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, L β T2. 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 GATT 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 LH β mRNA expression. The number of gonadotrope and thyrotrope 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 α T31 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 L β T2 cell line. Furthermore, we characterize the functional importance of these factors on expression of the LH β -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 LH β reporter construct used for these studies contained 207 bp of the 5'-flanking sequence of the rat LH β 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 L β T2 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 L β T2 cells were generously provided by Dr P L Mellon (University of California, San Diego, CA, USA).

Regular PCR reactions contained 1 $\times$ PCR buffer (Invitrogen) with 1.8 mM MgCl₂ concentration, 200 μ M dNTP, 1 U Taq DNA polymerase (Invitrogen), 200 nM of each primer, and 2–5 μ l cDNA to a total volume of 50 μ l in an Icyler (Bio-Rad). PCR conditions were as follows: 95 °C $\times$ 2 min, 35 cycles $\times$ (95 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min), and 72 °C $\times$ 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 *LH β*) with universal cycling conditions. Each reaction was run in 15 μ l 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

Table 1 PCR Primers used in the present study

	Sequence (5'-3')	Expected product size (bp)
Mouse primers		
<i>Gata2</i>		
Forward	CTAGCTACCATGGGCACCCAG	359
Reverse	CAATTTCACAACAGGTGCCC	
<i>Gata3</i>		
Forward	TGCCAGATAGCATGAAGCTGGAGA	527
Reverse	TCGGGTCTGGATGCCTTCTTTCTT	
<i>Gata4</i>		
Forward	TGTCATCTCACTATGGGCACAGCA	260
Reverse	TCCCAAGTCCGAGCAGGAATTTGA	
<i>Fog1</i>		
Forward	TCCTAACTGAAGAAGCCGCCAACT	244
Reverse	ATGACTGCGGTAGCAAGGATGGAT	
<i>Fog2</i>		
Forward	TGCTGGTCCTAAATGGCTTCTGGA	359
Reverse	ACATCAAATGGGCTTGCAAGTTCC	
Rat primers		
<i>Gata2</i>		
Forward	AACACACCACCCAATACCCACCTA	451
Reverse	TTCTTCTTCATGGTCAGTGGCCT	
<i>Gata3</i>		
Forward	AACGCCTGCGGACTCTACCATAAA	253
Reverse	ACATCTTACGGTTTCGGGTCTGGA	
<i>Gata4</i>		
Forward	AGGGATAAGGGTGCTGGGTTTCTT	314
Reverse	AGGGCTGCTATGTCTAGTCCACAA	
<i>Fog1</i>		
Forward	ACATGTCCAGGAGGAAACAGAGCA	1340
Reverse	TGACTTCGGCCAAGGCTTTACTGT	
<i>Fog2</i>		
Forward	TGACGTCATCGCGAAAGGAGACTT	399
Reverse	ACTCCTTGCCAAGTCACATCCAGA	
Primers used after ChIP		
Pair 1		
F1	AATGTCAGCTAAGCCCTGACACCT	270
R1	TTTGGTGGGATTGGGAAGTACCCT	
Pair 2		
F2	TTCAGCGAGCAGCCTGCAGTGGCCTCCCCT	203
R2	CCACTAAGTAGTGGCTACAGGCTTGGGTAA	

System (Promega). Nuclear extracts and cytosol were obtained from adult mouse pituitaries and testis (as a positive control tissue for GATA4 expression) and L β T2 cells using the method described by Andrews & Fallér (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 L β T2 cells were prepared in RIPA buffer, briefly sonicated and quantified by BCA assay (Pierce Biotechnology, Inc., Rockford, IL, USA). The expression of GATA2, LH β , and α -tubulin was detected following the protocol described above. The antibodies used for LH β and α -tubulin detection were rabbit anti-rat LH β (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 LH β and α -tubulin was evaluated by Kodak 1D Image Analysis Software (Eastman Kodak Company, Rochester, NY, USA). The amount of LH β 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 × 10⁴ cells/well) were cultured overnight in 12-well plates. Using the calcium phosphate precipitation method, each well was transfected with 400 ng of the LH β 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 L β T2 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 × 10⁵ cells/well) were grown overnight in antibiotic-free media in 12-well plates. The cells were then transfected with 400 ng/well LH β 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 $\pm$ S.E.M. from at least three experiments with three replicates performed within each experiment.

siRNA interference in L β T2 cells

For siRNA experiments, L β T2 cells were seeded to 12-well plates at 2.5 × 10⁵ 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 L β T2 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-1 Reduced Serum Medium (Invitrogen), complexed with 3 μ l 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 LH β 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-001810-10-05) were transfected into L β T2

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 LH β -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 $\pm$ 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 - 32 P]-ATP and purified over a Quick Spin G-25 Sephadex Column (Roche Applied Science). The nucleotide sequence of the rat LH β 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'-AGAGATTAGTGTC-TAGGTTACCCA-3'; and 5'-GATAM, 5'-AGAAT-TCAGTGTCTAGGTTACCCA-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 injected with a peptide corresponding to amino acids 24–52 of mouse Pitx1 conjugated to keyhole limpet hemocyanin (Covance Research, Richmond, CA, USA) provided by U B Kaiser (Jiang *et al.* 2005). Where indicated, 200-fold molar excess of unlabeled wild-type or mutated oligonucleotide probes or 1 μ l of antisera were added to the protein samples 20 min prior to the addition of labeled probe. To observe the competition of GATA and Pitx1 binding for LH β promoter, increasing amount of *in vitro* translated GATA2 or GATA4 was mixed with equal amount of L β T2 nuclear extracts. The protein–DNA complexes were resolved on a 4.5% nondenaturing polyacrylamide gel in 0.5 $\times$ Tris–borate–EDTA buffer. The gel was dried and subjected to autoradiography. For the quantification of the band density of Pitx1–DNA complex, the films were photographed using a Kodak digital camera, and the net band density was evaluated by Kodak 1D Image Analysis Software. Data are shown as the mean $\pm$ S.E.M. from three experiments.

Chromatin immunoprecipitation

Approximately 1–1.5 $\times$ 10 7 L β T2 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 μ l 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 μ l) was used for each immunoprecipitation, which was diluted with 900 μ l 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 μ l protein A/G Plus agarose bead slurry (sc-2003, Santa Cruz) and 2 μ g 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 μ l was aliquoted as input sample (kept at –80 °C for later processing). The samples were immunoprecipitated with 5 μ g 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 μ l 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-40, 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 NaHCO $_3$) 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 μ l TE buffer and used in PCR reactions.

The PCR reactions were conducted in 50 μ l volume containing 200 nM of each primer, 1.5 mM MgCl $_2$, 1 $\times$ PCR buffer, 200 μ M dNTP, 1 U Taq DNA polymerase (Invitrogen), and 5 μ l of the purified ChIP DNA in an Icyler (Bio-Rad). The sequences of the primers for mouse LH β promoter region are listed in Table 1. The PCR products were then separated on

Figure 1 Expression of transcripts encoding GATA transcription factors (left panels) and the GATA cofactors FOG1 and 2 (right panels) in the pituitary. Total RNA from adult rat pituitary (A), adult mouse pituitary (B), and the LβT2 gonadotrope cell line (C) was reverse transcribed in the presence (+) or absence (–) of reverse transcriptase (RT) and analyzed by PCR with intron-spanning rat and mouse GATA-specific or FOG-specific primers.

agarose gels and photographed as described previously. The experiments were performed a total of six times. Finally, all the ChIP DNA samples were further analyzed by qPCR in a 384-well plate on a 7900HT Sequence Detection System using SYBR Green PCR Master Mix, 400 nM forward and reverse primers (Table 1, pair 2), and 5 μl ChIP DNA in a 15 μl total reaction volume in triplicates. The relative DNA levels in the IgG and specific antibody precipitated samples were calculated using the comparative *C_T* method as described in Applied Biosystems User Bulletin No. 2.

Statistical analysis

Statistical analysis was performed using the SigmaStat Software package (SPSS Science, Chicago, IL, USA). Data were analyzed for normality followed 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

Figure 2 Expression of GATA proteins in the mouse pituitary and in LβT2 cells by western blot analysis. Following separation on an SDS-PAGE gel, samples were transferred to PVDF membranes and incubated with polyclonal rabbit anti-GATA2 (A), polyclonal goat anti-GATA4 (B), or monoclonal mouse anti-GATA3 (C) antibodies and then with peroxidase-labeled secondary antibodies. Signals were visualized with SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL, USA). *In vitro* translated GATA2, GATA4, and GATA3 proteins served as positive controls (lane 2 in A and B, lane 1 in C), whereas reticulocyte lysate (RL, lane 1 in A and B, and lane 2 in C) served as a negative control. Mouse testis (lanes 6 and 7 in B) was used as an additional positive control for GATA4 expression. Nuclear extracts from CV1 cells transfected with a GATA3 expression vector or an empty expression vector were also used as a positive control for GATA3 (lanes 5 and 6 in C). Nonspecific binding was determined in parallel blots that omitted the primary antibodies (lanes 6–9 in A and lanes 8–9 in B).

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

Figure 3 GATA2 and GATA4 stimulate transcriptional activity of the *LHβ* gene promoter. (A) CV1 cells were cotransfected with region –207/+5 of the rat *LHβ* gene promoter linked to a luciferase reporter and the indicated amount of expression vector for GATA2 (left panel) or GATA4 (right panel). Bars with different letters significantly vary ($P < 0.05$). (B) CV1 cells were transfected with the –207/+5 rat *LHβ* gene reporter and expression vectors for GATA2 (left panel) or GATA4 (right panel) in the presence or absence of FOG2. * $P < 0.05$ versus GATA factor alone. (C) CV1 cells were transfected with the 207/+5 rat *LHβ* gene reporter construct present as the wild type or with a mutation in the putative GATA site. * $P < 0.05$ versus response in the wild-type construct. For all experiments, the CV1 cells also received a β -galactosidase vector as an internal control for differences in transfection efficiency. Luciferase activity was normalized to β -galactosidase activity and expressed as fold-change relative to cells receiving the empty expression vector. All experiments were performed a minimum of three times in triplicate with data expressed as the mean $\pm$ S.E.M.

Figure 4 CV1 cells were transfected with the –207/+5 rat *LHβ* gene reporter present as the wild type or with mutations in either the GATA/Pitx1 site at position –101 or in the gonadotrope-specific element (GSE) sites previously shown to bind both steroidogenic factor-1 (SF-1) and liver receptor homologue-1 (LRH-1; see Fig. 8A). Cells were cotransfected with expression vectors for GATA2 and/or SF-1 (A), GATA2 and/or LRH-1 (B), or GATA2 and/or Pitx1 (C). Data for each reporter were independently analyzed for each treatment group. Within the same reporter construct and treatment group, bars with different letters significantly vary ($P < 0.05$). Transfection control and data calculation were the same as in Fig. 3.

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 negative control 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 α -tubulin.

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

Figure 6 Both endogenous and *in vitro* translated GATA proteins bind to position –101 of the rat *LHβ* gene promoter. (A) Electrophoretic mobility shift assay (EMSA) evaluated the ability of *in vitro* translated GATA2 (lanes 2 and 6), GATA3 (lanes 3 and 7), or GATA4 (lanes 4 and 8) to bind to oligonucleotide probes present as either the wild type (lanes 1–4) or with a mutation in the –101 region of the promoter (lanes 5–8). Reticulocyte lysate (RL) served as negative control (lanes 1 and 5). (B) *In vitro* translated GATA2 (left panel) or GATA4 (right panel) were mixed with the wild-type *LHβ* oligonucleotide probe. The addition of the corresponding antibody blunted formation of the GATA2 complex (lane 2) and supershifted the GATA4 complex (lane 6). The unlabeled wild-type oligonucleotide (lanes 3 and 7) successfully competed for GATA binding, while the mutated oligonucleotide had limited effect (lanes 4 and 8). (C) LβT2 nuclear extract bound to the wild-type oligonucleotide (lane 1) to produce two complexes indicated by the arrows. Addition of a GATA2-specific blocking antibody decreased the intensity of the upper portion of this doublet (lane 2), whereas a GATA4-specific antibody (lane 4) supershifted the lower band. Addition of both GATA2 and GATA4 antibodies eliminated this complex (lane 6). Addition of a GATA3 antibody did not change the binding pattern when present alone (lane 3) or in combination with the other antibodies. Pitx1 also binds to the probe to form a faster migrating complex.

with *LHβ* gene promoter sequences and are consistent with the lack of GATA3 expression in these cells when analyzed by western blot analysis (Fig. 2C).

GATAs and Pitx1 compete for DNA binding at the GATA cis-element

As the identified GATA cis-element overlaps with a previously reported DNA regulatory site for the homeodomain transcription factor, Pitx1, we asked whether GATA2 or GATA4 could compete with Pitx1 for binding to this region (Fig. 7). For unclear reasons, Pitx1 protein cannot be expressed using a standard *in vitro* translation approach. We, therefore, chose to look at the effect of increasing amounts of *in vitro* translated GATA on DNA-binding by endogenous Pitx1. The presence of Pitx1 in LβT2 nuclear extract was confirmed by supershift with a Pitx1-specific antibody (Fig. 7A, lane 2). As shown in Fig. 7B, increasing amounts of GATA2 (left panel) or GATA4 (right panel) competed for Pitx1 DNA binding in a dose-dependent manner. These results suggest that the relative level of GATA and Pitx1 expression is likely important for determining *LHβ* gene promoter activity.

GATA2 and GATA4 bind to the proximal region of the *LHβ* gene promoter

We then investigated the ability of GATAs to interact with the endogenous *LHβ* gene promoter using ChIP assay in

the LβT2 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 *LHβ* gene promoter in living cells.

GATA blunts *LHβ* gene expression in the LβT2 cell line

LβT2 cells were transiently transfected with region −207/+5 of the rat *LHβ* 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 *LHβ* 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

Figure 7 GATA2 and GATA4 compete with Pitx1 for binding to the −101 region of the *LHβ* gene promoter. (A) LβT2 nuclear extract interacted with the wild-type *LHβ* probe to produce a complex that migrated at faster mobility than the GATA2/4 complex (lane 1). Addition of a Pitx1-specific antibody nearly eliminated this band (lane 2). (B) GATA2 (left panel) and GATA4 (right panel) compete with Pitx1 for binding to the −101 *LHβ* promoter region. *In vitro* translated GATA2 or GATA4 was added alone (lanes 2 and 7) or at increasing concentrations with LβT2 nuclear extract. A representative gel from three experiments is shown. Note that GATA2 binding alone is under exposed in order to optimize the ability to observe the inhibition of Pitx1 binding. The intensity of Pitx1 binding was calculated for all the three experiments and depicted in graphical form below the EMSA panel.

Figure 8 GATA2 and GATA4 bind to the endogenous *LHβ* gene promoter as detected by chromatin immunoprecipitation (ChIP). (A) Position of primers used in the PCR reactions following immunoprecipitation. The putative GATA cis-element and two previously characterized gonadotrope-specific elements (GSEs) known to bind steroidogenic factor-1 (SF-1) are shown. (B) ChIP results using a GATA2-specific antibody (primer pair 1). (C) ChIP results using a GATA4 antibody (primer pair 2). An antibody directed against SF-1 was used as a positive control. (D) Quantitative PCR results of the ChIP DNA using primer pair 2. Bars with different letters differ significantly ($P < 0.05$).

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, α T31 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 α T31 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 α T31 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 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.

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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