GATA transcription factors regulate LHβ gene expression

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Abstract

The GATA family of transcription factors are critical determinants of cell differentiation as well as regulation of adult gene expression throughout the reproductive axis. Within the anterior pituitary gland, GATA factors have been shown to increase glycoprotein α-subunit gene promoter activity; however, nothing has been known about the impact of these factors on expression of the gonadotropin β-subunits. In this study, we demonstrate expression of both GATA2 and GATA4 in primary mouse gonadotropes and the gonadotrope cell line, LIT2. Based on the transient transfection in fibroblast cells, GATA factors increase LH β-subunit gene (LHβ) promoter activity alone and in synergy with the orphan nuclear receptors steroidogenic factor-1 (SF-1) and liver receptor homologue-1 (LRH-1). The GATA response was localized to a DNA regulatory region at position −101 in the rat LHβ gene promoter which overlaps with a previously described cis-element for pituitary homeobox-1 (Pitx1) and is flanked by two SF-1/LRH-1 regulatory sites. As determined by gel shift, GATA and Pitx1 can compete for binding to this element. Furthermore, mutation analysis revealed a requirement for both the GATA/Pitx1 and the SF-1/LRH-1 cis-elements in order to achieve synergy. These studies identify a novel role for GATA transcription factors in the pituitary and reveal additional molecular mechanisms by which precise modulation of LHβ gene expression can be achieved.

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Introduction

Normal functioning of the reproductive axis requires precise interactions among the hypothalamus, pituitary, and gonads through the complex interplay of GnRH, gonadal steroids, and other regulatory factors. In the anterior pituitary gland, the gonadotrope subpopulation biosynthesizes and secretes the gonadotropins, LH, and FSH. LH and FSH share a common α-glycoprotein subunit with their β-subunits conferring unique functionality. Data from our laboratory and many others have identified transcription factors that regulate expression of the LH β-subunit gene (LHβ), including steroidogenic factor-1 (SF-1; NR5A1), liver receptor homologue-1 (LRH-1; NR5A2), pituitary homeobox-1 (Pitx1), early growth response-1, and selective promoter factor-1 (Sp1; Halvorson et al. 1998, Dorn et al. 1999, Kaiser et al. 2000, Weck et al. 2000, Quirk et al. 2001, Zheng et al. 2007). These factors have been shown to act alone and in concert to regulate basal, tissue-specific, and hormonally mediated expression of the LHβ gene.

A number of studies have suggested a role for the GATA family of transcription factors in the regulation of reproductive function (LaVoie 2003, Viger et al. 2008). There are six identified GATA factors in vertebrates. The GATA members are highly conserved with nearly 80% homology in the zinc finger DNA-binding domains. GATA factors bind to the consensus motif (A/T)GATA(A/G), although they have also been reported to bind to sequences containing core GATC or (A/T)GATA(A/G) elements (Merika & Orkin 1993, Nakagawa et al. 2001). Functional specificity of the GATA members is believed to be achieved through differences in tissue expression as well as unique protein–protein interactions provided by divergent N-terminal zinc fingers (Pedone et al. 1997). Furthermore, GATA transcriptional activation may be modified by interaction with co-factors, including Friend of GATA (FOG1 and FOG2; Robert et al. 2002, Cantor & Orkin 2005). Despite their name, FOG proteins have been found to either augment or blunt GATA effects dependent on promoter and cell context (Cantor & Orkin 2005). Of note, GATA members have been shown to interact with multiple transcription factors with known importance for gonadotropin and GnRH receptor gene expression, including SF-1, LRH-1, Sp1, and AP1 (Kawana et al. 1995, Fluck & Miller 2004, Bouchard et al. 2009).

Although first characterized in the hematopoietic and cardiac systems, GATA factors are now known to have widespread expression in adrenal, thyroid, placental, intestinal, and pancreatic cells, as well as...
at all levels of the reproductive axis (LaVoie 2003, Viger et al. 2008). GATA family members have been shown to be critical for both embryonic development and adult function. With the exception of GATA5, GATA knockout results in embryonic death due to abnormalities in heart tube formation and extraembryonic endodermal development (Tsai et al. 1994, Pandolfi et al. 1995, Fujiwara et al. 1996, Kuo et al. 1997, Molkentin et al. 1997, 2000, Morrissey et al. 1998). Within the central nervous system, it has been shown that migrating GnRH neurons express high levels of GATA4 and that GATA factors are essential for activity of the neuron-specific GnRH gene enhancer (Lawson & Mellon 1998, Lawson et al. 1998). In the ovary and testis, GATA factors are key regulators of gonadal germ cell development and steroidogenesis. GATA factors, in particular GATA4 and GATA6, stimulate expression of multiple gonadal genes including StAR, aromatase (CYP19), inhibin α-subunit, and Müllerian-inhibiting substance (MIS) genes, both alone and in synergy with SF-1 (Watanabe et al. 2000, Tremblay & Viger 2001, Tremblay et al. 2001, Robert et al. 2006).

GATA factors have also been shown to direct the differentiation of cell lineages within the developing anterior pituitary gland through reciprocal interactions of GATA2 and the transcription factor Pit1. As reported by Dasen et al. (1999), overexpression of GATA2 directs cell fate toward the gonadotrope lineage and away from the thyrotrope lineage, as measured by an increase in glycoprotein α-subunit and LHβ mRNA expression. The number of gonadotrope and thyrotrhope cells was observed to be decreased in neonates from a second pituitary-specific GATA2 knockout mouse model (Charles et al. 2006). Although adult animals in this study were fertile, they expressed lower levels of FSH basally and in response to castration. Circulating LH levels were not reported in this study; however, the observed decrease in seminal vesicle weight may suggest a loss of LH-stimulated testosterone production. Partial retention of normal function was attributed to markedly elevated GATA3 expression, which may have compensated for the loss of GATA2 effects.

A limited number of studies have investigated the role of GATA factors on expression of gonadotrope-specific genes. Steger et al. (1994) have reported the presence of a cis-element in the gonadotropin α-subunit gene promoter that mediates GATA responsiveness in the gonadotrope-derived zF31 cell line. In addition, the GnRH receptor gene promoter has been shown to contain a cis-element, which binds an unspecified GATA factor (Pincas et al. 2001). In the results reported here, we further define GATA mRNA and protein expression in both primary pituitary gonadotropes and the mature gonadotrope LβT2 cell line. Furthermore, we characterize the functional importance of these factors on expression of the LHβ-subunit gene.

Materials and methods

Animal and pituitary tissue collection

Experimental mice and rats were housed in the University of Texas Southwestern Medical Center Animal Resource Center on a 12 h light:12 h darkness cycle. Food and water were available ad libitum. After brief CO2 exposure, the animals were decapitated and pituitaries were collected in RNAlater (Ambion, Austin, TX, USA) and stored at −80 °C for RNA preparation. All animal procedures were performed in accordance with guidelines established by the Institutional Animal Care and Use Committee.

Plasmids

The rat LHβ reporter construct used for these studies contained 207 bp of the 5′-flanking sequence of the rat LHβ gene and the first 5 bp of the 5′-untranslated region fused to a luciferase reporter gene, pXP2. The corresponding DNA sequence can be found on PubMed (accession number AF020505). Mutations were introduced into the promoter construct using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA).

The mouse GATA expression vectors contain either the 6-5 kb fragment of mouse Gata2 (cDNA provided by D F Gordon; Charles et al. (2006)), the full-length Gata3 (cDNA provided by R S Viger; Tremblay & Viger (2001)), or Gata4 (cDNA provided by S Hammes). The SF-1 expression vector contains 2-1 kb of the mouse SF-1 cDNA (provided by K L Parker, UT Southwestern Medical Center, Dallas, TX, USA). The LRH-1 expression vector contains 1-7 kb of the mouse LRH-1 cDNA (provided by D J Mangelsdorf, UT Southwestern Medical Center, Dallas, TX, USA; Lu et al. (2000)). The Pitx1 expression vector contains the mouse Pitx1 open reading frame (provided by U B Kaiser, Brigham and Women’s Hospital, Boston, MA, USA; Zakaria et al. (2002)). The Fog2 expression vector contains the full-length Fog2 coding region (provided by E N Olson, UT Southwestern Medical Center, Dallas, TX, USA; Lu et al. (1999)). All cDNAs were subcloned into pcDNA3.1 (+) (Invitrogen).

RNA extraction, reverse transcription, and PCRs

Total RNA was prepared from adult mouse or rat pituitaries or from cultured LβT2 cells using TRI Reagent (Ambion) according to the manufacturer’s instructions. Total RNA samples were DNase treated using the Turbo DNA-free kit (Ambion). DNase-treated total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) primed with random hexamer. A parallel reaction lacking the reverse
transcriptase was prepared as a negative control. The LβT2 cells were generously provided by Dr P L Mellon (University of California, San Diego, CA, USA).

Regular PCR reactions contained 1×PCR buffer (Invitrogen) with 1.8 mM MgCl₂ concentration, 200 μM dNTP, 1 U Taq DNA polymerase (Invitrogen), 200 nM of each primer, and 2–5 μl cDNA to a total volume of 50 μl in an iCycler (Bio-Rad). PCR conditions were as follows: 95°C × 2 min, 35 cycles × (95°C, 30 s; 55°C, 30 s; 72°C, 1 min), and 72°C × 5 min. Sequences of the intron-spanning primers are listed in Table 1. The PCR products were separated on agarose gels and photographed using a ChemiImager 4400 Imaging System (Alpha Innotech, Santa Clara, CA, USA). The identity of the PCR products was confirmed by DNA sequencing.

Quantitative real-time PCR (qPCR) was performed with the above reverse-transcribed cDNA in a 384-well plate on a 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using Taqman Universal PCR Master Mix and gene-specific Taqman Gene Expression Assay primer/probe sets (Mm00492300_m1 for *Gata2*, Mm00484689_m1 for *Gata4*, and Mm00656868_m1 for *LHβ*) with universal cycling conditions. Each reaction was run in 15 μl total volume in triplicates. The expression of each target gene was normalized to 18S transcript expression in each sample. The relative target gene expression levels among treatments were calculated using the comparative C_T method as described in Applied Biosystems User Bulletin No. 2.

**Western blot analysis**

*In vitro* translated GATA2, GATA3, and GATA4 were generated using the TNT-Coupled Reticulocyte Lysate

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**Table 1** PCR Primers used in the present study

<table>
<thead>
<tr>
<th>Mouse primers</th>
<th>Sequence (5′–3′)</th>
<th>Expected product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gata2</em> Forward</td>
<td>CTAGCTACCATGGGCACCCAG</td>
<td>359</td>
</tr>
<tr>
<td><em>Gata2</em> Reverse</td>
<td>CAATTTGCACAACAGGTGCC</td>
<td></td>
</tr>
<tr>
<td><em>Gata3</em> Forward</td>
<td>TGCCAGATAGCATGAAAGCTGAGA</td>
<td>527</td>
</tr>
<tr>
<td><em>Gata3</em> Reverse</td>
<td>TCGGGTCTGGATGCTTCCTT</td>
<td></td>
</tr>
<tr>
<td><em>Gata4</em> Forward</td>
<td>TGTCATCTCTACTTGGCCACAGCA</td>
<td>260</td>
</tr>
<tr>
<td><em>Gata4</em> Reverse</td>
<td>TCCCAAGTCCAGAGAGGAATTTGA</td>
<td></td>
</tr>
<tr>
<td><em>Fog1</em> Forward</td>
<td>TCCTAACTGAAGAAGCCGCCAACT</td>
<td>244</td>
</tr>
<tr>
<td><em>Fog1</em> Reverse</td>
<td>TCTAACCTGAAGAAGCCGCCAATCT</td>
<td></td>
</tr>
<tr>
<td><em>Fog2</em> Forward</td>
<td>TGCTGGTCTCTTAAATGGGTCTTGGAGA</td>
<td>359</td>
</tr>
<tr>
<td><em>Fog2</em> Reverse</td>
<td>ACATCAAATGGGCTTCAGGTTC</td>
<td></td>
</tr>
<tr>
<td>Rat primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Gata2</em> Forward</td>
<td>AACACACACCACTAATTACCCACTA</td>
<td>451</td>
</tr>
<tr>
<td><em>Gata2</em> Reverse</td>
<td>TTCCCTTTCATGGTCAGTGGCCT</td>
<td></td>
</tr>
<tr>
<td><em>Gata3</em> Forward</td>
<td>AAGCCCTGCAGACTCTACCCAATAA</td>
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</tr>
<tr>
<td><em>Gata3</em> Reverse</td>
<td>ACATCTCTGTTGCTTGGGTCTGGA</td>
<td></td>
</tr>
<tr>
<td><em>Gata4</em> Forward</td>
<td>AGGGATAAGGGTGCTTGTTTCTT</td>
<td>314</td>
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<tr>
<td><em>Gata4</em> Reverse</td>
<td>AGGGCTGCTATGTCTAGTCCCAA</td>
<td></td>
</tr>
<tr>
<td><em>Fog1</em> Forward</td>
<td>ACATGTCCAGAAGGAACACAGAC</td>
<td>1340</td>
</tr>
<tr>
<td><em>Fog1</em> Reverse</td>
<td>TGACTTCGGCCAAGGCTTACTG</td>
<td></td>
</tr>
<tr>
<td><em>Fog2</em> Forward</td>
<td>TGACGTCTACGCAGAAAAGGAGACCT</td>
<td>399</td>
</tr>
<tr>
<td><em>Fog2</em> Reverse</td>
<td>ACTCCTTGCCAAGTCACATCCA</td>
<td></td>
</tr>
<tr>
<td>Primers used after ChIP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair 1 F1</td>
<td>AATGTCAGCTAAGCCCTGACACCT</td>
<td>270</td>
</tr>
<tr>
<td>Pair 1 R1</td>
<td>TTTGTTGGAATTGGGAAACTCCT</td>
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</tr>
<tr>
<td>Pair 2 F2</td>
<td>TTAGCCGACGACGCGCTGAGGCTCCCCTC</td>
<td>203</td>
</tr>
<tr>
<td>Pair 2 R2</td>
<td>CCACCTACAGTGGCACGGCCTTTGGTAA</td>
<td></td>
</tr>
</tbody>
</table>
System (Promega). Nuclear extracts and cytosol were obtained from adult mouse pituitaries and testis (as a positive control tissue for GATA4 expression) and LβT2 cells using the method described by Andrews & Faller (1991). The proteins were separated using 10% SDS-PAGE gel and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). After overnight blocking with 10% nonfat milk in PBS, the membrane was probed with rabbit anti-GATA2 (sc-9008), mouse anti-GATA3 (sc-268), or goat anti-GATA4 (sc-1237) antibodies for 2 h at room temperature. This was followed by incubation with HRP-conjugated anti-rabbit, anti-mouse, or anti-goat IgG secondary antibodies. All these antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The bound secondary antibody was visualized using the SuperSignal West Dura Extended Duration Substrate kit (ThermoScientific, Rockford, IL, USA) and photographed with the Fujifilm LAS-3000 (Quansys Biosciences, Logan, UT, USA), or exposed to X-ray films.

Whole cell lysates of GATA2 small interfering RNA (siRNA)-treated LβT2 cells were prepared in RIPA buffer, briefly sonicated and quantified by BCA assay (Pierce Biotechnology, Inc., Rockford, IL, USA). The expression of GATA2, Lhb, and α-tubulin was detected following the protocol described above. The antibodies used for Lhb and α-tubulin detection were rabbit antirat Lhb (A F Parlow, National Hormone and Pituitary Program, Harbor-UCLA, CA, USA) and mouse anti-α-tubulin (Sigma–Aldrich). The films were photographed using a Kodak digital camera (DC 290), and the net band density of Lhb and α-tubulin was evaluated by Kodak 1D Image Analysis Software (Eastman Kodak Company, Rochester, NY, USA). The amount of Lhb was normalized to that of α-tubulin in each sample. The experiment was repeated four times.

**Transient transfection of cell lines**

Green monkey kidney fibroblast cells (CV1) were maintained in monolayer culture in low glucose DMEM supplemented with 10% fetal bovine serum (FBS) (v/v) and 1% penicillin/streptomycin (v/v) at 37 °C in humidified 5% CO2/95% air. CV1 cells (6–7×10^4 cells/well) were cultured overnight in 12-well plates. Using the calcium phosphate precipitation method, each well was transfected with 400 ng of the Lhb promoter reporter construct and 10 ng of each expression vector, or as indicated in the dose–response experiments. Cotransfection of 48 ng/well of β-galactosidase plasmid allowed correction for differences in transfection efficiency within the experiments. Cells were harvested 48 h following transfection and the cell extracts were analyzed for luciferase and β-galactosidase activity with the Tropix Luciferase Assay Kit (Applied Biosystems) using a luminometer from Berthold Detection Systems (Pforzheim, Germany).

For the LβT2 transfection experiments, cells were maintained in high glucose DMEM supplemented with 10% fetal bovine serum (v/v), 1% penicillin/streptomycin (v/v), and 1 mM sodium pyruvate. Cells (2.5–3×10^5 cells/well) were grown overnight in antibiotic-free media in 12-well plates. The cells were then transfected with 400 ng/well Lhb luciferase reporter vector and various expression vectors at the indicated doses using the FuGENE 6 Transfection Reagent (Roche Applied Sciences) and OptiMEM-1 Reduced Serum Medium (Invitrogen) according to the manufacturer’s instructions. β-galactosidase plasmid (48 ng/well) was cotransfected to correct for differences in transfection efficiency and cell numbers. The day following transfection, cells received fresh antibiotic-free DMEM. The cells were harvested 48 h after FuGENE transfection and assessed for luciferase activity with the Promega Dual Luciferase Reporter Assay System using the luminometer. Luciferase activity was normalized to β-galactosidase activity and results were calculated as fold-change relative to the control wells that received the empty pcDNA3.1 expression vector. Data are shown as the mean ± S.E.M. from at least three experiments with three replicates performed within each experiment.

**siRNA interference in LβT2 cells**

For siRNA experiments, LβT2 cells were seeded to 12-well plates at 2.5×10^5 cells/well in DMEM containing 10% FBS (without antibiotics) 1 day before siRNA transfection. Dharmacon ON-TARGETplus SMARTpool siRNAs directed toward mouse GATA2 (L-062114-00-0005) or a nontargeting negative control (D-001810-10-05) were transfected into LβT2 cells using DharmaFECT-1 siRNA Transfection Reagent (Thermo Fisher Scientific, Lafayette, CO, USA) according to the manufacturer’s instructions. Each well was transfected with 1 ml of 100 nM siRNA diluted in OptiMEM-I Reduced Serum Medium (Invitrogen), complexed with 3 μl DharmaFECT-1. The cells were cultured overnight and the transfection mixture was replaced the next day with DMEM containing 10% FBS. The cells were harvested 48 h after transfection for total RNA isolation for qRT-PCR analysis or whole cell lysates for western blot analysis.

To analyze the effect of siRNA of GATA factors on the Lhb promoter, we modified a previously described technique of luciferase-based testing after siRNA treatment (Zhuang & Liu 2006). Dharmacon ON-TARGETplus SMARTpool siRNAs directed toward mouse Gata2 (L-062114-00-0005), Gata4 (L-040759-01-0005), Pitx1 (L-043250-01-0005), or a nontargeting negative control (D-001810-1005) were transfected into LβT2 cells maintained in high glucose DMEM supplemented with 10% fetal bovine serum (v/v), 1% penicillin/streptomycin (v/v), and 1 mM sodium pyruvate. Cells (2.5–3×10^5 cells/well) were grown overnight in antibiotic-free media in 12-well plates. The cells were then transfected with 400 ng/well Lhb luciferase reporter vector and various expression vectors at the indicated doses using the FuGENE 6 Transfection Reagent (Roche Applied Sciences) and OptiMEM-1 Reduced Serum Medium (Invitrogen) according to the manufacturer’s instructions. β-galactosidase plasmid (48 ng/well) was cotransfected to correct for differences in transfection efficiency and cell numbers. The day following transfection, cells received fresh antibiotic-free DMEM. The cells were harvested 48 h after FuGENE transfection and assessed for luciferase activity with the Promega Dual Luciferase Reporter Assay System using the luminometer. Luciferase activity was normalized to β-galactosidase activity and results were calculated as fold-change relative to the control wells that received the empty pcDNA3.1 expression vector. Data are shown as the mean ± S.E.M. from at least three experiments with three replicates performed within each experiment.
cells as aforementioned. The cells were cultured overnight, and on the next day washed and replaced with fresh antibiotic-free DMEM. Then, the cells were transfected with 400 ng LHβ-luciferase reporter construct and 48 ng β-galactosidase plasmid using the FuGENE 6 Transfection Reagent. On the following day, fresh antibiotic-free DMEM was added. The cells were harvested 48 h after the reporter construct transfection for luciferase activity analysis as described above. Data are shown as the mean ± S.E.M. from at least three experiments with three replicates performed within each experiment.

**Electrophoretic mobility shift assay**

Double-stranded oligonucleotide probes were end labeled with [γ-32P]ATP and purified over a Quick Spin G-25 Sephadex Column (Roche Applied Science). The nucleotide sequence of the rat LHβ gene promoter is based on sequencing data available at GenBank (accession number AF020505). The sense strands for the oligonucleotides containing the putative GATA binding site are 5'GATA, 5'-AGAGATTAGTGCTAGGTTACCCA-3'; and 5'-GATAM, 5'-AGAATTCAGTGTCTAGGTTACCCA-3' (the substituted bases are underlined). Nuclear extracts and in vitro translated proteins were prepared as described for western blot analysis. Except for the Pitx1 antibody, antibodies used were the same as those used for western blot analysis. The polyclonal Pitx1 antibody was generated in rabbits (Molecular Innovations). The antibodies were the same as those used for western blot analysis. The polyclonal Pitx1 antibody was generated in rabbits (Molecular Innovations). The antibodies used were the same as those used for western blot analysis.

**Chromatin immunoprecipitation**

Approximately 1–1.5 × 107 LβT2 cells were grown on 10 cm dishes in DMEM medium containing 10% FBS and 1% penicillin/streptomycin for 2 days. The cells were then cross-linked with 1% formaldehyde at room temperature for 10 min with gentle shaking. The reaction was stopped by addition of 2.5 M glycine solution to a final concentration of 0.125 M. Cells were washed twice with cold PBS, collected, and centrifuged. The cell pellets were then suspended in 200 μl SDS lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, and 1% SDS) on ice for 10 min. The cell lysates were sonicated on ice to fragment the genomic DNA into lengths of 200–1000 bp (Cell Disruptor, Model W-375; Heat Systems Ultrasonics, Inc., Plainview, NY, USA), then centrifuged at 14,000 g for 10 min at 4 °C. Chromatin solution (100 μl) was used for each immunoprecipitation, which was diluted with 900 μl chromatin immunoprecipitation (ChIP) dilution buffer (0.01% SDS, 1-1% Triton X-100, 1-2 mM EDTA, 16-7 mM Tris, pH 8.1, and 167 mM NaCl) with protease inhibitor. The mixture was pre-cleared with the addition of 80 μl protein A/G Plus agarose bead slurry (sc-2003, Santa Cruz) and 2 μg salmon sperm DNA, followed by rotation for 2 h at 4 °C. After brief spinning, the supernatant was transferred into a new tube, and 10 μl was aliquoted as input sample (kept at −80 °C for later processing). The samples were immunoprecipitated with 5 μg specific antibodies to SF-1 (rabbit anti-mouse polyclonal antibody, 07-618, Upstate Biotechnology, Lake Placid, NY, USA), GATA2, GATA4, or normal rabbit or goat IgG at 4 °C overnight. The immune complexes were collected by adding 60 μl protein A/G agarose slurry and incubating at 4 °C for 2 h followed by centrifugation. The complexes were washed sequentially for 10 min each at 4 °C in low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1, and 150 mM NaCl), high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1, and 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% Nonidet P-10, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris, pH 8.1), and twice in TE buffer (10 mM Tris, pH 8.1, 1 mM EDTA). The protein/DNA complexes were eluted from the beads with fresh elution buffer (1% SDS, 0.1 M NaHCO3) three times. The eluates were pooled, NaCl was added to a final concentration of 100 mM, and cross-linking was reversed by incubation at 65 °C overnight. Samples were then digested with proteinase K for 1 h at 37 °C and purified using Qiaquick PCR Purification Kit (Qiagen). The DNA was eluted from the column in 50 μl TE buffer and used in PCR reactions.

The PCR reactions were conducted in 50 μl volume containing 200 nM of each primer, 1-5 mM MgCl2, 1×PCR buffer, 200 μM dNTP, 1 U Taq DNA polymerase (Invitrogen), and 5 μl of the purified ChIP DNA in an iCycler (Bio-Rad). The sequences of the primers for mouse LHβ promoter region are listed in Table 1. The PCR products were then separated on
spanning rat and mouse GATA-specific or FOG-specific primers.

reverse transcriptase (RT) and analyzed by PCR with intron-
described in Applied Biosystems User Bulletin No. 2.

By RT-PCR analysis, rat (Fig. 1A) and mouse (Fig. 1B)
gonadotrope-derived cell line, LβT2 were analyzed for normality followed by ANOVA. The statistical analysis was performed using the SigmaStat Software package (SPSS Science, Chicago, IL, USA). Data were analyzed for normality by RT-PCR analysis, rat (Fig. 1A) and mouse (Fig. 1B) pituitaries and the LβT2 cells were also found to express transcripts encoding the GATA cofactors, FOG1 and FOG2 (right panels). The predicted size for these products can be found in Table 1.

As shown by western blot analysis, GATA2 protein (Fig. 2A) is expressed in the nuclear extracts of LβT2 cells (lane 5) and adult mouse pituitaries (lane 3) but is undetectable in the cytosolic fraction of the adult mouse pituitary (lane 4), consistent with its role as a transcription factor. The mobility of these proteins is comparable to that of the in vitro translated GATA2 protein (lane 2). Unprogrammed reticulocyte lysate (RL, lane 1) and omission of the first antibody (lanes 6–9) were used as negative controls. Similarly, GATA4 protein (Fig. 2B) is observed only in the nuclear extracts of LβT2 cells and mouse pituitaries (lanes 3 and 5), not in the cytosol of mouse pituitaries (lane 4). The mouse testis was used as a positive control tissue (lanes 6 and 7). Endogenous GATA4 was found to run at a slower mobility than in vitro translated GATA4 in both the pituitary and the testicular cells, potentially due to

Statistical analysis

Statistical analysis was performed using the SigmaStat Software package (SPSS Science, Chicago, IL, USA). Data were analyzed for normality by ANOVA. The Tukey or Student–Newman–Keuls test was used for post hoc comparisons. The $t$-test was used where only two groups were involved. Statistical significance was set at $P<0.05$.

Results

GATA expression in adult pituitaries and the gonadotrope-derived cell line, LβT2

By RT-PCR analysis, rat (Fig. 1A) and mouse (Fig. 1B) pituitaries were found to express transcripts encoding GATA2, 3, and 4 (left panels). As shown in Fig. 1C (left panel), LβT2 gonadotropes were found to express the transcripts encoding GATA2, 3, and 4. Both primary pituitary and LβT2 cells were also found to express transcripts encoding the GATA cofactors, FOG1 and FOG2 (right panels). The predicted size for these products can be found in Table 1.

As shown by western blot analysis, GATA2 protein (Fig. 2A) is expressed in the nuclear extracts of LβT2 cells (lane 5) and adult mouse pituitaries (lane 3) but is undetectable in the cytosolic fraction of the adult mouse pituitary (lane 4), consistent with its role as a transcription factor. The mobility of these proteins is comparable to that of the in vitro translated GATA2 protein (lane 2). Unprogrammed reticulocyte lysate (RL, lane 1) and omission of the first antibody (lanes 6–9) were used as negative controls. Similarly, GATA4 protein (Fig. 2B) is observed only in the nuclear extracts of LβT2 cells and mouse pituitaries (lanes 3 and 5), not in the cytosol of mouse pituitaries (lane 4). The mouse testis was used as a positive control tissue (lanes 6 and 7). Endogenous GATA4 was found to run at a slower mobility than in vitro translated GATA4 in both the pituitary and the testicular cells, potentially due to
post-translational modifications. Despite the presence of GATA3 transcripts, we were unable to detect GATA3 protein in this gonadotrope cell line (Fig. 2C).

**GATA2 and GATA4 stimulate LHβ gene promoter activity via a putative cis-element located at position −101**

Having demonstrated expression of GATA2 and GATA4 in gonadotrope cells, we then determined their ability to alter expression of the LHβ gene. CV1 fibroblast cells were cotransfected with a rat LHβ gene promoter-reporter construct and increasing amounts of GATA2 or GATA4 expression plasmid. As shown in Fig. 3A, GATA2 (left panel) and GATA4 (right panel) produced a dose-dependent increase in the promoter activity of region −207 to +5 of the rat LHβ gene. Neither GATA2 nor GATA4 significantly increased activity of the empty reporter vector, pXP2 (data not shown). Furthermore, the GATA response was approximately twofold less when tested in a longer construct containing region −797/ +5, suggesting the presence of an upstream inhibitory factor (data not shown). Interestingly, transactivation by SF-1 or LRH-1 is also greater in the shorter construct (personal observation).

The transcriptional cofactor FOG2 has been shown to either augment or blunt GATA effects on gene expression dependent on cell type and promoter context (Robert et al. 2002, Cantor & Orkin 2005). Having demonstrated FOG expression in gonadotrope cells (Fig. 1C), we asked whether FOG2 altered GATA-mediated stimulation of LHβ gene promoter activity. As shown in Fig. 3B, FOG2 blunts both GATA2 and GATA4 transactivation of this gene.
By sequence analysis, region −207 to +5 of the rat LHβ gene promoter contains a highly conserved putative GATA DNA regulatory site (GATTA) at position −101 relative to the transcriptional start site. Mutation of this cis-element significantly decreased GATA induction of LHβ promoter activity, confirming its importance for mediating the GATA response (Fig. 3C). Interestingly, this region overlaps a previously characterized cis-element for the bicoid-related homeodomain protein, Pitx1. Pitx1 is known to contribute to differentiation of pituitary cell lineages as well as transcriptional control of the gonadotropin subunits (Tremblay & Drouin 1999, Quirk et al. 2001, Jiang et al. 2005).

GATA functionally interacts with the orphan nuclear receptors SF-1 and LRH-1

The identified GATA cis-element is flanked by two DNA regulatory regions (gonadotrope-specific elements (GSEs)) for the closely related orphan nuclear hormone receptors, SF-1 and LRH-1 (Halvorson et al. 1996, Weck et al. 2000, Zheng et al. 2007). We evaluated the ability of GATA2 to functionally interact with both of these factors on either the wild-type rLHβ gene promoter, a reporter construct containing mutations in both GSE sites, or a construct containing a mutation in the GATA site (Fig. 4). As shown in Fig. 4A, GATA2 synergized with SF-1 to stimulate wild-type LHβ promoter activity. As expected, mutation of the GSE sites eliminated the SF-1 response while maintaining the response to GATA. Somewhat surprisingly, the GATA response was markedly decreased in the presence of SF-1 on this promoter sequence (5-5-fold for GATA2 alone versus 0-98-fold GATA2 + SF-1, P<0.05). Conversely, the GATA response was lost and the SF-1 response retained with mutation of the 101 GATA site. GATA2 significantly blunted the SF-1 effect on this mutated promoter sequence (23-fold for SF-1 alone and 11-fold for SF-1 + GATA2, P<0.05).

As shown in Fig. 4B, GATA2 and LRH-1 also acted together to increase LHβ gene expression. In contrast to SF-1, LRH-1 did not alter the GATA response in the GSE-mutated construct. Although there was a trend for GATA2 to decrease the LRH-1 response in the GATA-mutated reporter, this effect was not statistically significant.

We were curious as to whether there would be an observable interaction of GATA2 and Pitx1 on the LHβ gene promoter as both of these factors bind to the same cis-element. As shown in Fig. 4C, neither synergy nor inhibition was observed in the presence of equal amounts of expression vector for both of these factors. We tested a number of different ratios of these vectors and did not observe a clear change in promoter activity in the presence of these factors when present alone or together (data not shown). These results suggest that DNA binding by either GATA or Pitx1 produce essentially equivalent transcriptional responses that cannot be distinguished by the transient transfection approach.

Inhibition of endogenous GATA expression decreases expression of the LHβ gene

To further confirm the importance of GATA2 and GATA4 for gonadotrope function, we used the siRNA approach to knock down endogenous GATA expression in LβT2 cells. As increased GATA expression stimulates LHβ gene expression, we reasoned that decreasing the intracellular expression of the GATAs should blunt expression of these genes. For these experiments, a siRNA directed against Pitx1 was used as a control as this transcription factor is known to stimulate LHβ promoter activity via an overlapping cis-element. Transfection of siRNAs for Gata2, Gata4, or Pitx1 significantly decreased their corresponding mRNA expression by ~60–70% relative to expression in cells receiving a nontargeting control siRNA (Fig. 5A).

Figure 5 Inhibition of endogenous GATA expression by siRNA decreases LHβ gene expression in gonadotrope LβT2 cells. Cells were transfected with siRNA and harvested for RNA or whole cell extract for analysis using qPCR (A and C), luciferase activity (B), or Western blot (D). All results are shown as percentage relative to expression in cells receiving a nontargeting control vector and represent a minimum of three experiments. *P<0.05 versus control. (A) Effect of Gata2, Gata4, and Pitx1 siRNA (100 nM) on expression of their corresponding mRNAs 48 h after transfection. (B) Inhibition of endogenous GATA factors or Pitx1 (as a positive control) by siRNA decreases LHβ gene promoter activity. Cells were transfected first with siRNA (Gata2 (100 nM), Gata4 (300 nM), Pitx1 (100 nM), or nontargeting siRNA at matching doses), and the following day, with the rat LHβ gene promoter (region −207/+5) construct linked to a luciferase reporter and a β-galactosidase vector to serve as control. Cells were harvested 48 h later and analyzed as described in Fig. 3. (C) LHβ mRNA expression in LβT2 cells 48 h following treatment with Gata2 siRNA (100 nM). (D) LHβ protein expression in LβT2 cells treated with Gata2 siRNA (100 nM) and harvested 48 or 72 h following transfection. The western blots are representative of three or more independent experiments, C, control; G2, GATA2. (E) Quantitation of the density of the LHβ protein bands corrected to β-tubulin.

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We then transfected LβT2 cells with both siRNA and the LHβ-luciferase construct. LHβ gene promoter activity was significantly decreased with knockdown of Gata2, Gata4, or Pitx1 expression (Fig. 5B).

We were also interested in determining the effect of GATA knockdown on expression of the LHβ gene at the mRNA and protein levels. For these studies, we focused on the role of GATA2 as the Gata2 siRNA had been found to be more efficient than the GATA4 siRNA pool when tested on LHβ promoter activity. As shown in Fig. 5C, transfection of Gata2 siRNA into LβT2 cells decreased the expression of LHβ mRNA by a small, but statistically significant, amount. More impressively, LHβ protein expression was decreased by ~60% at 48 h and 40% at 72 h in the presence of the Gata siRNA compared with the nontargeting control (P<0.05). As expected, GATA2 protein expression was dramatically decreased compared with the control after siRNA transfection.

GATA proteins bind to the region spanning position −101 in the rat LHβ promoter

Having demonstrated that GATA2 and GATA4 upregulate LHβ promoter activity, we investigated whether this effect was due to direct interaction of these transcription factors with the functionally defined −101 cis-element (Figs 3 and 4). The ability of in vitro translated GATA proteins to bind to this region of the LHβ gene promoter sequence was evaluated by electrophoretic mobility shift assay. GATA proteins were found to bind to the wild-type but not the mutated probes (Fig. 6A). As predicted, the nonprogrammed RL used as a negative control did not bind to the wild-type 3′-GSE probe (lane 1). As shown in Fig. 6B, the presence of GATA in the observed complexes was confirmed by blocking or supershift with an antibody directed against the appropriate GATA factor (lanes 2 and 6). The specificity of these protein–DNA interactions was demonstrated by successful competition with unlabeled wild-type oligonucleotides (lanes 3 and 7), while the mutated oligonucleotide had limited effect (lanes 4 and 8).

We next tested the ability of endogenous GATA to bind to the −101 LHβ gene promoter (Fig. 6C). LβT2 nuclear extract bound to the wild-type probe to produce multiple bands, including a more slowly migrating doublet. Addition of a GATA2-specific antibody blocked the presence of the upper portion of this doublet (lane 2), while a GATA4 antibody supershifted the lower band in this doublet (lane 4). These results are consistent with the reported sizes of 50 and 46 kDa for GATA2 and GATA4 respectively. Addition of both the GATA2 and the GATA4 antibodies eliminated GATA binding (lane 6). In contrast, addition of a specific GATA3 antibody did not alter band intensity when added alone or in combination with GATA2 and/or GATA4. These results demonstrate the ability of endogenous GATA2 and GATA4 to interact with the −101 LHβ gene promoter.
with \( LH_b \) gene promoter sequences and are consistent with the lack of GATA3 expression in these cells when analyzed by western blot analysis (Fig. 2C).

**GATA2 and GATA4 bind to the proximal region of the \( LH_b \) gene promoter**

We then investigated the ability of GATAs to interact with the endogenous \( LH_b \) gene promoter using ChIP assay in the \( LbT2 \) gonadotrope cell line. Antibodies directed against GATA2 or GATA4 increased DNA pull down relative to the IgG-precipitated (control) samples as tested by standard RT-PCR (Fig. 8B and C) or real-time qPCR (Fig. 8D). An antibody directed against mouse SF-1 was used as a positive control as SF-1 is known to bind to two cis-elements that flank the putative GATA site (Fig. 8A). These data strongly support the ability of GATA proteins to bind to the \( LH_b \) gene promoter in living cells.

**GATA blunts \( LH_b \) gene expression in the \( LbT2 \) cell line**

\( LbT2 \) cells were transiently transfected with region \(-207/+5\) of the rat \( LH_b \) gene promoter–reporter construct and increasing amounts of expression vector for GATA2 (Fig. 9A) or GATA4 (Fig. 9B). In contrast to stimulation observed in the fibroblast CV1 cell line, believed to lack GATA expression (Robert et al. 2006), GATA overexpression decreased \( LH_b \) gene promoter activity by a relatively small, albeit statistically significant, amount in the gonadotrope cell line.

**Discussion**

The GATA family of transcription factors has been demonstrated to have important roles in both cell-type specification and adult function at all levels of the reproductive axis. Within the pituitary gland, Dasen et al. (1999) have reported that GATA factors direct cell
fate with GATA2 overexpression directing differentiation toward the gonadotrope lineage. In their study, GATA2 and GATA3 were observed early in development; however, GATA3 expression disappeared by embryonic day 13.5 while GATA2 expression persisted into adulthood in both gonadotropes and thyrotropes (Dasen et al. 1999).

Two immortalized cell lines, αT31 and LβT2, are widely used in studies on gonadotrope function (Windle et al. 1990, Alarid et al. 1996). The LβT2 cell line is considered to be more representative of mature gonadotropes as it expresses the LHβ- and FSHβ-subunits in addition to the glycoprotein α-subunit and GnRH-receptor genes, while αT31 cells lack β-subunits that are known to be expressed later in ontogeny (Japon et al. 1994). Steger et al. (1994) have demonstrated GATA2 expression in the αT31 cell line as well as expression of a ‘GATA4-related’ protein. We now definitely establish expression of both GATA2 and GATA4 mRNA and protein in the LβT2 cell line (Figs 1 and 2). Although we were able to detect GATA3 transcripts in this cell line, we were unable to detect GATA3 protein, consistent with the previously reported loss of expression in later stages of pituitary development. To our knowledge, our results are the first to report GATA4 expression in adult gonadotropes (Fig. 2). Furthermore, we demonstrate that transcripts for the important GATA cofactors, FOG1 and FOG2, are present in gonadotropes. The ability of FOG2 to modulate GATA-stimulated LHβ gene expression suggests that this cofactor may provide an additional mechanism for fine-tuning gonadotropin gene expression.

In the results reported here, we clearly establish the ability of in vitro and endogenous GATA2 and GATA4 to bind to a cis-element present at position −101 in the rat LHβ gene promoter (Figs 6 and 8). These GATA factors were shown to markedly upregulate wild-type LHβ gene promoter activity by transient transfection analysis in the CV1 cell line. Conversely, downregulation of GATA expression by siRNA decreased LHβ expression. Mutation of the putative GATA site nearly eliminated the GATA response, demonstrating the functional importance of this cis-element in this heterologous cell line (Figs 3 and 4).

Unexpectedly, transfection of GATA2 and GATA4 in the gonadotrope LβT2 cell line led to a small, although statistically significant, decrease in LHβ promoter activity in contrast to the stimulation observed in fibroblast CV1 cells (Fig. 9). Multiple mechanisms could potentially account for the apparently discrepant effect of GATA transfection in the two cell lines. For example, it is of note that the blunting effect in LβT2 cells was not observed until the introduction of relatively large amounts of expression plasmid. In the CV1 transfections, these high doses of GATA factors resulted in markedly less promoter activation than that observed at lower doses (Fig. 3A).

As we have shown, LβT2 cells express GATA2 and GATA4 protein by western blot analysis, in contrast to CV1 cells that are believed to lack GATA expression (Robert et al. 2006). Overexpression of GATA factors in the LβT2 cells may, therefore, lead to sequestration of cofactors away from DNA-bound GATA. GATA may also squelch promoter activity by competing for common

![Figure 8 GATA2 and GATA4 bind to the endogenous LHβ gene promoter as detected by chromatin immunoprecipitation (ChIP).](image1)

![Figure 8](image1)

![Figure 9 GATA2 and GATA4 blunt LHβ gene promoter activity in the LβT2 cell line. Cells were transiently transfected with the −207/+5 LHβ gene reporter construct and expression vectors for either GATA2 (A) or GATA4 (B) at the doses indicated. *P<0.05 versus the empty expression vector.](image2)
co-activators with other transcription factors known to be critical for LHβ gene expression in the gonadotrope. For example, CREB-binding protein is known to be an important co-activator for GATA as well as both SF-1 and LRH-1 (Monte et al. 1998, Robert et al. 2006, Jiang et al. 2008). As yet another possibility, GATA may be recruiting inhibitory FOG2 present in LβT2 cells to the promoter as proposed by Tremblay on the MIS promoter (Tremblay et al. 2001).

The rat LHβ gene promoter has been shown to contain two DNA regulatory regions for the transcription factor Pitx1 (Tremblay & Drouin 1999, Quirk et al. 2001, Jiang et al. 2005). The GATA cis-element identified in these studies overlaps with the 5′-Pitx1 site, whereas the 3′-Pitx1 cis-element does not have strong sequence homology with a GATA site and is not important for GATA transactivation on transfection analysis (data not shown). The observation that GATA is able to compete with Pitx1 for binding to the common promoter region suggests that LHβ promoter activity is likely modulated by the relative amounts of each of these factors in different physiologic states. Further complexity is added by the fact that, like GATA, Pitx1 interacts functionally with SF-1 to synergistically stimulate LHβ gene expression.

Our results demonstrate marked synergy between GATA and both SF-1 and LRH-1 on activation of LHβ promoter activity (Fig. 4). In prior investigations, GATA factors were found to functionally interact with these nuclear receptors on additional genes with importance for reproductive function, including the aromatase (CYP19), inhibin α-subunit, and MIS genes (Watanabe et al. 2000, Tremblay et al. 2001, Tremblay & Viger 2001, Robert et al. 2006). Through a series of elegant experiments, the Viger group has characterized the amino acid residues in the GATA4 protein that are required for heterodimer formation with SF-1 and LRH-1, as well as those residues required for DNA binding and transactivation (Tremblay et al. 2001, Bouchard et al. 2009). Similar detailed analyses regarding GATA2 protein–protein interactions have not been performed. Interestingly, GATA has been shown to increase expression of both the SF-1 and the LRH-1 genes, providing yet another mechanism for coordinate regulation by these factors (Tremblay & Viger 2001, Bouchard et al. 2005).

The complexity of GATA/SF-1/LRH-1 regulation of LHβ promoter activity was further revealed in transfection experiments using LHβ promoter–reporter constructs with mutations in either the GATA or the SF-1/LRH-1 DNA regulatory sites (Fig. 4). Synergy between GATA and either of the nuclear receptors was found to require the presence of intact binding sites for both of these groups of factors, implying a requirement for each of these factors to bind to their cognate cis-elements in order to exert their effects. This constraint differs from that reported for the MIS gene in which strong GATA-mediated activation could be indirectly obtained through GATA interactions with DNA-bound SF-1 as well as through direct interactions with the GATA cis-element (Tremblay et al. 2001).

Our current studies demonstrate that SF-1 blunts GATA-mediated increases in LHβ promoter activity in a reporter construct lacking an SF-1/LRH-1 binding site and, conversely, that GATA inhibits SF-1-mediated stimulation of a construct lacking the GATA site (Fig. 4A). These results are consistent with sequestration of common co-activators by the non-DNA-bound transcription factor limiting the transactivation potential of the DNA-bound factor. Interestingly, the same squelching phenomenon was not observed between GATA2 and LRH-1, suggesting the use of nonoverlapping co-factors. Precedent exists for SF-1 and LRH-1 to differentially interact with other transcription factors to modulate LHβ gene expression. In a recent report by our group, the transcription factor COUP-TF blunted SF-1-mediated effects while augmenting LRH-1 stimulation (Zheng et al. 2010). Thus, although SF-1 and LRH-1 bind to the same two cis-elements in the LHβ gene, distinct function is achieved via divergent interactions with COUP-TF and GATA transcription factors.

In summary, the data presented here characterize the ability of GATA2 and GATA4 to stimulate LHβ gene promoter activity, both alone and in synergy with SF-1 and LRH-1. The GATA factors act via a DNA regulatory region that overlaps a previously described Pitx1 binding site and is positioned between two SF-1/LRH-1 cis-elements. The array of potential interactions among these transcription factors provides multiple mechanisms for fine-tuning expression of this critical reproductive gene.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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