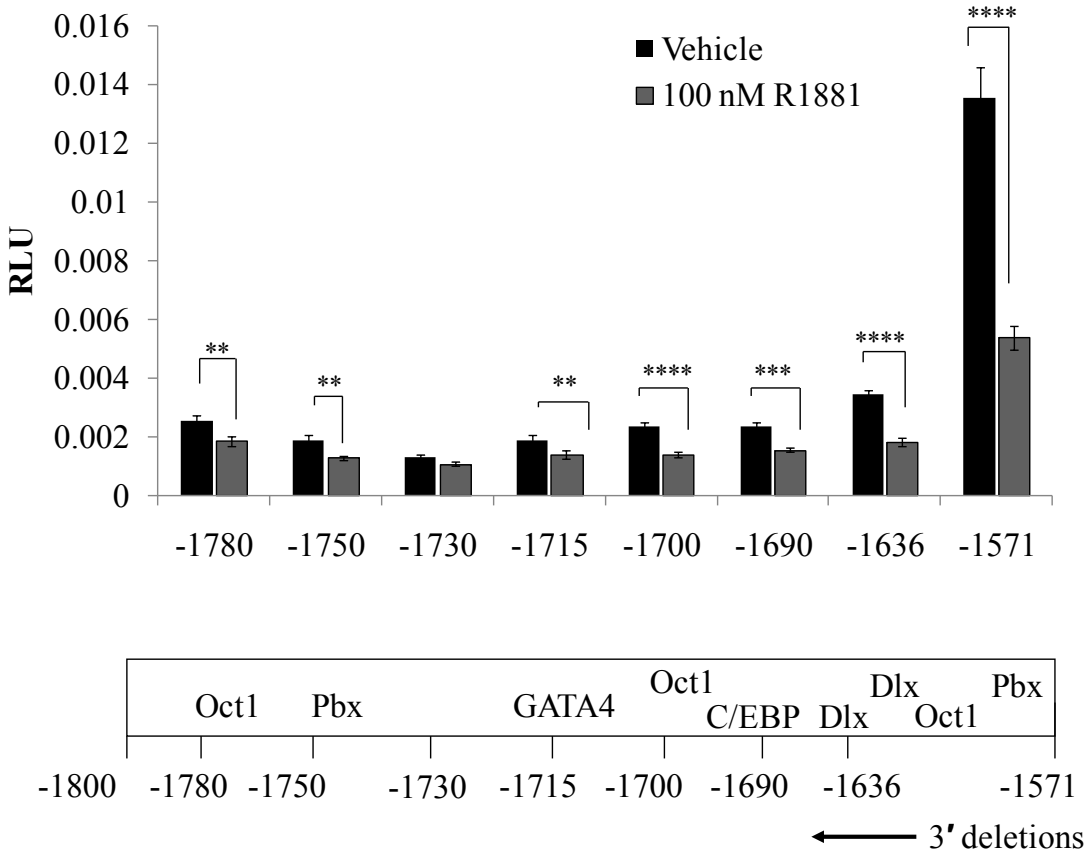

Figure 14: GnRH enhancer truncations from the 3′ end implicate sites between -1800 and -1780 in AR repression.

pGL3 RSVp reporter plasmids containing serial deletions from the 3′ end of the GnRHe were transiently transfected into GT1-7 cells. Cells were treated with 100 nM R1881 for 24 hours and assayed for luciferase and β-gal activity explained in *Materials and Methods*. pGL3 RSVp served as a negative control (not shown). Relative luciferase units (RLU) are luciferase units divided by β-gal units, relative to vehicle or R1881 treated pGL3 RSVp. All truncation deletions were significantly repressed by 24-hour treatment with R1881, except for the -1730 truncation. Data represent the mean, ± SEM of four experiments, each done in quadruplicate. Statistical significance between the indicated vehicle and R1881 treated reporter, indicated by (*), were determined by Student's t test. (P****<0.0001, P***<0.001, P**<0.01)

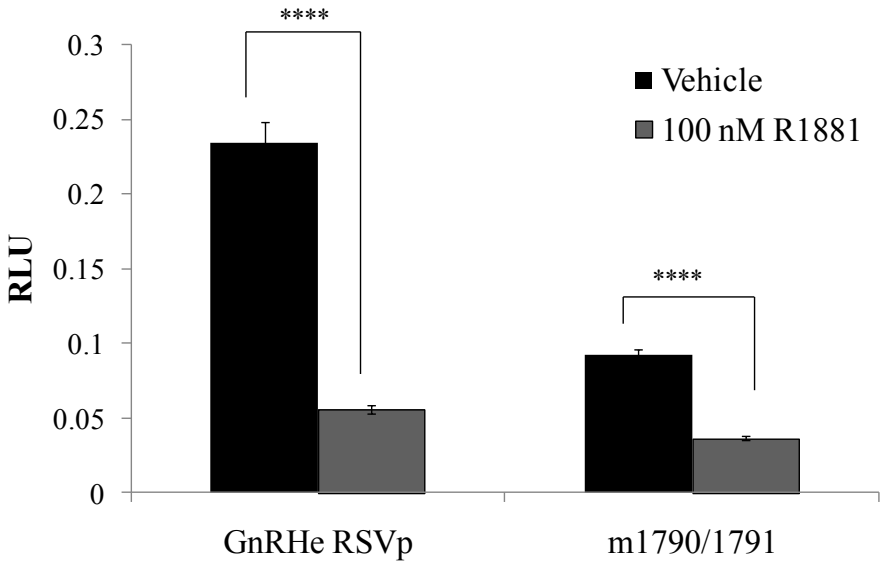

Figure 15: *Cis* elements at -1790 and -1791 are not required for AR-mediated repression. The GnRHe fused to RSVp luciferase backbone vector contained mutations at -1790 and -1791. Reporter plasmids were transiently transfected into GT1-7 cells. Cells were treated with 100 nM R1881 for 24 hours and assayed for luciferase and β-gal activity explained in *Materials and Methods*. pGL3 RSVp served as a negative control (not shown). Relative luciferase units (RLU) are luciferase units divided by β-gal units, relative to vehicle or R1881 treated pGL3 RSVp. Mutation of -1790 and -1791 resulted in a loss of basal activity, and was repressed by 24-hour treatment with 100 nM R1881. Data represent the mean, ± SEM of three experiments, each done in quadruplicate. Statistical difference between the corresponding vehicle and R1881 treated reporter, represented by (****) were determined by Student's t test with $P<0.0001$.

A 4x -1800/-1766 Multimer is Sufficient for AR-Mediated GnRH Repression

A luciferase reporter containing four copies of -1800/-1766, and thymidine kinase promoter (TKp) was transiently transfected into GT1-7 cells (Figure 16). R1881 significantly suppressed 4x -1800/-1766 reporter activity, confirming that *cis* elements within the -1800/-1766 region are sufficient for AR-mediated repression. The Oct-1a binding site was mutated in the 4x -1800/-1766 reporter and transiently transfected into GT1-7 cells. Basal expression fell dramatically, but repression was still present, indicating Oct-1 bound at that site is involved in basal activity, but not involved in the repression mechanism. When mutations were introduced into the 4x -1800/-1766 reporter between -1787 and -1789, basal activity was reduced and repression was lost. Thus, *cis* elements immediately upstream of the Oct-1a binding site are involved in AR-mediated GnRH repression; however, the factor(s) that binds to that region of the GnRHe remains to be determined.

Electromobility shift assay (EMSA) was utilized to determine whether a protein binds in that region. GT1-7 nuclear extracts were incubated with a radiolabeled probe spanning the -1802/-1778 region and AR antibody (Figure 17). A complex was shifted with an AR antibody. AR binds to palindrome sequences as a homodimer. Thus, AR could be binding to this region directly or indirectly through another protein. To identify the *cis* elements the complex could be binding to, double-stranded (d.s) oligonucleotide competitors with 3-bp mutations that scan the wild-type probe sequence were added as competitors (Figure 18). The oligonucleotide competitors do not compete with the

complex when -1795/-1790 is mutated, indicating the complex binds to the -1795/-1790 (GTCCTC) sequence of the GnRHe. Further confirming AR involvement, an ARE oligonucleotide competed with the complex. These results suggest that AR is interacting with the -1795/-1790 region of the GnRH enhancer.

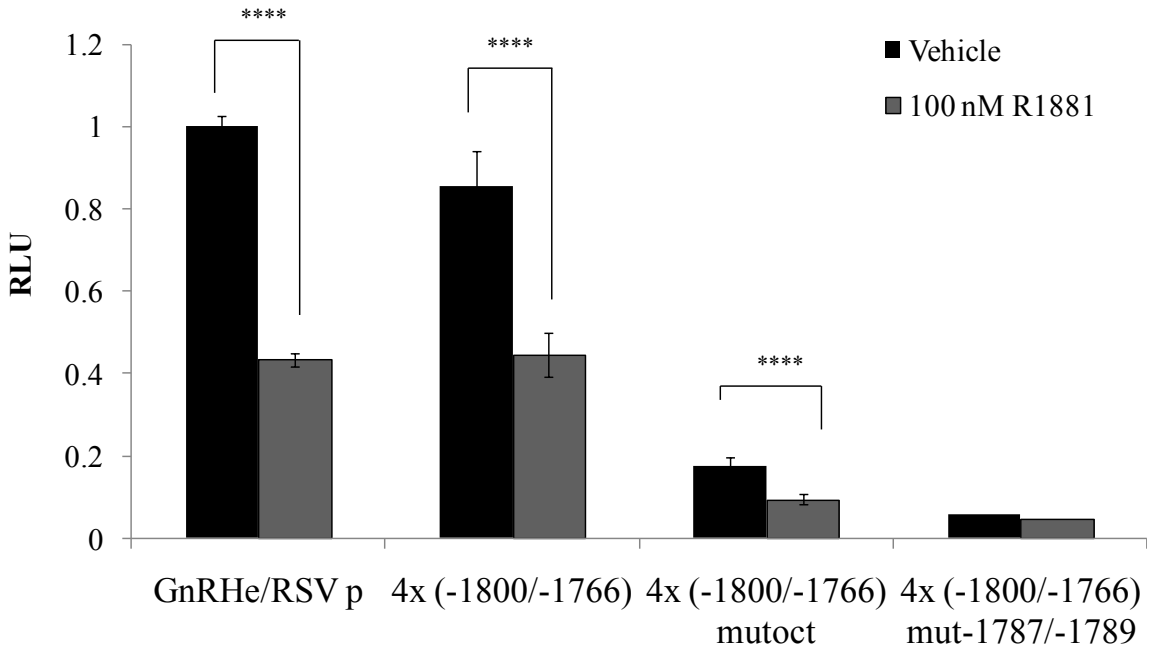

Figure 16: The -1800/-1766 region of the GnRH enhancer is sufficient, and the *cis* elements at -1787/-1789 are necessary, for AR-mediated repression.

A reporter construct containing four copies of the -1800/-1766 region of GnRHe, upstream of thymidine kinase promoter (TKp) [4x(-1800/-1766)], was transfected into GT1-7 cells. Basal activity of 4x(-1800/-1766) was approximately the same as GnRHe/RSVp, and repression upon treatment with R1881 was significant. Mutations that destroyed the Oct-1 binding site [4x(-1800/-1766)moct] affected basal expression, but repression by R1881 was still present. Mutation of -1787 and -1789 [4x(-1800/-1766)mut1787/1789] resulted in both a decrease in basal expression and a loss of repression by R1881. Relative luciferase units (RLU) are luciferase units divided by β-gal units, relative to vehicle treated GnRHe/RSVp. Data represent the mean, ± SEM of three experiments, each done in quadruplicate. (****) represents statistical significance between the corresponding vehicle and R1881 treated reporter as determined by Student's t test with $P<0.0001$.

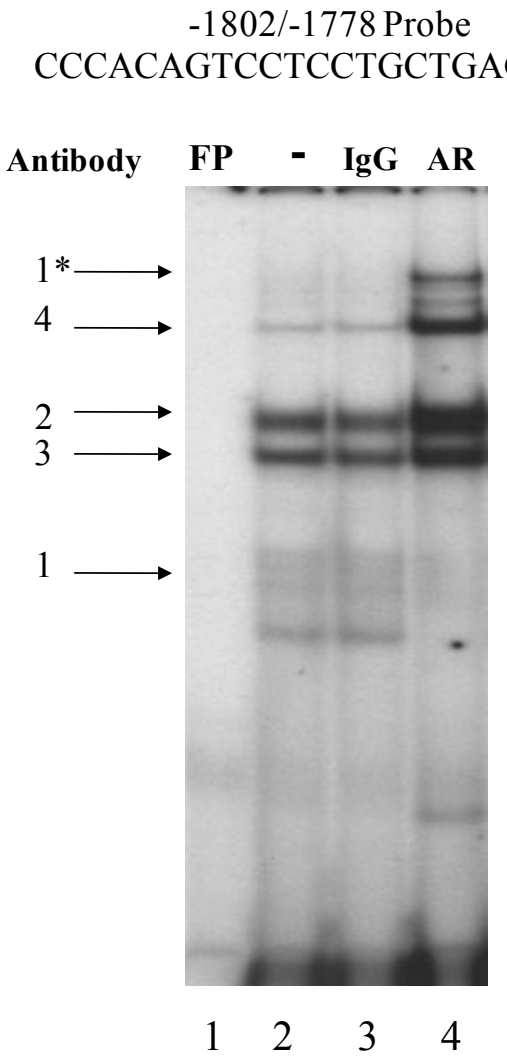

Figure 17: AR is part of a complex binding to the -1802/-1778 sequence of the enhancer.
An EMSA performed with GT1-7 cell nuclear extracts indicates binding of 2 complexes (labeled 1 and 2) and binding of a single protein (labeled 3) to a radiolabeled probe (-1802/-1778) representing a region of the GnRHe. Lane 1 contains free probe (FP) and lane 3 contains extracts incubated with rabbit immunoglobulin G (IgG), which served as a negative control. Lane 4 contains cell extracts incubated with AR antibody. Arrow labeled 1* indicates the protein complex supershifted with AR antibody. Arrow 2, 3, and 4 represent unknown proteins binding to the probe.

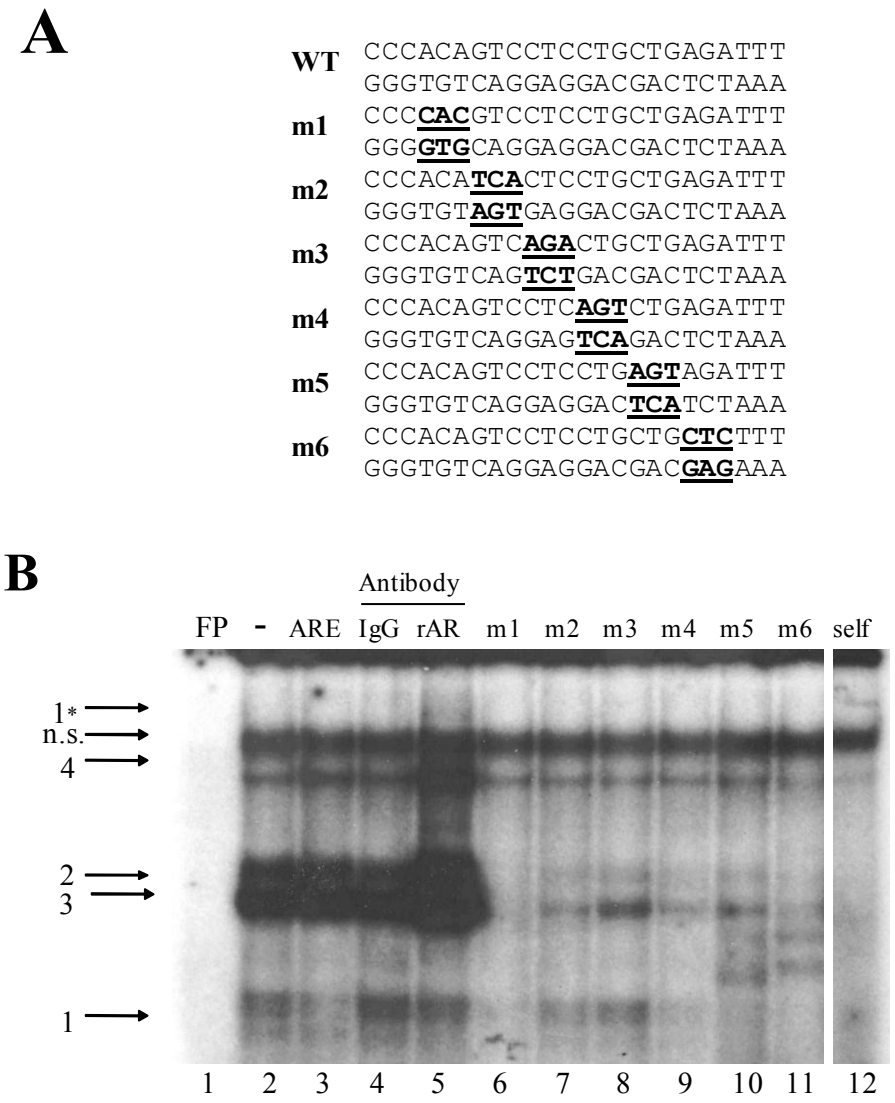

Figure 18: An AR-containing complex binds to -1795/-1790 of the GnRHe.
A. Representation of double-stranded oligonucleotides used for an EMSA competition experiment. Radiolabeled probe contained wild time (WT) sequence that represents the -1802/-1778 region of the GnRHe. Locations of 3-bp scanning mutations (m1-m6) are underlined and substituted nucleotides are in bold. **B.** GT1-7 nuclear extracts were incubated with radiolabeled WT or mutated -1802/-1778 probe. m2 and m3 do not compete complex 1 (labeled 1). m2-m5 do not compete complex 2 and 3 (labeled 2 and 3). An oligonucleotide sequence of a putative androgen-responsive element (ARE) also competes complex 1 (Lane 3). An antibody supershift was performed with antibodies for rat AR (rAR) (lane 5) and rabbit IgG as a control (lane 4). rAR results in a very slight supershift (1*) of complex 1. Lane 12 represents self-competition and identifies non-specific (n.s.) binding. Arrow 4 represents an unknown protein binding to the probe. Lane 1 contains free probe (FP).

Nkx2.1 is Potentially Involved in AR-Mediated GnRH Repression

AR could regulate gene expression through interactions with transcription factors that are known to be important for basal transcription of GnRH, such as Oct-1, Pbx/PREP, and Nkx2.1. One possibility is that AR tethers to these transcription factors. Alternatively, AR could recruit the factors away from the GnRH regulatory region. To test whether AR interaction with these transcription factors was involved in AR repression of GnRH transcription, reporter constructs were made that contained three copies of the consensus binding site for each transcription factor, placed between RSVe and RSVp. These reporters were transiently transfected into GT1-7 cells, with and without AR expression plasmid. The 3xOct-1 reporter was slightly, but significantly repressed by R1881 with and without exogenous AR (Figure 19A). Co-transfection of Oct-1 expression plasmid resulted in a small, but significant repression only when exogenous AR was expressed in the cells. The 3xPbx1 reporter was significantly repressed by 24-hour treatment with R1881, with or without exogenous AR (Figure 19B). Co-transfection with Pbx1 and AR expression plasmid resulted in a relief in repression. The reporter containing three copies of the consensus binding site for Nkx2.1 (3xNkx2.1) was repressed by R1881 when AR expression plasmid was co-transfected (Figure 19C). Cells transiently co-transfected with Nkx2.1 expression vector enhanced basal activity and resulted in approximately 62% repression. Therefore, R1881 caused a decrease in 3xOct-1 and 3xPbx1 reporter activity with and without exogenous AR and caused a decrease in 3xNkx2.1 reporter activity only with exogenous AR.

The -1800/-1780 region of the GnRHe is important for AR-mediated repression, as shown by prior transient transfections (Figure 14). Thus, EMSA was performed to test whether Nkx2.1 is able to bind to that region. Nuclear extracts form unknown complexes with a probe spanning the -1802/-1778 region and is competed off the radiolabeled probe with an unlabeled d.s. oligonucleotide containing three copies of the Nkx2.1 consensus-binding site (Figure 20). An unlabeled oligonucleotide containing a single copy of the Nkx2.1 binding site, but containing a 3 base pair mutation, did not compete the complexes from the radiolabeled probe. EMSA competition experiments were performed with oligonucleotides containing 3-bp scanning mutations (Figure 18). The same complexes were not competed by unlabeled probes containing mutations in the CTCCTGCTG region of the -1802/-1778 oligonucleotide, suggesting that Nkx2.1 is binding to the -1784/-1792 region of the GnRH enhancer.

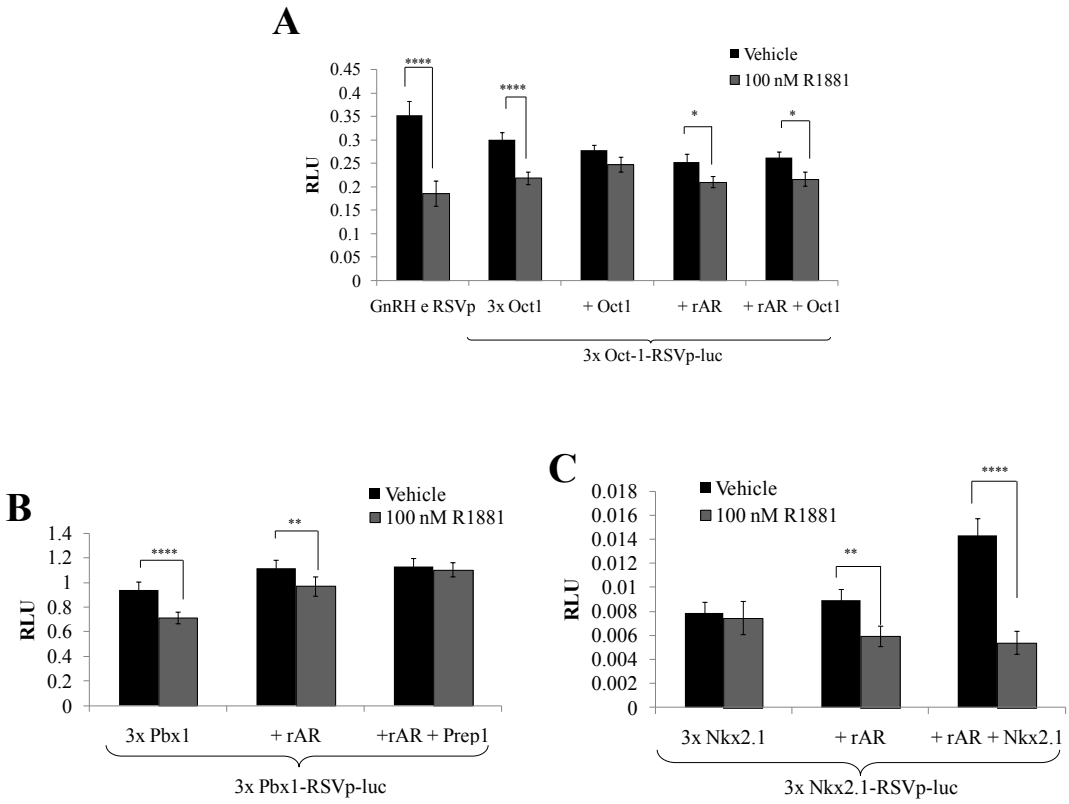

Figure 19: A reporter containing a trimer of the consensus-binding site for Oct-1, Pbx1, or Nkx2.1 is repressed by R1881.

Three copies of Oct-1, Pbx1, or Nkx2.1 consensus-binding sites were cloned upstream of the RSVp and downstream of pGL3 RSVe in a pGL3 luciferase reporter vector (3xOct-1-RSVp-luc, 3xPbx1-RSVp-luc, and 3xNkx2.1-RSVp-luc, respectively) and transiently transfected into GT1-7 cells then treated with vehicle (ethanol) or 100 nM R1881 for 24 hours. **A.** GnRHe RSVp served as a positive control. 3xOct-1-RSVp-luc reporter activity was significantly reduced with and without exogenous rAR. Co-transfection with Oct-1 expression plasmid (+Oct-1) resulted in a relief of repression with endogenous AR. **B.** 3xPbx1-RSVp-luc reporter activity was reduced with and without exogenous AR. No repression was seen when Prep1 was overexpressed in the cells. **C.** 3x Nkx2.1-RSVp-luc reporter activity was significantly repressed only when exogenous AR was present. Co-transfection with Nkx2.1 expression plasmid led to an increase in basal activity and approximately 62% repression by R1881. Relative luciferase units (RLU) are luciferase units divided by β-gal units, normalized to vehicle or R1881 treated pGL3 RSVe/p. pGL3 RSVe/p RLU values are not shown. Data represent the mean, ± SEM of three experiments, each done in quadruplicate. (*) indicates statistical difference between the corresponding vehicle and R1881 treated reporter as determined by Student's t test with P****<0.0001, P**<0.01 and P*<0.05.

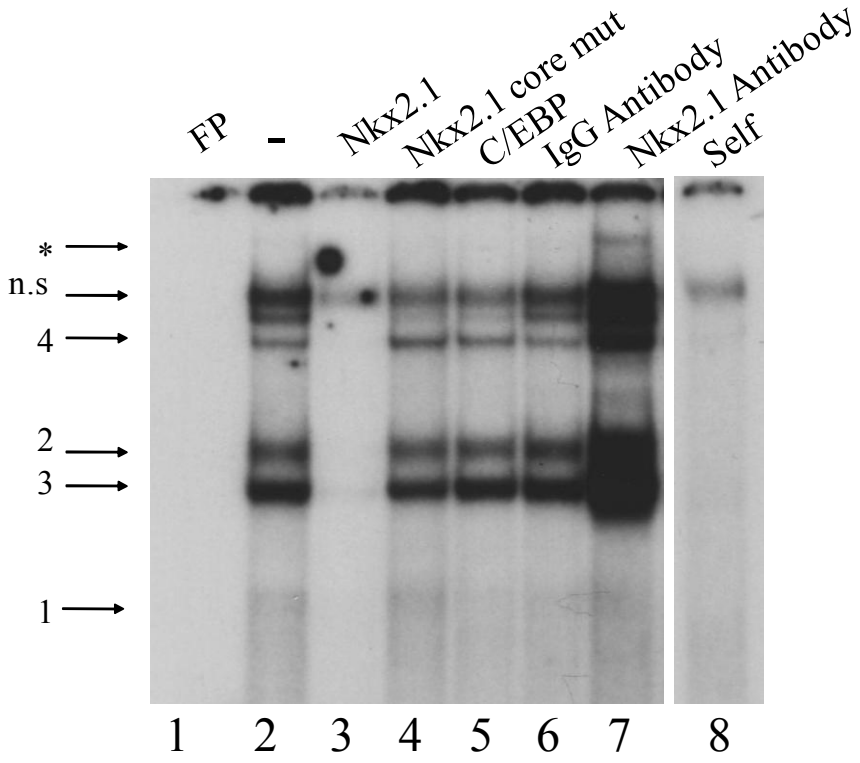

Figure 20: Unknown protein complexes binding to the -1802/-1778 probe may involve Nkx2.1.

GT1-7 nuclear extracts were incubated with radiolabeled probe containing WT sequence that represents the -1802/-1778 region of the GnRHe. Lane 1 contains free probe (FP) and lane 6 contains extracts incubated with rabbit immunoglobulin G (IgG), which served as a negative control. Arrow labeled * indicates a protein complex supershifted with Nkx2.1 antibody. Arrow 2, 3, and 4 represent unknown proteins binding to the probe. Arrow 1 is an unknown protein complex containing AR. All proteins are competed off the radiolabeled probe when extracts are pre-incubated with unlabeled probe containing 3 copies of the Nkx2.1 consensus b.s. n.s, non-specific Lane 5 contains extract pre-incubated with unlabeled probe representing the consensus sequence for C/EBP.

IV

Discussion

Proper GnRH synthesis is vital for reproductive function in both males and females and is regulated by a variety of factors, including androgens. Knowing the mechanisms by which androgens regulate GnRH would have significant implications for understanding potential causes of reproductive disorders and lead to the development of improved treatments. Belsham, et al., demonstrated that DHT treatment of GT1-7 cells results in approximately 55% repression of GnRH mRNA levels [31]. Repression proved to be AR dependent because reductions in GnRH mRNA levels were completely lost upon treatment with hydroxyflutamide, an AR antagonist.

Our *in vitro* studies utilizing GT1-7 cells further confirm AR-mediated GnRH repression on a transcriptional level. R1881, a synthetic AR agonist, reduced activity of a luciferase reporter containing the -5 kb GnRH promoter. Reporter activity decreased by approximately 53% upon treatment, which is consistent with the AR-mediated reduction of GnRH mRNA levels observed by Belsham, et al. [31]. We chose to use R1881 because it has a very high affinity for AR and is more stable in solution than DHT. R1881 can also bind to progesterone, mineralocorticoid, and glucocorticoid receptors [36-38]. However, we observed that the -5 kb GnRH luciferase reporter is significantly repressed only when the cells are co-transfected with AR expression plasmid (data not shown). Thus, the changes in reporter activities due to R1881 must largely be occurring through

AR in this system. Endogenous levels of AR were detected in GT1-7 cells by Western blot (Mellon Lab, unpublished data), but were not sufficient for repression. GT1-7 cells lose endogenous AR protein expression as they are passaged, and levels are especially low in higher-passage cells. Thus, it was necessary to over-express AR in our *in vitro* experiments.

The GnRH gene contains three well-characterized regulatory regions: GnRHp, GnRHe, and GnRHue. Our results demonstrated that AR represses GnRH expression through GnRHp and GnRHe, and not through GnRHue. However, the specific mechanism remains unknown. AR regulation classically involves direct binding of AR to DNA, followed by the recruitment of co-repressors or co-activators. We observed an increase in AR interaction with endogenous GnRHp and GnRHe after R1881 treatment. Therefore, AR could potentially be binding directly to DNA, but GnRHp and GnRHe do not contain sequences resembling classical AREs. Alternatively, AR could be binding to non-classical AREs. AR has also been shown to repress gene transcription independent of direct DNA binding [39]. One possibility is that AR interacts with transcription factors that are important for basal activity thereby tethering to the regulatory regions [20, 21, 40]. GnRHp and GnRHe contain binding sites for a variety of different factors, such as Oct-1, Pbx/PREP, Dlx, Msx, Nkx2.1, and C/EBP. Therefore, it is possible that AR is interacting with GnRHp and GnRHe indirectly through DNA-bound transcription factors to repress GnRH transcription.

We performed transient transfections with luciferase reporters containing serial deletions of the GnRHp and GnRHe. If AR is repressing GnRH by interacting with a

DNA-bound transcription factor, removing that factor's binding site should abrogate repression. We mapped repression to the -101/-86 region via truncation analysis of the -173 bp GnRH proximal promoter. Analysis of this region shows binding sites for Oct-1, Pbx/PREP, Nkx2.1, and AP-1. On the other hand, repression through GnRHe was mapped to the -1800/-1780 region, which contains an Oct-1 binding site (ATa). Since both sites contain Oct-1 binding sites, we hypothesized that repression involves Oct-1. Also GR, another member of the steroid hormone receptor family, represses GnRH through interaction with DNA-bound Oct-1 [28]. AR also has been shown to interact with Oct-1, in the context of the MAFbx gene [21]. Thus, we hypothesized that AR-mediated GnRH repression acts through a similar mechanism. However, 5′ truncation to -101 of the GnRHp removed part of the Oct-1 binding site and repression was still present. In the context of the enhancer, mutating both Oct-1 binding sites (ATa and ATb) decreased basal activity by more than 80%, but repression remained (Figure 13). Thus, Oct-1 binding to the -110/-88 site in GnRHp and to the ATa and ATb sites in GnRHe was not required for AR-mediated repression, suggesting that Oct-1 is not involved. Also, GnRHue contains nine Oct-1 binding sites. If Oct-1 were involved, liganded AR should also repress through GnRHue, but our findings prove otherwise. To further investigate Oct-1 involvement, we created luciferase reporters containing three copies of the Oct-1 consensus-binding site upstream of RSVp. R1881 treatment resulted in a significant reduction in reporter activity, with and without exogenous AR. Either endogenous AR levels were sufficient for reporter repression, or R1881 was activating a different nuclear

hormone receptor, such as PR or GR. Collectively, our results suggest that Oct-1 may not be involved in AR-mediated GnRH repression.

There are three AT-rich repeats in the first 126 bp of GnRHp that are not required for AR-mediated repression. However, they are important for basal activity. The second repeat (R2) is part of an Oct-1 binding site, which is vital for GnRH expression [11]. Therefore, the dramatic decrease in basal activity is due to the mutation in the Oct-1 binding site. Thus, our results are consistent with the role of Oct-1 in GnRH transcription, but we show it is not required for AR-mediated repression.

We next investigated whether we could map AR-mediated repression to a specific transcription factor-binding site. Six-base-pair scanning mutations were made in the -126/-86 region in the context of the -126 GnRHp luciferase reporter. Repression was significantly decreased when the -91/-86 region was mutated. Introducing mutations to the -103/-98 region of GnRHp resulted in a fold repression of about 23% while the wild type resulted in a repression of approximately 35%. Introducing mutations into both the -103/-98 and -91/-86 regions did not result in an additive de-repression. Rather, the double mutation resulted in approximately the same amount of repression compared to -91/-86 alone (approximately 18%). Thus, the -91/-86 region is vital for repression, but the exact mechanism of this repression remains to be elucidated. It is likely that AR is interacting with a transcription factor that is binding at that site. Pbx/PREP, Nkx2.1, and AP-1 bind to that region, suggesting that AR is interacting with one of these factors.

Pbx bound to the bovine prolactin gene (PRL3) was found to be required for GR-mediated PRL3 repression [41]. Thus, it would be of interest to know if AR-mediated

repression of GnRH is acting through the same mechanism. When a luciferase reporter containing three copies of the consensus Pbx binding site (upstream of RSVp) was analyzed, reporter activity was reduced by R1881 treatment. This repression was observed either with or without exogenous AR. Thus, R1881 could be binding to endogenous AR, or to other steroid hormone receptors also known to bind R1881. Repression was lost when Prep1 protein, a co-factor for Pbx, was over-expressed. Thus, it is possible that AR competes Pbx or Prep1 away from GnRHp. AR has been shown to interact with Prep2 in the GT1-7 cell line by co-immunoprecipitation (Mellon Lab, unpublished data). Alternatively, R1881 may be activating endogenous GR and competing Pbx from the reporter.

Interestingly, the -126/-86 region of GnRHp was not sufficient for repression, while the -173/-86 and -101/-45 regions were sufficient. Thus, the -91/-86 region is not the only site involved in AR-mediated GnRH repression through the proximal promoter. Rather, other regions upstream of -126 or downstream of -86 are also involved. Basal activities of the heterologous reporters were higher than the WT promoters, suggesting regions downstream of -45 contain binding sites for transcriptional repressors. It would be of interest to know if AR interacting with the -91/-86 region changes the conformation of AR. A conformational change could promote cooperative interaction with co-repressors or with factors bound to other sites in the GnRHp. For instance, binding of AR to Oct-1 in the mouse sex-limited protein gene causes a conformational change in AR, resulting in the recruitment of the co-activator, SRC-1 [42]. It is important to note that although the -173/-86 and -101/-45 regions were sufficient, fold repression was not as

robust compared to the entire proximal promoter. Therefore, we conclude that multiple sites across the entire proximal promoter are required for AR-mediated GnRH repression. Since sites throughout the proximal promoter appear to be necessary, it could be possible that AR is competing multiple transcription factors and co-activators away from the proximal promoter.

Since similar factors have been shown to bind to GnRHe as to GnRHp, we hypothesized that AR functions through the same mechanism in both regions. After 3′ truncation analysis, we observed that repression was lost when the GnRHe was truncated to -1730. However, basal activity dropped to less than 1% of the full-length enhancer. Therefore, the relief in repression could be attributed to this dramatic decrease in basal activity. Truncation from -1715 to -1730 resulted in the removal of a GATA4 binding site, an activator of GnRH transcription. Ultimately, we mapped repression to the -1800/-1780 region. The only transcription factor known to bind to that region is Oct-1. However, when we introduced mutations into the Oct-1 (ATa) binding site, repression was still present. Thus, AR could be binding to non-putative AREs or interacting with other unknown elements in the -1800/-1780 region.

To test sufficiency, we used a luciferase reporter containing four copies of the -1800/-1767 region of the GnRHe upstream of RSVp. Our results demonstrated that the -1800/-1767 region of the GnRHe was sufficient for AR-mediated repression. Mutation of the Oct-1 binding site in the 4x(-1800/-1767) reporter resulted in a dramatic decrease in basal activity to about 20% of wild type, but repression was still present. Thus, Oct-1 was not involved, which was consistent with results discussed earlier. Interestingly, when

a mutation was introduced into the -1789/-1787 region, basal activity dropped to 6% of basal levels and repression was lost. A decrease in basal activity to 6% of wild type was not necessarily sufficient to abolish R1881 suppression, since truncation from -1563 to -1571 reduced enhancer activity by 93%, and strong R1881-mediated repression of more than 50% was still observed. Therefore, it is unlikely that the loss of repression was due solely to low basal expression levels.

It remains unknown as to what factor binds the -1789/-1787 site, but it is very important for enhancer-specific GnRH expression. If this site is also involved in AR-mediated repression, AR could be binding directly to this region or indirectly through an unknown DNA-bound factor. Another possibility is that AR competes away a transcription factor that is required for GnRH expression. To test these possibilities, we utilized EMSA competition experiments. Our EMSA results demonstrated the binding of an unknown complex at the -1792/-1784 region. 12-O-tetradecanoyl phorbol-13-acetate (TPA), a protein kinase C (PKC) pathway activator, represses GnRH and requires an unknown protein complex binding at -1793 [35]. It is probable that we detected the same protein complex. The protein complex was not competed from the radiolabeled probe with an oligonucleotide containing an ARE, nor was it supershifted with an AR antibody, suggesting that AR is not a member of this complex. Thus, it is possible that liganded AR competes for binding of factors that are a part of this complex. Another possibility is that membrane-associated AR is activating the PKC pathway. Future experiments could utilize PKC inhibitors to investigate if AR-mediated repression involves the PKC pathway. However, Belsham, et al., observed that membrane AR is not involved in

GnRH repression [25]. Furthermore, when mutations were introduced into nucleotides -1791/-1790 in the GnRHe/RSVp luciferase reporter, basal activity decreased and repression by R1881 was still present. Assuming that the two-base pair mutation abrogates the binding of this complex to DNA, this complex may not be involved in repression, but it is important for GnRHe expression.

We also demonstrated the binding of an AR-containing complex to the -1795/-1790 region of GnRHe. The complex was competed off the radiolabeled probe with an unlabeled oligonucleotide containing an ARE and was supershifted with an AR antibody. The presence of two complexes containing AR suggests a variety of possibilities. One possibility is that the complexes represent unphosphorylated and phosphorylated AR. Alternatively, since AR binds to DNA as a homodimer, it is possible that the bottom band is a single DNA-bound AR and the other band is the homodimeric form. A third possibility is that the complexes include AR bound to a DNA-bound transcription factor and the larger complex represents the same protein complex but with an additional co-factor. Future EMSA competition experiments could be utilized to test these possibilities. The AR antibody caused a supershift of the complex and a release of an unknown protein. It is possible that the binding of the antibody changed the conformation of AR and resulted in a release of an interaction partner.

R1881 reduced activity of a luciferase reporter containing three copies of the Nkx2.1 consensus-binding sequence upstream of RSVp. Repression was only present when the cells were co-transfected with AR expression plasmid, indicating that repression was AR dependent. When GT1-7 cells were co-transfected with Nkx2.1

expression plasmid, basal activity increased. This is consistent with the fact that Nkx2.1 transactivates GnRH promoter activity in a concentration-dependent manner [30]. There have been conflicting results regarding the presence of Nkx2.1 protein in GnRH neurons. Lee, et al., used double immunohistochemistry to detect the presence of Nkx2.1 immunoreactive material in some, but not all, GnRH neurons in the preoptic region of female prepubertal mice [30]. Lee, et al., also utilized *in vitro* analysis to observe Nkx2.1 cDNA in the GT1-7 cell line. However, a more recent study using GT1-7 cells could not detect Nkx2.1 expression [43].

The unknown protein complex binding to -1792/-1784 of GnRHe was competed off the radiolabeled probe with an unlabeled oligonucleotide containing three copies of the Nkx2.1 consensus binding site. This suggests that Nkx2.1 protein is expressed in our GT1-7 cells and that Nkx2.1 protein is a part of the unknown protein complex. An alternative possibility is that a protein other than Nkx2.1 recognizes the consensus Nkx2.1 binding motif. Furthermore, the -91/-86 region, which is important for GnRH repression through the proximal promoter, also contains part of an Nkx2.1 binding site. Nkx2.1 plays a role in sexual maturation, and its expression is markedly increased before puberty [30]. Furthermore, Mastronardi, et al., observed delayed puberty, reduced reproductive capacity, and a short reproductive span in mice when Nkx2.1 was ablated from differentiated neurons [44]. Thus, Nkx2.1 plays an active role before and after sexual development. Future experiments could be performed to confirm the binding of Nkx2.1 to the -1792/-1784 region of GnRHe and whether or not Nkx2.1 is involved in AR-mediated GnRH repression.

The direct effect of androgens on GnRH neurons *in vivo* has been controversial. AR has not been shown to be present in GnRH neurons *in vivo*, although it may be present in low amounts [22, 23]. But GT1-7 cells, a model for GnRH neurons, have been shown to contain AR mRNA, protein, and ARA70, a co-activator of AR [31]. The inconsistency could be due to the scarcity and wide distribution of GnRH neurons *in vivo.* Alternatively, AR may be expressed in a subset of GnRH neurons.

In conclusion, we find that AR-mediated GnRH repression occurs through the proximal promoter and distal enhancer. Repression through the proximal promoter is likely to be independent of direct AR binding to DNA. Rather, it may require the interaction of AR with other transcription factors. Repression through GnRHe was found to be through the -1800/-1766 region, and we demonstrated the binding of an unknown protein complex, possibly containing Nkx2.1, and also an AR-containing complex to this region. These complexes are likely playing a role in AR-mediated repression.

Dysregulation of the GnRH gene leads to improper levels of the GnRH peptide hormone and thus abnormal communication between the hypothalamus, pituitary, and gonads. Our studies map AR-mediated GnRH repression to certain parts of the GnRH gene. Future studies could be utilized to confirm the exact players involved. Identifying the tissue-specific proteins involved in AR-mediated GnRH repression could lead to novel protein targets for the treatment of reproductive disorders.

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