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ORIGINAL PAPER

Multiple core homeodomain binding motifs differentially contribute to transcriptional activity of the murine gonadotropin-releasing hormone receptor gene promoter

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Abstract Multiple homeodomain (Hbox) proteins have been shown to organize expression of key markers of gonadotropes. Nine putative Hbox-binding sites, characterized by the homeospecific TAAT motif, are located within the proximal 600 bp of the murine GnRHR promoter. Homeoproteins bind separate Hbox sites within this promoter, supporting basal- and endocrine-directed transcription. The function of the most proximal sites (Hbox1 and Hbox2) in the murine GnRHR is unknown; thus, understanding of the global contribution of homeospecific TAAT sites to promoter function is incomplete. Site-directed mutagenesis revealed that loss of Hbox2 reduced promoter activity in a cell-specific manner, having no effect in αT3-1 cells but reducing promoter function in LβT2 cells, another gonadotrope-derived cell line representing a later developmental stage. In contrast, eliminating Hbox1 reduced basal activity in both lines. This region displayed specific binding to homeoprotein Oct-1. Mutagenesis of a previously identified Oct-1-binding site in concert with Hbox1 led to further reduction in activity. We suggest that the two most proximal homeodomain-binding sites in the murine GnRHR promoter may regulate the promoter in a developmentally dependent fashion and that Oct-1 acts at multiple but distinct TAAT sites to support basal transcription.

Keywords GnRH receptor · Transcription · Pituitary · Gene regulation · Homeodomain

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Introduction

The pulsatile discharge of GnRH from hypothalamic neurons is absolutely necessary for the synthesis and secretion of LH, and to a lesser extent, FSH from gonadotrope cells in the anterior pituitary [1–4]. Upon binding to specific, high-affinity receptors on gonadotrope cells, GnRH stimulates the expression of three genes necessary for the production of LH and FSH, the common α-subunit, the specific LH-β, and FSH-β subunits [5–8]. Thus, the GnRH receptor (GnRHR) exists as the functional link for hypothalamic input to the anterior pituitary in regard to gonadotropin secretion, and is therefore a central point for regulation of reproductive function. Changes in the number of receptors for GnRH in the pituitary are correlated with changes in gonadotropin secretion and have been implicated as an important mechanism underlying the regulation of LH secretion [9–11]. Thus, the regulation of LH secretion is dependent not only on the amount of GnRH released from the hypothalamus, but also on the number of GnRH receptors available for binding, and consequently the sensitivity of the pituitary to a given dose of GnRH [3, 10, 12–15]. In this regard, the availability of the cDNA

encoding the mammalian Type I GnRHR [16, 17] has provided new insight into changes in gene expression associated with regulation of reproductive function.

During the last decade, a number of regulatory sites have been identified in the murine GnRHR gene promoter. Most studies have focused predominantly on transcription factors that interact either directly or indirectly at three primary sites; the gonadotropin receptor-activating sequence (GRAS) [18–20], an activator protein-1 (AP-1) element [21, 22], and the gonadotrope-specific element which binds the nuclear orphan receptor steroidogenic factor-1 (SF-1) [23]. Other regulatory sites have been identified as well, including the sequence underlying responsiveness to GnRH-1 (SURGE-1) [24], and most recently, the down-stream activin response element (DARE) [25, 26]. While these are clearly important to promoter function, the role that homeodomain proteins play in regulating promoter activity in gonadotrope cells has become increasingly compelling. At least nine different homeodomain proteins (LHX2 [27], LHX3 [28], PitX1 [29], PitX2 [30, 31], Otx1 [32], Oct-1 [33], Pbx [34], Prep-1 [34], and Dlx3 [35]) have been implicated in regulating the expression of the common α, the unique LH and FSH β, and GnRHR genes. In regard to the latter, there are nine putative homeobox (Hbox) sites, which we arbitrarily numbered Hbox1 through 9 (Fig. 1), evident in the 600-bp proximal promoter. These Hbox sites possess the core homeodomain recognition sequence TAAT [36], and several have been found to be important for promoter function. One or more homedomain proteins appear to interact at DARE (Hbox7 and Hbox6) [25, 37], which is critical to the activin-responsive phenotype of the murine GnRHR promoter [26]. Binding of Oct-1 at Hbox6 can contribute to both basal- and GnRH-stimulated promoter activity [33]. Currently however, the potential contribution of the two most proximal Hbox sites (Hbox2 and Hbox1) to promoter function has yet to be determined. In this study, we hypothesized that these Hbox sites contribute to the transcriptional regulation of the GnRHR gene. We find that the homeodomain protein Oct-1 acts at multiple Hbox sites to regulate transcriptional activity of the murine GnRHR promoter.

Results

Hbox1 supports transcriptional activity of the GnRHR promoter in both cell lines

The TAAT motifs represent the core DNA-binding site of multiple homeodomain DNA-binding proteins [38]. A number of these Hbox sites exist within the proximal 600 bp of the murine GnRHR promoter, and have been determined to play a role in promoter activity [33, 37]. Given this, we hypothesized that the putative homeodomain DNA-binding site at Hbox1, which has not yet been evaluated, would play a role in regulating function of the murine GnRHR gene promoter. The native sequence of ATTAGG was replaced with a 6-bp block replacement of CCGGTT. This eliminated the core homeodomain DNA-binding site from the putative transcriptional element. The resultant mutant was transiently transfected into αT3-1 and LβT-2 gonadotrope-derived cell lines. As expected, mutation of Hbox1 had a substantial impact on promoter function. A significant reduction ($P < 0.05$) in promoter activity was observed when μHbox1 was compared to the wild-type promoter in αT3-1 cells (Fig. 2a). Similarly, eliminating the Hbox1 DNA-binding site resulted in a decrease ($P < 0.05$) in transcriptional activity of the GnRHR promoter in LβT-2 cells as well (Fig. 3).

To better understand the global contribution that homeodomain DNA-binding sites make on activity of the murine GnRHR promoter, we also evaluated a second

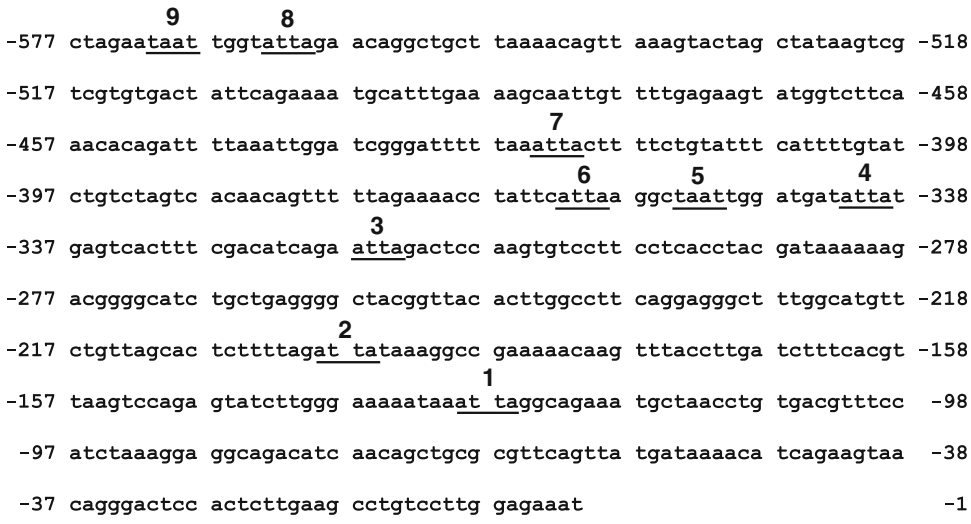

Fig. 1 Location of core homeodomain motifs in the proximal promoter of the murine GnRHR gene

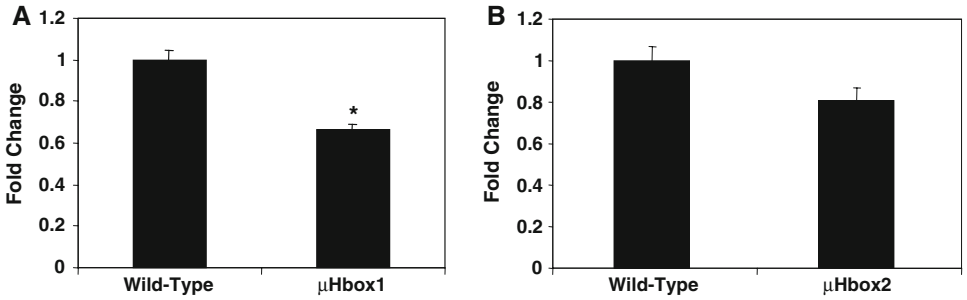

Fig. 2 Mutation of Hbox1 reduces basal activity of the murine GnRHR promoter in αT3-1 cells. Vectors expressing luciferase from either a wild-type promoter, or promoters in which **a** Hbox1 or **b** Hbox2 elements were mutated, (μHbox1, μHbox2) were transfected into αT3-1 cells, and activity measured after 48 h. Activity of each mutation was tested through separate experiments. Each vector was tested in triplicate. Values represent the mean + SEM of at least three experiments with different vector preparations, normalized for transfection efficiency by evaluating activity from cotransfected CMV-β-galactosidase vector. * ($P < 0.05$)

previously uncharacterized Hbox site, Hbox2. The native sequence of AGATTA in the homeodomain core motif at −196 was replaced with a *Nae*I recognition sequence (GCCGGC) to eliminate the core homeospecific TAAT motif of Hbox2. The resulting promoter construct was transfected into αT3-1 cells. In contrast to Hbox1, mutation of Hbox2 did not affect GnRHR promoter activity in αT3-1 cells (Fig. 2b); however, the elimination of the core homeodomain motif in Hbox2 led to a reduction ($P < 0.05$) of promoter activity in LβT2 cells (Fig. 3). As Hbox1 appears to contribute more globally to promoter function (i.e., attenuates promoter activity in both of the gonadotrope derived cell lines), we focused our efforts on the potential identity of the protein that may mediate the functional contribution of Hbox1 to GnRHR promoter activity.

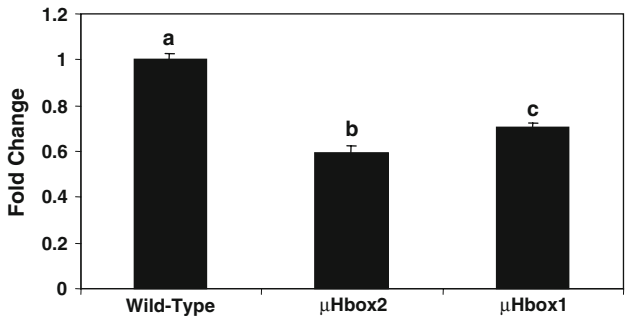

Fig. 3 Mutation of Hbox1 and Hbox2 reduces basal activity of the murine GnRHR promoter in LβT2-1 cells. Vectors expressing luciferase from either a wild-type promoter, or promoters in which the Hbox1 or Hbox2 elements were mutated, (μHbox1, μHbox2) were transfected into LβT2-1 cells, and activity measured after 48 h. Each vector was tested in triplicate. Values represent the mean + SEM of at least three experiments with different vector preparations, normalized for transfection efficiency by evaluating activity from cotransfected CMV-β-galactosidase vector. Letters (a, b, c) indicate statistical difference ($P < 0.05$)

A transcriptional complex forms at Hbox1 which contains oct-1 protein

We sought to use EMSA to determine if specific protein–DNA complexes formed at Hbox1. Two specifically bound complexes were observed with the Hbox1 probe, (Fig. 4a). Homologous competition displaced binding of both the complex A and complex B (Fig. 4b). When a probe that contained a mutation in the TAAT core motif was used (μHbox1), complex A was lost. Binding of complex B was retained but with weaker affinity (Fig. 4a). This may represent the loss of formation of some homeodomain–heterodimeric complex that requires the TAAT core motif to remain intact. Increasing concentrations of μHbox1 failed to competitively displace binding of complex A from the Hbox1 probe (Fig. 4b). In contrast, μHbox1 was capable of displacing some of the binding of complex B (Fig. 4b). This is consistent with the observation that the μHbox1 probe contains complex B but not complex A (Fig. 4a).

We next sought to identify a homeodomain protein contained in the complexes formed with the Hbox1 probe. LHX3 and Oct-1 are homeodomain proteins previously shown to bind the GnRHR promoter [33, 37]. Given that they are expressed in the mature pituitary [39] as well as αT3-1 and LβT-2 cells [33, 37, 40], we viewed these as candidate proteins. Binding of LHX3 typically requires a tandem TAAT repeat [41] whereas Oct-1 can bind at a single TAAT site [42]. Thus, we chose to focus our attention on Oct-1. Radiolabeled probe containing a consensus Oct-1 site formed the specific complexes that comigrated with those observed on the Hbox1 probe (Fig. 5a). Homologous competition with unlabeled Oct-1 displaced both the complex A, and to a lesser extent, complex B (Fig. 5b). Heterologous competition with increasing concentrations of unlabeled Hbox1 displaced formation of complex A (Fig. 5b). In contrast, formation of complex B was reduced only slightly by heterologous

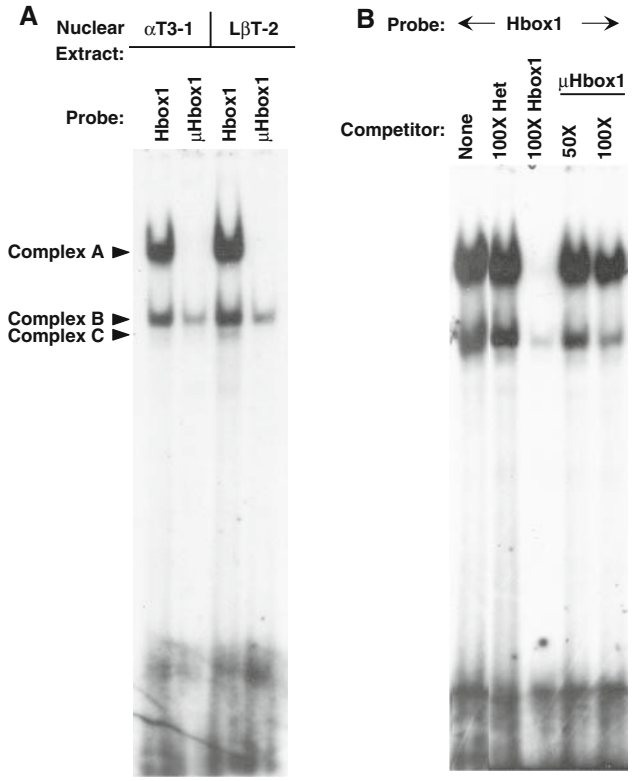

Fig. 4 The Hbox1 element binds specific protein complexes. **a** Nuclear extract from both αT3-1 and LβT2-1 cells contain protein complexes that bind to the Hbox1 region. Nuclear extracts were incubated with labeled probe corresponding to the sequence of Hbox1, or the mutation of Hbox1 used in transfections (μHbox1). Complexes were visualized by electrophoresis and autoradiography. Data shown is of a representative experiment. **b** A specific complex is formed in αT3-1 cells. The Hbox1 probe was incubated with nuclear extract from αT3-1 cells in the absence or presence of 50- or 100-fold molar excess of unlabeled competitor representing Hbox1, μHbox1, or heterologous competitor (Het), and resulting complexes visualized by electrophoresis and autoradiography. Data shown are those of a representative experiment

competition with unlabeled Hbox1. To further confirm that the protein–DNA complexes which formed at Hbox1 contain Oct-1, an Oct-1-specific antibody or an equal mass of IgG was added to the binding reaction. Inclusion of Oct-1 antibody resulted in a clear supershift, revealing the formation of a higher molecular weight complex (Fig. 5c). The inclusion of an antibody for LHX3, another homeodomain DNA-binding protein known to bind the GnRHR receptor [37], did not result in a supershift, nor did the addition of antiserum for LHX2.

Oct-1 protein regulates transcriptional activity of the murine GnRHR promoter at multiple homeodomain sites

Within the proximal murine GnRHR promoter, Oct-1 has been found to bind to the Hbox5 site and support transcriptional activity of the promoter [33]. The results of this study indicate that Oct-1 can also bind to the most proximal Hbox1 site to support basal transcription. The action of Oct-1 at both binding sites in concert may be important to support basal transcription of the GnRHR gene. Thus, we assessed the effect of eliminating both Oct-1-binding sites on transcriptional activity of the promoter. The core TAAT homeodomain recognition sequence was eliminated from Hbox5 and Hbox1 of the murine GnRHR promoter by block replacement. The double Oct-1 mutant was transfected into αT3-1 and Lβ T-2 cells in combination with each mutation alone as well as the wild-type promoter. Loss of Oct-1 recognition site at Hbox5 resulted in a decrease ($P < 0.05$) in promoter function in both αT3-1 and Lβ T2 cells (Fig. 6A, B), a result consistent with our previous report [25]. In comparison to mutation of Hbox5 alone, a further reduction promoter activity by mutation of both Hbox5 and Hbox1 was observed only in Lβ T2 cells (Fig. 6B). In contrast, the double mutation caused a greater ($P < 0.05$) decrease in promoter activity when compared to eliminating Hbox1 alone (Fig. 6) in both cell lines.

Discussion

The GnRHR is the site for stimulatory input by GnRH to gonadotropes in the anterior pituitary. As such, GnRHR represents a critical protein regulating synthesis and secretion of LH and FSH. The primary amino acid sequence of the Type I GnRHR is highly conserved across multiple species including the mouse and human. As expected, cell and tissue distributions of the GnRHR are virtually identical. Considerable progress has been made in regard to defining the molecular mechanisms that mediate cell-specific expression and hormonal regulation of the murine GnRHR gene. To this end, many important cis-regulatory elements have been identified in the murine GnRHR gene promoter, which are important for its expression. Recently, a number of homeodomain proteins have been implicated in regulating expression of gonadotrope-specific genes [28–32, 34, 35]. Genomic analysis reveals that at least nine putative homeodomain DNA-binding sites, characterized by the core TAAT motif, are located within the proximal 600 bp of the murine GnRHR promoter. The role that the more distal homeodomain sites play in transcriptional activity of the promoter has been investigated. Both LHX3 and Oct-1 are homeodomain proteins that have been shown to interact with the GnRHR promoter and direct its transcription [33, 37]. LHX3 binds to Hbox6, and is located in the DARE element. Mutation of Hbox 6 leads to a decrease in basal transcription [25]. Whether or not LHX3 plays a role in mediating activin

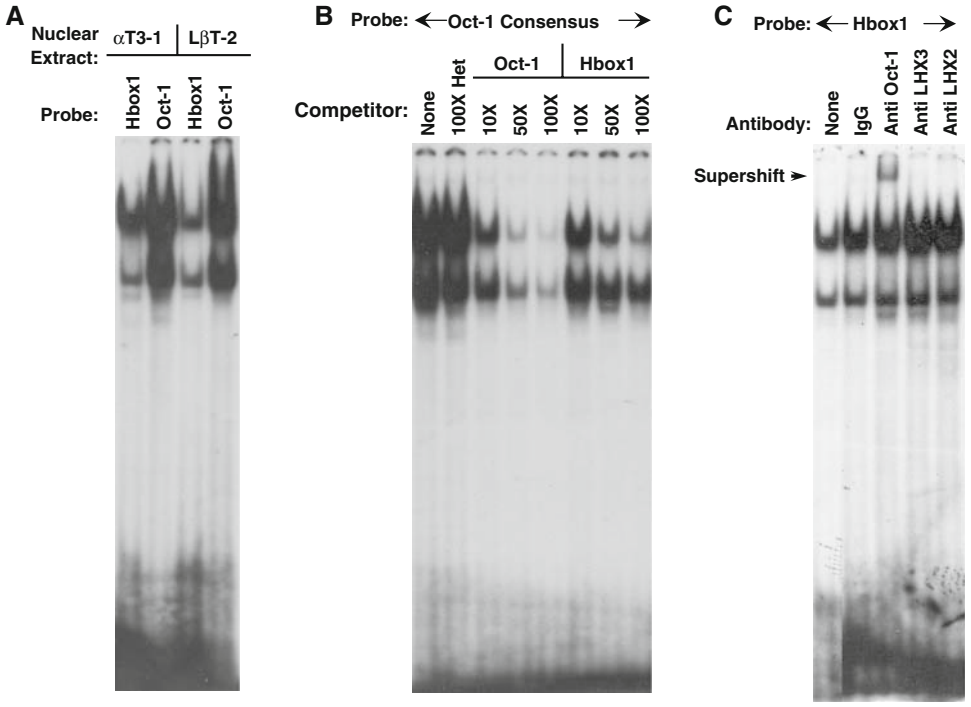

Fig. 5 Hbox1 is capable of binding Oct-1. **a** Oct-1 and Hbox1 probes bind to similar complexes in both αT3-1 and L*β*T2-1 cells. Nuclear extracts from these cell lines were incubated with Hbox1 or Oct-1 probe, and complexes formed were visualized by electrophoresis and autoradiography. **b** Hbox1 can compete for Oct-1 binding. A probe containing the Oct-1 consensus-binding site was incubated with αT3-1 nuclear extract, in the absence or presence of unlabeled competitor at 10-, 50-, or 100-fold excess. Competitors represented homologous Oct-1 competitor, Hbox1, or a heterologous competitor (Het). Complexes were visualized by electrophoresis and autoradiography. **c** Oct-1 antibody supershifts the protein complex formed on Hbox1. Hbox1 probe was incubated with αT3-1 nuclear extract in the absence or presence of nonspecific antiserum (IgG), or antibodies raised against Oct-1, LHX3, or LHX2, and resulting complexes visualized by electrophoresis and autoradiography. Data shown are those of representative experiments

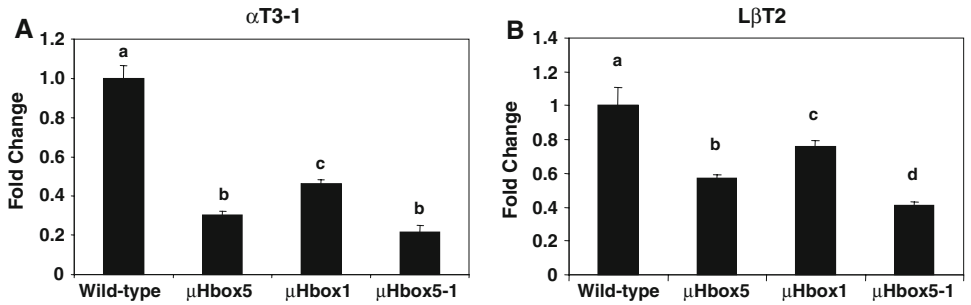

Fig. 6 Double mutation of Hbox1 and Hbox5 differentially affects GnRHR promoter function in αT3-1 and L*β*T2 cells. Wild-type mGnRHR promoter activity was compared with that of promoters mutated in the Oct-1 sites at Hbox1, Hbox5, or both together. **A** αT3-1 cells were transfected with promoters driving the luciferase reporter gene and luciferase activity evaluated 48 h after transfection. Each vector was tested in triplicate. Values represent the mean + SEM of at least three experiments with different vector preparations, normalized for transfection efficiency by evaluating activity from cotransfected CMV-*β*-galactosidase vector. **B** L*β*T2-1 cells were similarly transfected. Letters indicate statistical difference (a, b, c, d: $P < 0.05$)

regulation of the GnRHR promoter is unclear at this time [26]. Oct-1 binds to Hbox5 and can direct basal transcription of the promoter [33]. It can also, in part, confer GnRH responsiveness to the GnRHR promoter [33]. We find that Oct-1 can bind at a second homeodomain DNA-binding site, Hbox1, at −116 from the translational start site. Furthermore, mutation of Hbox1 leads to reduced transcriptional activity of the promoter. Thus Oct-1 appears to have multiple binding sites within the GnRHR gene promoter through which to affect transcription of the gene. This underscores the need for caution when assigning the action of such a ubiquitous transcription factor [43, 44] to a single binding site within a promoter that has multiple TAAT core homeospecific motifs.

Neither Hbox1 nor the previously defined Oct-1-binding site at Hbox5 in the murine GnRHR promoter represents a consensus Oct-1-binding element. This is not surprising given that Oct-1 has been shown to bind degenerate sequences [42, 45] in different promoters. Three types of Oct-binding sequences have been identified: [1] the canonical Oct-1 octamer site; [2] non-canonical octamer-related sites; and [3] TAAT core motif sites [42]. Using chromatin immunoprecipitation, binding of Oct-1 to a non-canonical octamer-related site at Hbox5 has been reported [33]. Such an approach would not necessarily distinguish between Oct-1 binding at non-canonical octamer-related site vs. a TAAT core homeospecific motif located in close proximity, as is the case with the murine GnRHR promoter. Plasmid immunoprecipitation has been used to study transcriptionally active complexes in gonadotrope cell lines [46, 47]. Transfecting a vector containing a mGnRHR construct harboring mutated homeobox-binding sites adjacent to the Hbox1 element is an approach we could use in future studies to address this technical challenge.

The affinity of Oct-1 for Hbox1 was less than that for a canonical Oct-1 site, which is not unexpected. The Oct-1 protein contains a POU domain which has two regions; the POU specific region and the POU homeobox region [48–50]. Binding of Oct-1 to prototypical octamer-related sites [49] involves both regions, whereas binding of Oct-1 to a TAAT core motif site may involve only the POU homeobox region [42, 51, 52]. Thus, the affinity of Oct-1 for canonical Oct-1-binding sites is greater than the affinity to core TAAT homeoprotein sites [51]. In the case where Oct-1 recognition sites contain the TAAT core motif, the two 3′ flanking nucleotides determine the strength of the interaction between the Oct-1 protein and the promoter [42, 52] and can also determine the formation of heterodimeric complexes between Oct proteins and other transcription factors. The TAATTT sequence that observed binding Oct-1 in the GnRHR promoter is present in the IL-5 [53] and Oct-1 [42] promoters, and has been shown to have greater affinity for Oct proteins than for other nucleotide combinations [42, 52]. Inclusion of an Oct-1-specific antibody in the binding reaction results in a clearly identifiable supershift, further substantiating that the transcriptional complex which forms at the core TAAT homeodomain motif at Hbox1 does indeed contain Oct-1. Other proteins which may contribute to the transcriptional complex that forms at this portion of the promoter and any contribution that they may have at this site or in combination with other sites within the GnRHR promoter in regulating transcriptional activity remains to be determined.

This report serves to further document the effects of homeodomain DNA-binding proteins on activity of the GnRHR promoter. Using a "reverse biology" approach (i.e., regulatory element to protein), we have determined that Oct-1 protein binds to a previously uncharacterized TAAT core homeodomain DNA-binding motif in the murine GnRHR gene promoter. Given the clear role of multiple homeodomain proteins in organizing expression of key markers of the gonadotrope transcriptome, we recognized a fundamental deficiency with respect to analysis of the murine GnRHR gene. That is, the functional contribution of all TAAT core homeodomain DNA-binding motifs present in this promoter have not been fully characterized. Elucidating the contribution of the homeodomain DNA-binding protein Oct-1 at Hbox1 represents a first step in correcting such a deficiency.

At present, there is a single TAAT core motif (Hbox2) in the murine GnRHR gene promoter that remains to be fully characterized. It is interesting to note that while mutating Hbox2 does not affect GnRHR promoter activity in αT3-1 cells, the same mutation led to a significant reduction in promoter activity in Lβ T2 cells. Given the differential roles of homeodomain proteins in directing gene expression in the pituitary at various developmental stages [39, 54, 55], it is intriguing to speculate that the protein(s) mediating the functional activity of Hbox2 in Lβ T2 cells may uniquely contribute to GnRHR promoter function at a later stage of development. In contrast to the αT3-1 cell line, Lβ T2 cells are capable of expressing both the LH and FSHβ subunit genes and are generally considered to reflect immortalization of a more developmentally "mature" gonadotrope lineage [56]. Also, in light of the differential impact of the combined mutation of Hbox1 and Hbox5 (Fig. 6), it is possible that the contribution of homeodomain proteins to expression of the GnRHR gene during development may not be confined to single sites but rather reflect changes in the combinatorial interaction of multiple sites. If correct, then it may be possible to further utilize both cell lines in future studies to more completely characterize potential developmental switches in the contribution of homeodomain proteins to expression of the GnRHR gene and, more globally, to the terminal phenotype of gonadotropes [26].

Materials and methods

Materials

Dr. Pamela Mellon (University of California San Diego) generously provided the αT3-1 and Lβ T-2 cells. Oligodeoxynucleotides were obtained from Invitrogen (Carlsbad, CA). Oct-1 oligodeoxynucleotide sequences were those used by Kam et al. [33]; for all others see Table 1. DNA sequencing was conducted by Macromolecular Services at the University of California Davis or the Sequencing and Synthesis core facility at the University of Georgia. Restriction enzymes and DNA-modifying enzymes were

Table 1 Oligodeoxynucleotides used for cloning and EMSA

Name	Sequence 5′ to 3′
RV3	CTAGCAAAATAGGCTGTCCC
GL2	CTTTATGTTTTTGGCGTCTTCCA
Hbx2NaeIs	gccggcTAAAGGCCGAAAAACAAGTTTACCT
Hbx2NaeIas	gccggcAAAAGAGTGCTAACAGAACATGC
Hbx1AgeIs	TAaccggtTCAGAAATGCTAAC
Hbx1AgeIas	ATTTCTGAaccggtTATTTTTCCCAAGA
Hbox1	TGGGAAAAATAAATTAGGCAGAAATG
μHbox1	TGGGAAAAATAAccggttCAGAAATG

Changes from native sequence are underlined and in lower case

obtained from Fermentas (Hanover, MD) and New England Biolabs (Beverly, MA), respectively. Hyperfilm and G-25 Microspin columns were obtained from Amersham Biosciences (Piscataway, NJ). Radionucleotide was obtained from MP Biomedicals (Irvine, CA; Catalog No. 35001X). Dr. Mark Roberson (Cornell University, Ithaca, NY) magnanimously provided the LHX2 antiserum [35]. The Oct-1 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, Catalog No. sc-232), and the LHX3 antibody was purchased from USBiological (Swampscott, MA, Catalog No. L2078).

Vector construction

The plasmid pMGR-600LUC consists of 600 bp of the 5′ flanking region of the murine GnRHR gene fused to the cDNA-encoding luciferase in the pGL3-Basic vector (Promega Corp., Madison, WI). The control vector used to test for transfection efficiency in all the experiments contained the Rous sarcoma virus promoter linked to the cDNA-encoding β-galactosidase (RSV-βgal). These two plasmids are further described elsewhere [57].

The plasmid pμHbox2 was constructed using two pieces in which the native sequence was replaced with a *Nae*I restriction site. Sequence-specific primers and pGL3-flanking primers were used in individual PCR reactions with pMGR-600LUC as a template. The upstream piece (RV3 and Hbx2NaeIrev) and the downstream piece (Hbx2NaeIfwd and GL2) were gel-purified and subcloned into pGEM-T Easy (Promega Corp.) and the sequence confirmed by sequence analysis. The upstream piece was liberated from pGEM-T Easy with *Sph*I/*Kpn*I/*Nae*I while the downstream piece was excised with *Nae*I/*Nco*I. The two pieces were combined in a concurrent ligation and inserted into *Kpn*I/*Nco*I sites of pGL3-Basic.

The plasmid pμHbox1 was constructed using two pieces in which the native sequence was replaced with an *Age*I restriction site. Sequence-specific primers and pGL3 flanking primers were used in individual PCR reactions with pMGR-600LUC as a template. The upstream piece (RV3 and Hbx1AgeIrev) and the downstream piece (Hbx1AgeIfwd and GL2) were gel-purified and subcloned into pGEM-T Easy and the sequence confirmed by sequence analysis. The upstream piece was liberated from pGEM-T Easy with *Kpn*I/*Age*I while the downstreatm piece was excised with *Age*I/*Nco*I. The two pieces were combined in a concurrent ligation and inserted into *Kpn*I/*Nco*I sites of pGL3-Basic. The mutation was verified by sequence analysis.

The block replacement construct pBR15 was described previously [25] and contains the 8-bp *Not*I restriction site with essentially eliminates the Oct-1-binding site [33]. The plasmid pM-600μOct-1 was made using pBR15 as a template in PCR. A fragment was generated using the Hbx1AgeIrev primer and the pGL3-flanking primer RV3 and was subsequently cloned into pGEM-T Easy. This product was excised with *Kpn*I/*Age*I and subcloned into the same sites in pμHbox1. The presence of the BR15 and the Hbox1 double mutation was confirmed with sequence analysis.

Transient transfections

Culture of αT3-1 and LβT2-1 cells, and transient transfections using SuperFect (Qiagen, Valencia, CA) were preformed as previously described [25, 58]. In brief, 0.8 μg of the test luciferase plasmid and 0.2 μg of RSV-βgal were used. After 3 h of incubation with the SuperFect/DNA mixture, the media were removed from cells and then replaced with growth media. Cells were harvested 48 h after transfection and assayed for luciferase and β-galactosidase activity as previously described [19, 58]. Values were normalized for transfection efficiency by dividing the luciferase values by β-galactosidase values. Within a transfection, all the treatments and vectors were tested in triplicate. All the transfections were repeated at least three times using different plasmid preparations.

Electromobility shift assays

Electromobility shift assays (EMSA) were conducted as previously described [18, 21, 25, 57, 58]. In brief, nuclear extracts of αT3-1 and LβT-2 cells were prepared [59] and incubated with binding buffer (24 mM HEPES, pH 7.9, 84 mM KCl, 5 mM DTT, 10% glycerol), polydeoxyinosinic deoxycytidylic acid (2 μg), radiolabeled probe (100,000 cpm), and appropriate unlabeled competitor for 15 min on ice, and then 15 min at room temperature. For supershifts, specific antibody, antiserum, or an equal mass of IgG was incubated for an additional 15 min at room temperature before being placed on ice for 15 min. Complexes were resolved by electrophoresis for 2–2.5 h at 35 mA through a

6% polyacrylamide gel which had been pre-run at 100 V for 1 h in TBS. Gels were transferred to blotting paper, dried, and exposed to hyperfilm MP for approximately 16 h at −70°C. In all the EMSA, radiolabeled probes were prepared using polynucleotide kinase and [γ-^{32}P]ATP (4500 Ci/mmol). Double-stranded radiolabeled DNA probes were separated from free radionucleotide by column chromatography using G-25 Microspin columns.

Statistical analysis

In every transfection, each treatment and vector was analyzed in triplicate, and the experiments were replicated three times using different plasmid preparations. Transfection data are expressed as means ± standard error. Data were analyzed by one-way analysis of variance using the GLM procedure of SAS (SAS/STAT 9.1, SAS Institute Inc., Cary, NC). When a significant ($P < 0.05$) F-test was observed, multiple comparisons were made with Tukey's studentized range test.

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