Pituitary homeobox 1 (Pitx1) stimulates rat LHβ gene expression via two functional DNA-regulatory regions

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Abstract

Luteinizing hormone (LH) plays a central role in the reproductive axis, stimulating both gonadal steroid biosynthesis and the development of mature gametes. Over the past decade, significant progress has been made in characterizing the transcription factors and associated DNA-regulatory sites which mediate expression of the LH β-subunit gene (LHβ). One of these factors, pituitary homeobox 1 (Pitx1), has been shown to stimulate LHβ gene promoter activity, both alone and in synergy with the orphan nuclear receptor, steroidogenic factor-1 (SF-1), and the early growth response gene 1 (Egr-1). Prior reports have attributed the Pitx1 response to a *cis*-element located at position −101 in the rat LHβ gene promoter. While investigating the role of Pitx1 in regulating rat LHβ gene expression, we observed a small, but significant, residual Pitx1 response despite mutation or deletion of this site. In the studies presented here, we identify the presence of a second functional Pitx1 region spanning positions −73 to −52 in the rat LHβ gene promoter. Based on electrophoretic mobility shift assay, Pitx1 binds to both the initially described 5′Pitx1 site as well as this putative 3′Pitx1 region. In transient transfection analysis, mutation of the LHβ-3′Pitx1 site significantly blunted Pitx1 responsiveness, with elimination of the Pitx1 response in a construct containing mutations in both Pitx1 *cis*-elements. We also analyzed the importance of each of these Pitx1 sites for providing functional synergy with SF-1 and with Egr-1. We observed a markedly decreased synergistic response with mutation of the 5′Pitx1 site with further loss following mutation of the 3′Pitx1 site. In contrast, functional interaction between Pitx1 and Egr-1 persisted with mutation of both Pitx1 regions. We conclude that Pitx1 stimulates the rat LHβ gene promoter via two Pitx1 DNA-regulatory regions. These results further our understanding of the molecular mechanisms that regulate expression of this critical reproductive gene promoter.

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Introduction

The gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are critical modulators of sexual development and reproductive function, acting on the gonads to stimulate both steroidogenesis and gametogenesis. Produced by the gonadotrope subpopulation of the anterior pituitary gland, LH and FSH are heterodimeric glycoproteins composed of a common α-subunit linked non-covalently to one of two unique β-subunits (LHβ and FSHβ, respectively). These β-subunits provide the functional specificity that distinguishes LH from FSH via interactions with distinct G-protein-coupled receptors. Biosynthesis of the β-subunits is believed to be the rate-limiting step in the generation of physiologically active heterodimers.

Over the past decade, substantial progress has been made in the identification of the transcription factors which are required for basal, tissue-specific, and gonadotropin-releasing hormone (GnRH)-activated expression of the LHβ-subunit gene, including steroidogenic factor-1 (SF-1), early growth response gene 1 (Egr-1) and Sp1 (Halvorson *et al.* 1996, 1998, Keri & Nilson 1996, Parker *et al.* 1996, Wolfe 1999, Kaiser *et al.* 2000, Zhao *et al.* 2001). More recent investigations have demonstrated a critical role for the transcription factor pituitary homeobox 1 (Pitx1) in both anterior pituitary development as well as the regulation of a broad array of pituitary-specific genes in the adult (Lamonerie *et al.* 1996, Lanctot *et al.* 1997, Drouin *et al.* 1998, Tremblay *et al.* 1998, Kurotani *et al.* 1999, Lanctot *et al.* 1999, Quirk *et al.* 2001, Quentien *et al.* 2002, Zakaria *et al.* 2002, Jeong *et al.* 2004).

Pitx1, and the closely related proteins Pitx2 and Pitx3, are members of the bicoid-related homeodomain protein family. During embryonic development, Pitx1 and Pitx2 are expressed in the epithelia of the oral cavity and the first branchial arch, structures which subsequently develop into the anterior pituitary gland, nasopharynx, palate, tongue, and olfactory and dental epithelium. In later development, Pitx1 contributes to the differentiation of anterior pituitary lineages through synergism

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with cell-restricted transcription factors such as SF-1 (gonadotropes), Pit1 (lactotropes and somatotropes), and bHLH NeuroD1/Pan1 (corticotropes; Szeto *et al.* 1999, Tremblay & Drouin 1999, Tremblay *et al.* 1999).

Mice homozygous for deletion of the Pitx1 gene undergo normal early pituitary organogenesis, perhaps due to the compensatory effects of Pitx2; however, subsequent pituitary development is markedly abnormal. Both mRNA and protein levels of LHβ, FSHβ, and thyroid-stimulating hormone (TSH)β are substantially reduced in Pitx1-null animals with a less-marked decrease in glycoprotein α-subunit levels (Szeto *et al.* 1999).

Pitx1 expression persists in the adult pituitary gland. Based on analysis of pituitary-derived cell lines, Pitx1 mRNA and protein are expressed in all pituitary lineages, with particularly high expression levels in cell lines which express the glycoprotein α-subunit (i.e. gonadotropes and thyrotropes) (Tremblay *et al.* 1998, Kurotani *et al.* 1999). Within gonadotropes, Pitx1 has been demonstrated to stimulate expression of the genes that encode the α-subunit, LHβ, FSHβ, and GnRH receptor (Tremblay *et al.* 1998, Quirk *et al.* 2001, Zakaria *et al.* 2002, Jeong *et al.* 2004). Pitx1-mediated transactivation of the LHβ gene has been shown to be enhanced in the presence of the orphan nuclear hormone receptor, SF-1, as well as Egr-1 (Tremblay *et al.* 1998, 1999, Quirk *et al.* 2001).

We were interested in investigating the effect of Pitx1 and known transcriptional partners in regulation of the rat LHβ gene promoter. In early experiments, we observed persistent Pitx1-stimulated promoter activity despite mutation of the previously described Pitx1 *cis*-element at position −101. In the studies reported here, we identify a second region in the proximal rat LHβ gene promoter which binds Pitx1 and confers Pitx1 responsiveness. Furthermore, we characterize the importance of both of these sites for synergy between Pitx1 and SF-1 or Egr-1.

Materials and methods

Plasmids used in transfection studies

The LHβ reporter constructs used for these studies contain 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 (Nordeen 1988). 5′ deletions were created by subcloning PCR products containing the LHβ promoter sequences into the pXP2 vector using BamHI/HindIII sites that were introduced by the primers (Kaiser *et al.* 1998). In a subset of experiments, the LHβ promoter sequence was excised using BamHI and HindIII restriction enzymes and inserted into pGL3-Basic at BglI/HindIII (Promega, Madison, WI, USA). Mutations were introduced into the LHβ promoter region using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA).

The mouse Pitx1 expression vector contains the Pitx1 open reading frame subcloned into pcDNA3/Amp (Invitrogen, Carlsbad, CA, USA; kindly provided by U B Kaiser, Brigham and Women's Hospital, Boston, MA, USA; Jeong *et al.* 2004). The SF-1 expression vector contains 2·1 kb of the mouse SF-1 cDNA driven by the cytomegalovirus (CMV) promoter in the vector pCMV5 (provided by K L Parker, Southwestern University School of Medicine, Dallas, TX, USA; Lala *et al.* 1992).

Transient transfection of cell lines

Green monkey kidney fibroblasts (CV-1) and mouse gonadotrope-derived cells (LβT2 and αT3–1) were maintained in monolayer culture in low-glucose (CV-1) or high-glucose (LβT2) Dulbecco's modified Eagle's medium supplemented with 10% (v/v) certified fetal calf serum and 1% (v/v) penicillin/streptomycin at 37 °C in humidified 5% CO_2/95% air. The immortalized gonadotrope cell lines were generously provided by Dr P L Mellon (University of California, San Diego, CA, USA; Windle *et al.* 1990, Thomas *et al.* 1996). Cells were transfected at 50–80% confluence in 12-well plates using the calcium phosphate precipitation method (CV-1 cells) or Lipofectamine reagent (LβT2 cells; Invitrogen; Sambrook *et al.* 1989). Cells received 0·4 μg/well of reporter vector and 0·1 μg/well of expression vector. Co-transfection with a Rous sarcoma virus (RSV)-β-galactosidase plasmid allowed correction for differences in transfection efficiency between wells in all experiments. Cells were harvested approximately 48 h following transfection and the cell extracts analyzed for luciferase activity and β-galactosidase activity using the Galacto-Light assay system (Applied Biosystems, Foster City, CA, USA; Edlund *et al.* 1985, deWet *et al.* 1987). Luciferase activity was normalized to the level of β-galactosidase activity and results calculated as fold-change relative to expression in the control wells. Data are shown as the mean ± S.E.M. from 3–7 independent experiments.

Electrophoretic mobility shift assay (EMSA)

The nucleotide sequence of the rat LHβ gene promoter is based on sequencing data available at GenBank accession number AF020505. Double-stranded oligonucleotide probes were created by T4 polynucleotide kinase end-labeling with [γ-^{32}P]ATP followed by purification over a Quick Spin G-25 Sephadex column (Roche Applied Science, Indianapolis, IN, USA).

The following oligonucleotide sequences were utilized (substituted nucleotides underlined): (5′Pitx1) 5′-AGAG ATTAGTGTCTAGGTTACCCA-3′; (5′Pitx1 M) 5′-AGA ATTCAGTGTCTAGGTTACCCA-3′; (5′SF1-Pitx1) 5′-CTTTCTGACCTTGTCTGTCTCGCCCCCAAAGA

GATTAGTGTCTA-3′; (5′SF1-Pitx1 M) 5′-CTTTCT GACCTTGTCTGTCTCGCCCCCAAAGAATTCAG TGTCTA-3′; (5′SF1 M-Pitx1) 5′-CTTTCTGAAATTGT CTGTCTCGCCCCCAAAGAGATTAGTGTCTA-3′; (5′SF1 M-Pitx1 M) 5′-CTTTCTGAAATTGTCTGTC TCGCCCCCAAAGAATTCAGTGTCTA-3′; (3′Pitx1) 5′-CCTGTAGCCTCTGCTTAGTGGCCTTGCCA C-3′; (3′Pitx1 MA) CCTGAATTCTCTGCTTAGTG GCCTTGCCAC; (3′Pitx1 MB) 5′-CTGTAGCCTCTG AATTCTGGCCTTGCCAC-3′; (3′Pitx1 MC) 5′-CCT GTAGCCTCTGCTTAGTGGAATTCCCAC-3′.

10 μg nuclear protein or 3 μg glutathione-S-transferase (GST) or GST–Pitx1 were incubated with 60 000 c.p.m. oligonucleotide probe in DNA-binding buffer (20 mM Hepes (pH 7·9), 60 mM KCl, 5 mM $MgCl_2$, 10 mM PMSF, 10 mM dithiothreitol, 1 mg/ml BSA, and 5% (v/v) glycerol). Where indicated, an excess of unlabeled oligonucleotide or 2 μl of antisera was added 20 min prior to the addition of labeled probe. Protein–DNA complexes were resolved on a 5% nondenaturing polyacrylamide gel in 0·5 × Tris/borate/EDTA buffer and subjected to autoradiography.

The GST–Pitx1 plasmid was generated by insertion of the Pitx1 cDNA downstream of the GST coding sequence in the pGEX-4T-2 expression vector (Amersham Biosciences Corp, Piscataway, NJ, USA) in the BamHI/NotI sites (construct provided by U B Kaiser; Jeong *et al.* 2004). This plasmid was introduced into a BL21 bacterial stock followed by induction with isopropyl β-D-thiogalactosidase to induce expression of the fusion protein, which was purified on a GST-affinity column (Amersham Biosciences Corp).

Nuclear proteins were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents and total protein concentration determined by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). *In vitro*-translated SF-1 was generated from a plasmid containing 2·1 kb pairs of the mouse SF-1 cDNA (provided by Dr K L Parker) using the TNT Coupled Reticulocyte Lysate System (Promega).

The polyclonal Pitx1 antibody was generated in rabbits against a peptide corresponding to amino acids 24–52 of mouse Pitx1 conjugated to keyhole limpet hemocyanin (Covance Research, Richmond, CA, USA; provided by UB Kaiser; Zakaria *et al.* 2002).

To provide quantitation, autoradiographs were photographed using a Kodak digital camera (DC 290) and the net density of signals was evaluated by Kodak 1D Image Analysis software (Eastman Kodak Company, Rochester, NY, USA).

Statistical analysis

Statistical calculations was performed using the SigmaStat statistical software package (SPSS Science, Chicago, IL, USA). Data were analyzed for normality followed by calculation of analysis of variance (ANOVA) or the Kruskal–Wallis ANOVA on ranks for non-parametric data. The Student–Newman–Keuls method was utilized for *post-hoc* comparison, except for experiments with different sample sizes in which case Dunn's test was employed. Statistical significance was set at $P<0·05$.

Results

LHβ gene expression is activated by Pitx1 and SF-1 acting alone and in synergy

We first analyzed the effect of Pitx1 and/or SF-1 on transactivation of the LHβ gene promoter using transient transfection experiments in two cell lines, a gonadotrope-derived cell line (LβT2) and a fibroblast cell line (CV-1). Cells were transfected with a reporter construct containing region −207/+5 of the rat LHβ gene promoter upstream of the luciferase reporter vector, pXP2. Cells were co-transfected with expression vectors for Pitx1 and/or SF-1. In CV-1 cells, which lack both transcription factors, LHβ gene promoter activity was increased significantly with the addition of Pitx1 or SF-1 (17- and 55-fold, respectively; $P<0·05$ versus the empty control vector) (Fig. 1A). The addition of both factors produced a synergistic response of over 110-fold ($P<0·05$ versus control and versus Pitx1 or SF-1 alone). In LβT2 cells, overexpression of Pitx1 or SF-1 modestly, but significantly, increased LHβ promoter activity relative to control wells (1·8- and 2-fold, respectively; $P<0·05$ versus control; Fig. 1B). Once again, a synergistic response was observed with the overexpression of both factors (7·5-fold; $P<0·05$ versus control and versus Pitx1 or SF-1 alone). The comparatively small magnitude of the response in the gonadotrope LβT2 cell line is likely due to the high level of endogenous Pitx1 expression reported in these cells. Therefore, in order to increase our ability to identify subtle changes in expression, we chose to continue our analysis in the CV-1 cell line.

Residual Pitx1 response with mutation or deletion of the previously identified Pitx1 element

We next evaluated the response to Pitx1 following deletion or mutation of the previously identified Pitx1 site (Fig. 2A). A statistically significant residual Pitx1 response was observed in the mutated construct (7·6-fold) and in the 5′ deleted constructs (3·6- and 2-fold for the −82/+5 and −68/+5 constructs, respectively; $P<0·05$ versus response in the empty reporter vector, pXP2). In order to confirm this observation, additional constructs were generated which contained rat LHβ

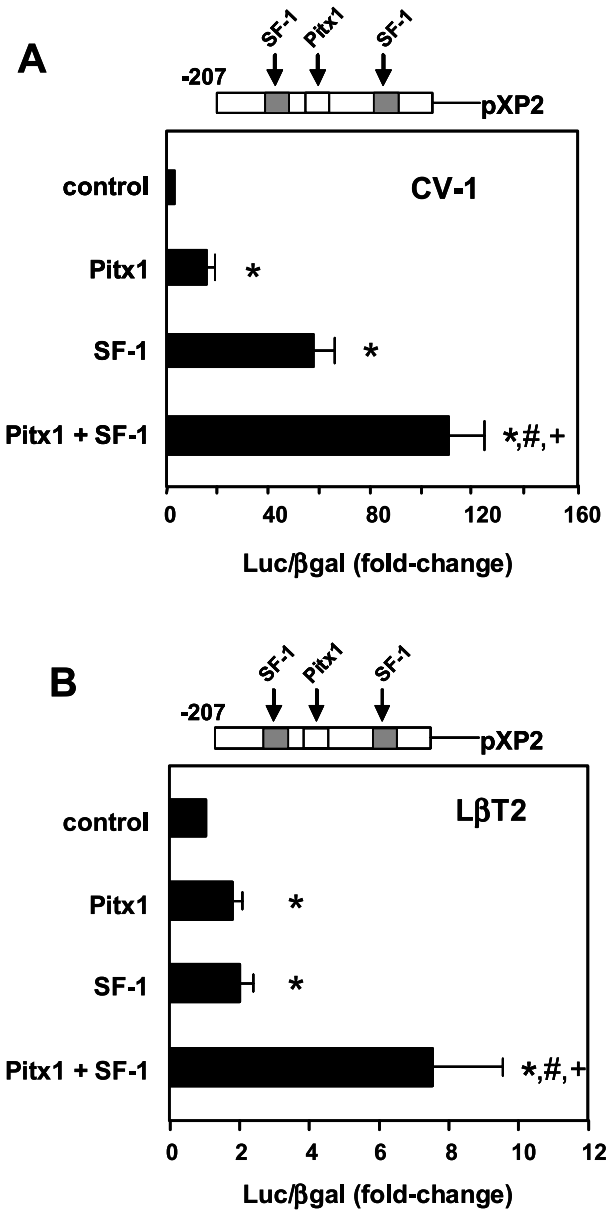

Figure 1 Pitx1 and SF-1 act alone and in synergy to increase LHβ gene promoter activity. Fibroblast CV-1 cells (A) and gonadotrope-derived LβT2 cells (B) were transiently transfected with region −207/+5 of the rat LHβ gene promoter linked to a luciferase reporter construct, pXP2. Cells were co-transfected with CMV-driven expression vectors encoding Pitx1 and/or SF-1. Control wells received equivalent amounts of 'empty' expression vector. All cells were co-transfected with an RSV-β-galactosidase vector to control for potential differences in transfection efficiency. Approximately 48 h after transfection, cells were analyzed for luciferase and β-galactosidase activity. Luciferase activity was normalized to β-galactosidase activity and promoter activity was calculated as fold-change over expression in the control wells. *$P<0{\cdot}05$ versus control, #$P<0{\cdot}05$ versus Pitx1 alone, +$P<0{\cdot}05$ versus SF-1 alone.

gene sequences in a second luciferase reporter vector, pGL3. As shown in Fig. 2B, a residual Pitx1 response was again observed in a construct containing a mutation in the −101 Pitx1 site (2·8-fold; $P<0{\cdot}05$ versus control). Taken together, these results suggested strongly the presence of a second functional Pitx1 *cis*-element in the LHβ gene promoter.

Pitx1 present in gonadotropes binds to the LHβ gene promoter

EMSA was used to characterize the site(s) at which Pitx1 interacts with the rat LHβ gene promoter. As shown in Fig. 3A, nuclear extracts from the gonadotrope-derived αT3–1 cell line bound to an oligonucleotide probe which spans the −101 Pitx1 *cis*-element to produce a dominant band (Fig. 3A, lane 1). Formation of this complex was effectively competed by the addition of excess cold wild-type oligonucleotide (Fig. 3A, lane 2), but not by a mutated oligonucleotide (Fig. 3A, lane 3) or by an unrelated oligonucleotide containing the LHβ 3′Egr-1 site (Fig. 3A, lane 4). Gonadotrope nuclear extract interacted weakly with the mutated oligonucleotide when used as a probe, demonstrating specificity of the protein–DNA interaction (Fig. 3A, lane 5).

The identity of the dominant band was confirmed by the addition of a Pitx1-specific antiserum which blunted the Pitx1-containing complex (Fig. 3B, lanes 1 and 2). In contrast, a pre-immune antibody had no effect on this complex (Fig. 3B, lane 3). Nuclear extract from the differentiated LβT2 cell line was also demonstrated to contain endogenous Pitx1 which bound to the LHβ 5′Pitx1 region (Fig. 3B, lanes 5 and 6).

Pitx1 binds to a second proximal site in the rat LHβ gene promoter

Analysis of the proximal rat LHβ gene promoter sequence identified three regions with homology to the consensus Pitx1 *cis*-element (TAA(T/G)CC). These putative *cis*-elements within the 3′Pitx1 region were designated sites A, B and C (Fig. 4). As shown in Fig. 5A, a GST–Pitx1 fusion protein bound with high affinity to the previously identified 5′Pitx1 site on EMSA (Fig. 5A, left-hand panel). This complex was effectively supershifted by a Pitx1-specific antibody. Pitx1 also bound specifically to an oligonucleotide probe which spans all three putative 3′ Pitx1 sites in the 3′Pitx1 region (Fig. 5A, right-hand panel).

Comparison of Pitx1 affinity for the 5′Pitx1 and 3′Pitx1 regions

Pitx1 was noted to produce a less-intense complex with the 3′Pitx1 probe than with the probe spanning the

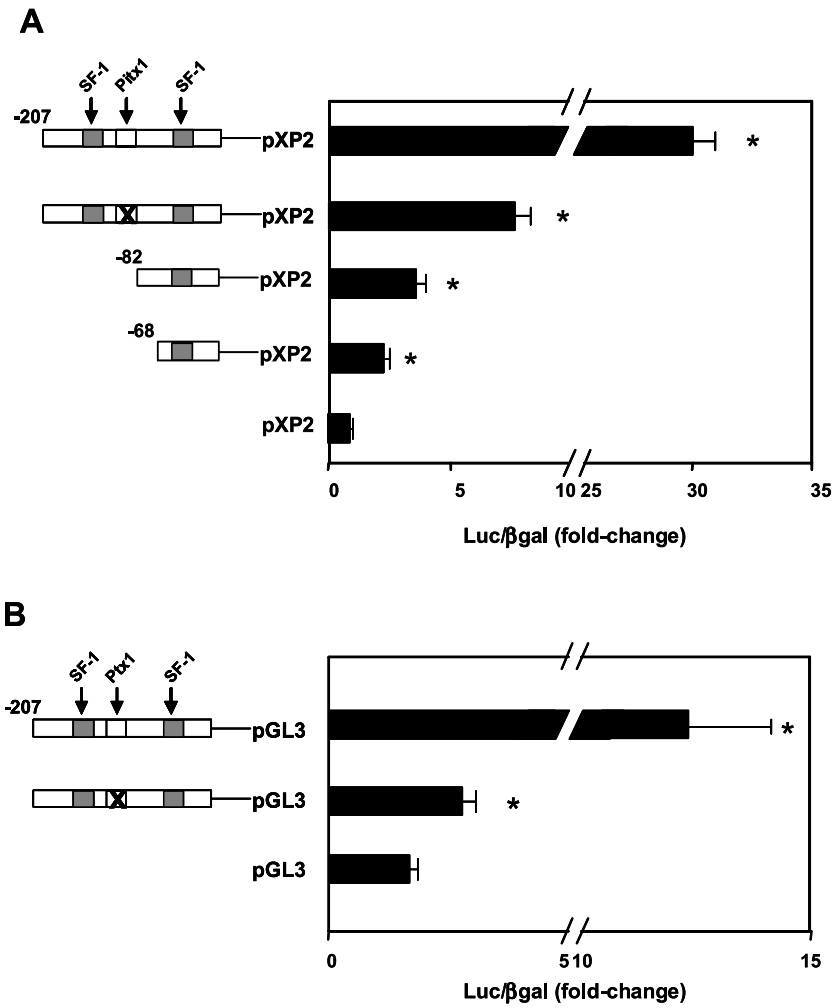

Figure 2 Identification of a residual Pitx1 response following mutation or deletion of the −101 Pitx1 *cis*-element. CV-1 cells were transfected with the indicated luciferase reporter construct and a Pitx1 expression vector or empty expression vector. Cells were harvested and results calculated as described in Fig. 1. (A) CV-1 cells were transfected with luciferase constructs containing region −207/+5 of the rat LHβ gene promoter present as the wild-type or with mutation in the previously identified Pitx1 *cis*-element at position −101. Alternative wells were transfected with 5′ deleted LHβ promoter constructs or with the empty reporter vector, pXP2. *$P<0{\cdot}05$ versus Pitx1 response in pXP2. (B) CV-1 cells were transfected with reporter constructs containing region −207/+5 of the wild-type or Pitx1-mutated rat LHβ gene promoter linked to the luciferase reporter vector, pGL3. *$P<0{\cdot}05$ versus Pitx1 response in pGL3.

more distal Pitx1 *cis*-element. In order to estimate the relative efficiency of the Pitx1–DNA interaction in these two regions, a competitive EMSA experiment was performed (Fig. 5B). Pitx1 fusion protein was added to the oligonucleotide probe containing the 3′Pitx1 region. Increasing amounts of unlabeled 5′ or 3′Pitx1 oligonucleotide were added and the intensity of the generated complex quantified. Based on results from three independent experiments, the Pitx1 binds the 5′Pitx1 site with approximately 6-fold greater affinity than the 3′Pitx1 region. This result is consistent with the relative functional importance of these two sites as assessed in transfection experiments.

Pitx1 binds with greatest intensity to site B within the putative 3′Pitx1 region of the proximal LHβ gene

We next evaluated the ability of Pitx1 to bind to each of the putative 3′Pitx1 DNA-binding sites identified in the

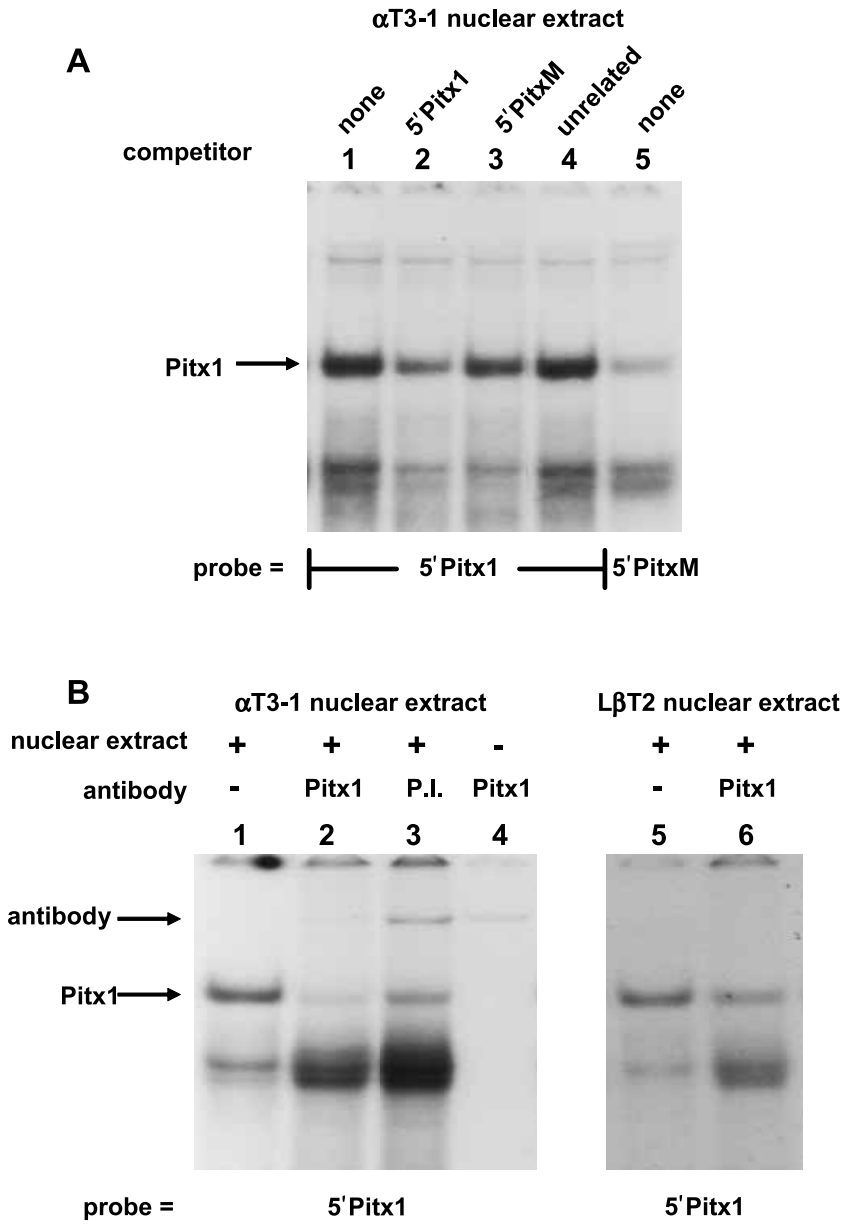

Figure 3 Endogenous Pitx1 in gonadotrope cell lines binds to the LHβ gene promoter. Nuclear extracts from αT3–1 or LβT2 gonadotrope cells were incubated with ^{32}P-labeled oligonucleotide probes and the reaction mixture run on a 5% non-denaturing polyacrylamide gel, dried, and subjected to autoradiography. (A) αT3–1 nuclear extract was added to a ^{32}P-labeled oligonucleotide probe spanning region −103/−80 of the rat LHβ gene promoter as the wild-type sequence (lanes 1–4) or with a mutation in the −101 Pitx1 (5′Pitx1) site (lane 5). Where indicated, a 500-fold excess of wild-type, 5′Pitx1-mutated, or unrelated unlabeled oligonucleotide was added to the reaction mixture (lanes 2, 3, and 4 respectively). (B) αT3–1 (lanes 1–3) or LβT2 nuclear extract (lanes 5 and 6) were added to the wild-type 5′Pitx1 probe. As indicated, a Pitx1-specific antibody or a pre-immune antibody (P.I.) was added 20 min prior to the addition of probe. In lane 4, Pitx1 antibody was added to probe without extract.

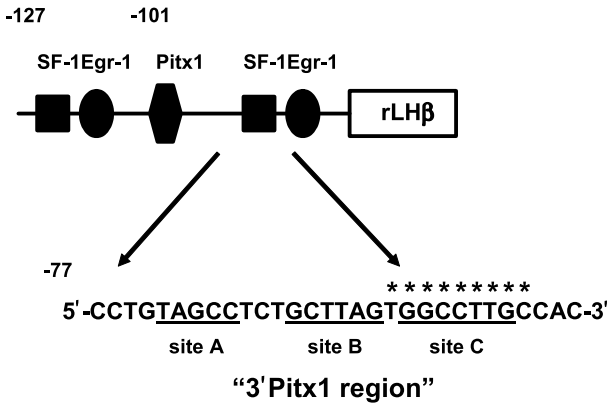

Figure 4 Schematic of the proximal rat LHβ gene promoter sequence indicating DNA-regulatory elements for SF-1, Egr-1, and Pitx1. By sequence analysis, region −77 to −48 contains three sites with homology to the consensus Pitx1 *cis*-element. These putative *cis*-elements are named 3′Pitx1 sites A, B, and C. Note that the putative 3′Pitx1C overlaps the 3′SF-1-binding site (TGGCCTTGC).

rat LHβ gene promoter. Oligonucleotide probes were generated which contained mutations in each of these Pitx1 sites individually. As shown in Fig. 6A, mutation of site B essentially eliminated the ability of Pitx1 to bind to the probe. In contrast, substantial amounts of protein binding persisted following mutation of the A or C sites. The numerical data presented are based on the cumulative results from four assays, with binding to the wild-type probe set at 100%. Of interest, binding to site A was somewhat decreased in the experiment shown in this figure, raising the possibility that it may contribute to Pitx1 effects.

The mutated oligonucleotides were also tested in competition experiments. Due to the relative subtlety of these differences by visual analysis, this experiment was repeated three times and the intensity of the complexes formed quantified. These data were pooled and expressed as a percentage of the 'no competition' control band. As shown in Fig. 6B, excess oligonucleotide containing either the wild-type sequence or a mutation in site A or site C decreased complex formation between Pitx1 protein and the wild-type 3′Pitx1 probe by approximately 30–50%. In contrast, the site B mutant oligonucleotide was unable to compete for Pitx1 binding. Taken together, these EMSA data demonstrate the ability of Pitx1 to bind to site B located at position −66/−60 in the rat LHβ gene promoter.

The 3′Pitx1 region contributes to Pitx1-induced activation of the rat LHβ gene

In order to test the functional importance of site B, additional luciferase reporter constructs were generated which contained mutations in this site, either alone or

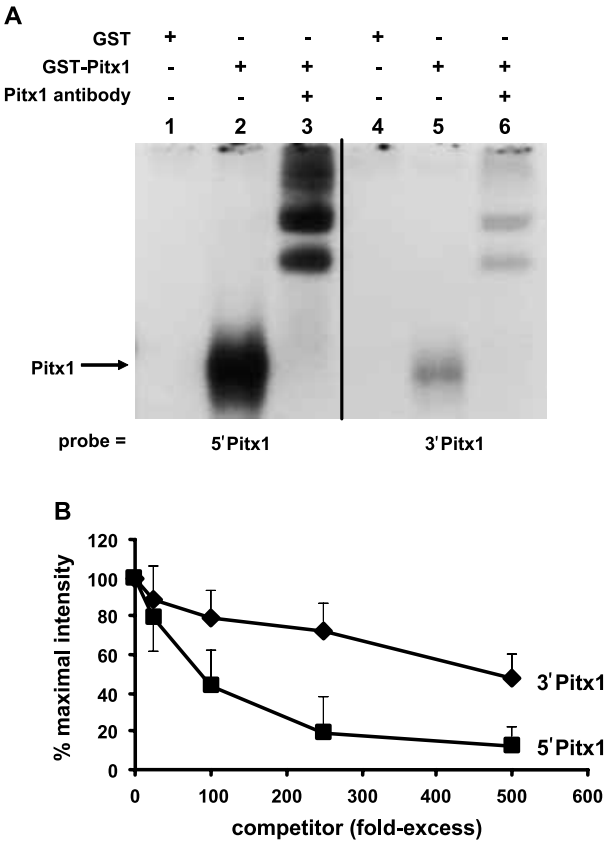

Figure 5 Pitx1 binds to the 3′Pitx1 region in the LHβ gene promoter. (A) GST–Pitx1 fusion protein was added to ^{32}P-labeled oligonucleotide probes spanning the 5′Pitx1 or 3′Pitx1 regions. A Pitx1-specific antibody was added as described in Fig. 3 (lanes 3 and 6). (B) The 3′Pitx1 probe was mixed with GST–Pitx1 and increasing amounts of 3′Pitx1 or 5′Pitx1 oligonucleotide. The intensity of the resulting complexes was quantified with the mean±S.E.M. from three experiments shown.

in conjunction with the mutated 5′Pitx1 *cis*-element. As shown in Fig. 7A, mutation of site B in the 3′Pitx1 region significantly blunted the Pitx1 response in the rat LHβ gene promoter, with complete loss of Pitx1-responsiveness in the presence of the double mutation.

Functional analysis of the role of site A was determined by 5′ truncation as already shown in Fig. 2A, with deletion of this site occurring with 5′ truncation from position −82 to −68. The Pitx1 response decreased from 3·6-fold in the presence of the complete 3′ region to 2·0-fold in the shorter construct ($P<0{\cdot}05$ for construct −82/+5 versus −68/+5), suggesting that site A may contribute to the Pitx1 response in the presence of an intact site B.

We also were interested in investigating the role of each of the Pitx1 *cis*-elements in mediating the synergistic response with SF-1 (Fig. 7B). The addition of

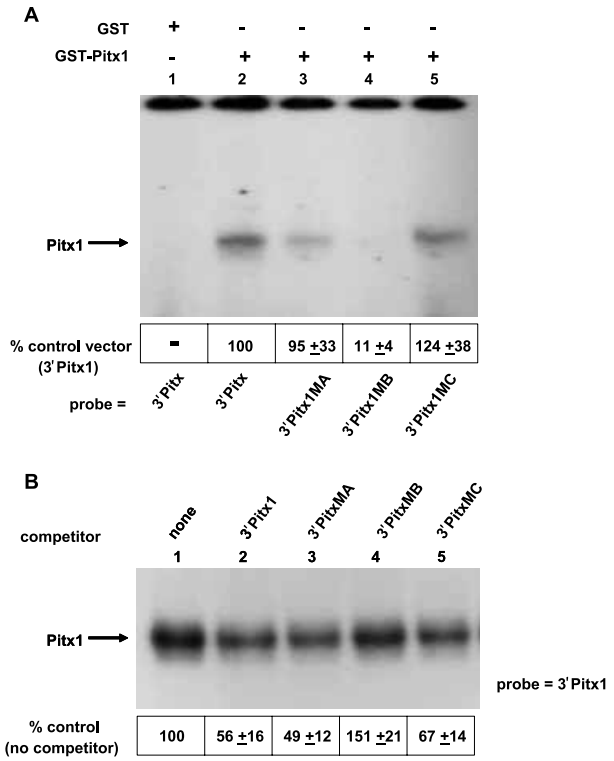

Figure 6 Pitx1 binds to site B within the 3′Pitx1 region. (A) GST–Pitx1 protein was added to ^{32}P-labeled oligonucleotide probes spanning the 3′Pitx1 regions as the wild-type sequence or with mutation in putative sites A, B, or C. The intensity of the resulting complexes was quantified and results expressed as a percentage of binding to the wild-type probe (n=4). (B) GST–Pitx1 protein was added to the 3′Pitx1 probe. 500-fold excess of wild-type or mutated 3′Pitx1 oligonucleotide was added as indicated. Results from three independent experiments were quantified and results expressed as the percentage intensity of the control band (i.e. no competitor).

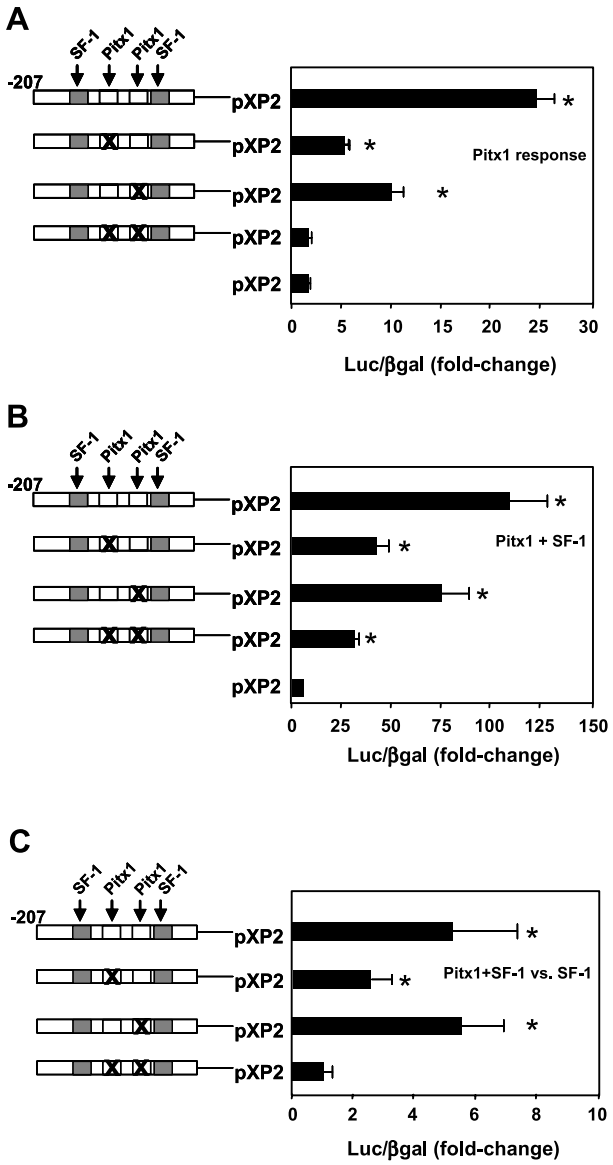

Figure 7 Effect of mutation of the Pitx1 sites on the Pitx1 response and Pitx1–SF-1 synergy. CV-1 cells were transiently transfected with the −207/+5LHβ gene promoter present as wild-type or with mutation in the 5′Pitx1 *cis*-element, 3′Pitx1B *cis*-element, or both. Cells were co-transfected with the Pitx1 and/or SF-1 expression vectors. Cell extracts were harvested and assayed as described in Fig. 1. (A) Analysis of the Pitx1 response. Results shown as promoter activity in response to the Pitx1 expression vector relative to promoter activity with addition of the control expression vector. *$P<0{\cdot}05$ versus the response in pXP2. (B) Analysis of Pitx1–SF-1 synergy. Results shown as corrected luciferase activity in response to the Pitx1 and SF-1 expression vectors relative to luciferase activity with addition of the control expression vector. *$P<0{\cdot}05$ versus the response in pXP2. (C) LHβ gene promoter activity in response to Pitx1 and SF-1 together relative to addition of SF-1 alone. *$P<0{\cdot}05$ for the response to SF-1+Pitx1 versus response to SF-1 alone.

both Pitx1 and SF-1 increased luciferase activity by over 100-fold in the wild-type rat LHβ gene promoter. In the 5′Pitx1-mutated construct, transfection of Pitx1 and SF-1 produced a response that was greater than additive for the two factors alone (5-, 17-, and 43-fold for Pitx1, SF-1, and Pitx1+SF-1, respectively), but was diminished relative to the wild-type promoter. Mutation of the 3′Pitx1 *cis*-element had minimal impact on the synergistic response.

As an alternative method for analyzing the degree of synergy in the various constructs, the results were also calculated as the response to both transcription factors relative to the response to SF-1 alone. As shown in Fig. 7C, Pitx1–SF-1 synergy was decreased markedly with mutation of the 5′Pitx1 site in this series of experiments. Pitx1 augmentation of the SF-1 response was unchanged with mutation of the 3′Pitx1 site alone; however, synergy was eliminated with mutation of both Pitx1 *cis*-elements suggesting that the 3′Pitx1 site may

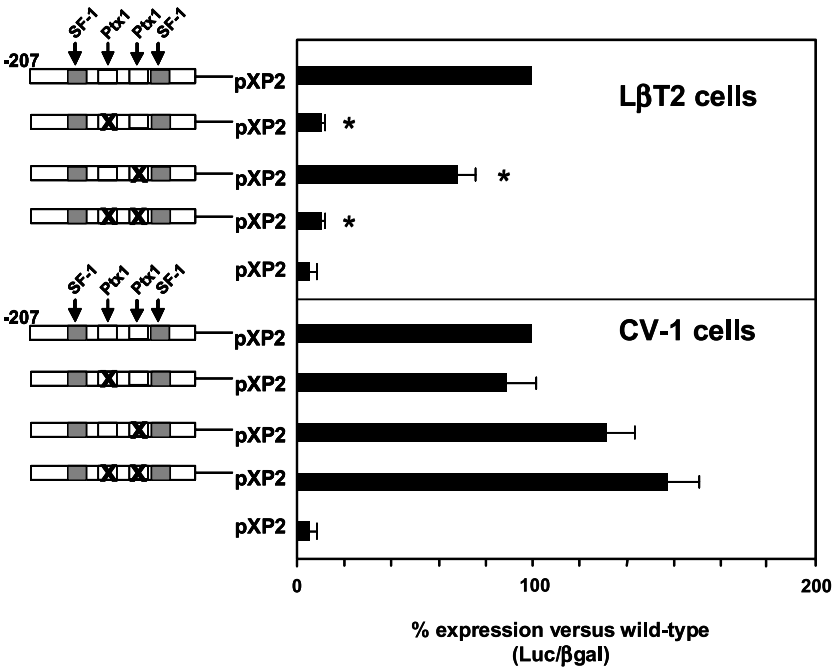

Figure 8 Mutation of the Pitx1 sites blunts rat LHβ promoter expression in the gonadotrope LβT2 cell line. LβT2 cells (upper panel) or CV-1 cells (lower panel) were transiently transfected with region −207/+5 of the rat LHβ gene promoter linked to a luciferase reporter construct, pXP2, present as the wild-type or with mutations in the putative 5′ or 3′Pitx1 sites or both. Cells were analyzed for reporter activity approximately 48 h after transfection. The data shown represent three independent experiments with expression in the wild-type construct set at 100%. *$P<0{\cdot}005$ versus wild-type for all mutant constructs.

play a minor role in the interaction between SF-1 and Pitx1.

The importance of the 3′Pitx1 site was also evaluated in the gonadotrope LβT2 cell line. As shown in Fig. 8 (upper panel), mutation of the putative 3′Pitx1 site blunted LHβ-driven luciferase activity in LβT2 cells, although not to the extent observed with mutation of the 5′ site ($P<0{\cdot}005$ versus wild-type for all mutant constructs). This result is consistent with an inability of endogenous Pitx1 to transactivate the mutated sequences. Quirk and colleagues (2001) similarly have shown that mutation of the 5′Pitx1 element blocks activation of the bovine LHβ promoter in LβT2 cells. Our interpretation of these data is further supported by equivalent analysis in Pitx1-deficient CV-1 cells in which mutation of the Pitx1 sites did not blunt basal expression (Fig. 8, lower panel).

Evidence that Pitx1 and SF-1 bind simultaneously to the 5′Pitx1 region but not to the 3′Pitx1 region

Our transfection data demonstrated that the 5′Pitx1 site is more important than the 3′Pitx1 site in conferring transcriptional synergy by Pitx1 and SF-1. We investigated whether this differential response could be explained by differences in the ability of SF-1 and Pitx1 to bind simultaneously to adjacent Pitx1 and SF-1 *cis*-elements.

As shown on the left-hand side of Fig. 9A, SF-1 and Pitx1 bound independently to a probe spanning the 5′SF-1 and 5′Pitx1 *cis*-elements (Fig. 9A, lanes 2 and 4, respectively). A third, larger complex was generated with the addition of both proteins together (Fig. 9A, lane 5), suggesting concurrent binding of SF-1 and Pitx1 to this DNA region. This result cannot distinguish direct DNA binding by both factors from DNA binding by a single factor that has formed a complex with a non-DNA binding partner. To further investigate this issue, oligonucleotide probes were utilized which contained mutations in the Pitx1 region (Fig. 9A, lanes 6–10), or in the SF-1 site (Fig. 9B, lanes 1–5), or in both sites (Fig. 9B, lanes 6–10). Mutation of the 5′Pitx1 region maintained SF-1 binding (Fig. 9A, lane 7) but nearly eliminated Pitx1 binding (Fig. 9A, lane 9), whereas mutation of the 5′SF-1 site had the converse effect (Fig. 9B, lanes 2 and 4). No evidence of a higher-order

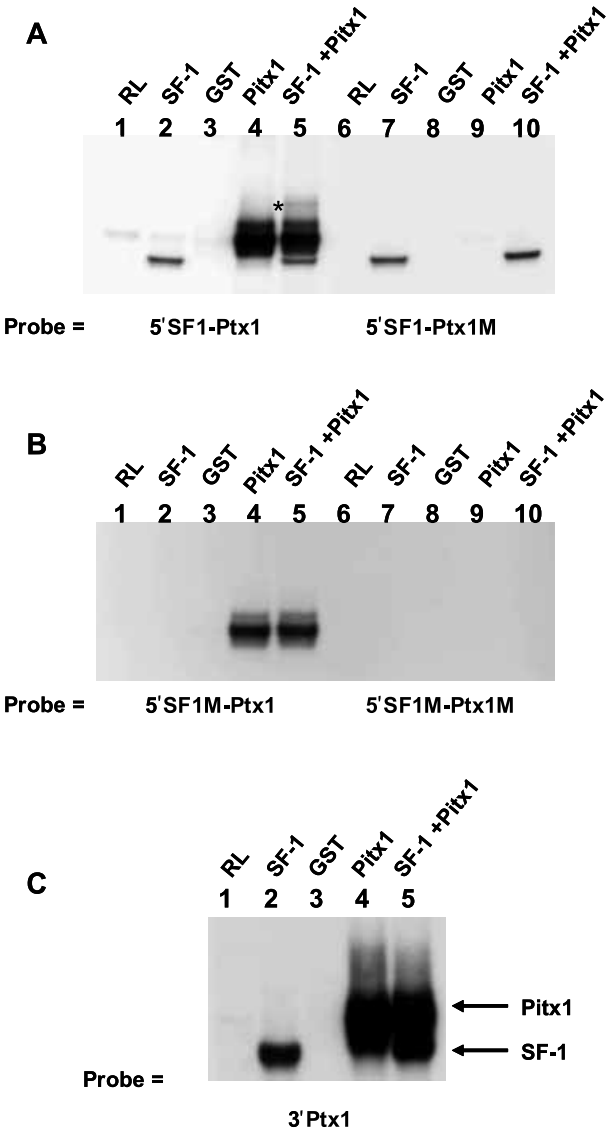

Figure 9 SF-1 and Pitx1 bind simultaneously to the 5′SF1-Pitx1 region but not to the 3′SF1-Pitx1 region. EMSA was utilized to evaluate the ability of Pitx1 and SF-1 to bind to the 5′ and 3′ SF1–Pitx1 regions. (A) Pitx1 fusion protein and *in vitro*-translated SF-1 were added alone (lanes 2 and 4) or in combination (lane 5) to an oligonucleotide probe spanning the 5′Pitx1 and 5′SF-1 sites (left panel). * Indicates complex containing both SF-1 and Pitx1. Mutation of the Pitx1 site prevented binding by Pitx1 either alone (lane 9) or in the presence of SF-1 (lane 10). (B) SF-1 (lanes 2 and 7) and Pitx1 (lanes 4 and 9) were added to a probe containing a mutation in the 5′SF-1 site (left) or in both the 5′SF-1 and 5′Pitx1 sites (right). SF-1 and Pitx1 were added in combination in lanes 5 and 10. (C) SF-1 (lane 2) and Pitx1 (lane 4) bind to a probe spanning the 3′SF-1 and 3′Pitx1 sites. No additional complexes were noted in the presence of both proteins (lane 5). RL, reticulocyte lysate.

complex was observed with either of the mutated probes, arguing against the ability of either SF-1 or Pitx1 to bind indirectly to the 5′ region.

In Fig. 9C, we investigated the ability of Pitx1 and SF-1 to bind to an oligonucleotide probe spanning the 3′SF-1 and Pitx1 sites (probe 3′Pitx1). In contrast to the results obtained with the 5′ region, we did not observe cooperative binding to the 3′ region (Fig. 9C, lane 5). In conjunction with the transfection data, these results are consistent with a model in which DNA binding at adjacent Pitx1 and SF-1 sites, as occurs in the 5′ region of the LHβ gene, is important for functional synergy between these two transcription factors.

Both Pitx1 regions contribute to synergy with Egr-1 on LHβ gene expression

Previous reports have demonstrated functional synergy between Pitx1 and Egr-1, an immediate early gene which is highly induced by GnRH. Our group and others have characterized two Egr-1 *cis*-elements in the LHβ gene promoter located near the SF-1 and Pitx1 sites (Halvorson *et al.* 1998, 1999, Dorn *et al.* 1999, Tremblay & Drouin 1999, Wolfe & Call 1999). We investigated the importance of the two Pitx1 DNA-regulatory regions in conferring functional synergy using transfection experiments that paralleled those shown for Pitx1 and SF-1 in Fig. 7. As demonstrated previously, Pitx1 and Egr-1 interact cooperatively to stimulate LHβ gene promoter activity (Fig. 10A). Next, the response to Pitx1 and Egr-1 together was evaluated in the wild-type and Pitx1 mutation constructs (Fig. 10B). Mutation of the 5′ and/or 3′ Pitx1 regions significantly decreased the Pitx1–Egr-1 response relative to the wild-type promoter sequence ($P<0{\cdot}05$), consistent with a contribution by both regions to this response. Interestingly, the response to both transcription factors exceeded the response to either transcription factor alone in the double mutant construct (Fig. 10B and C). This result suggests that Pitx1–Egr-1 synergy does not require binding by Pitx1, unlike the result observed for the Pitx1–SF-1 interaction.

Discussion

Regulation of gonadotropin biosynthesis and secretion is a critical step in the maintenance of normal reproductive function in both males and females. Investigations from a number of laboratories have begun to elucidate the complex interactions which mediate expression of the LHβ gene. *In vivo* and *in vitro* experiments have established a role for the homeobox gene product, Pitx1, in mediating LHβ promoter activity, both alone and in

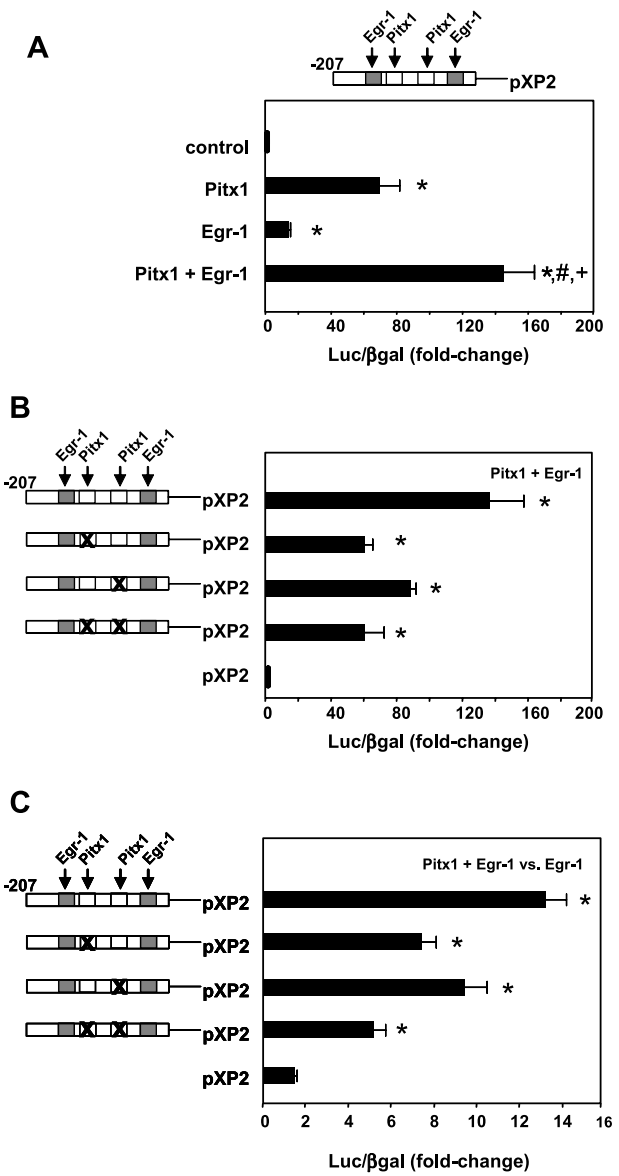

Figure 10 Effect of mutation of the Pitx1 sites on Pitx1–Egr-1 synergy. CV-1 cells were transiently transfected with the −207/+5LHβ gene promoter present as wild-type or with mutation in the 5′Pitx1 *cis*-element, 3′Pitx1B *cis*-element, or both. Cells were co-transfected with the Pitx1 and/or Egr-1 expression vectors, as indicated. (A) Response of the wild-type rat LHβ gene promoter to Pitx1, Egr-1, or both transcription factors. Promoter activity calculated as fold-change over expression in the control wells which received 'empty' expression vector. *$P<0{\cdot}001$ versus control, #$P<0{\cdot}01$ versus Pitx1 alone, +$P<0{\cdot}001$ versus Egr-1 alone. (B) Analysis of Pitx1–Egr-1 synergy. Results shown as corrected luciferase activity in the presence of both the Pitx1 and Egr-1 expression vectors relative to luciferase activity with addition of the control expression vector. *$P<0{\cdot}005$ versus response in pXP2. (C) LHβ gene promoter activity in response to Pitx1 and Egr-1 together relative to addition of Egr-1 alone. *$P<0{\cdot}005$ for the response to Pitx1+Egr-1 versus response to Egr-1 alone.

synergy with the transcription factors SF-1 and Egr-1 (Tremblay *et al.* 1998, Tremblay & Drouin 1999, Quirk *et al.* 2001). While previously believed to occur through a single Pitx1 DNA-regulatory site located at position −101 in the rat LHβ gene promoter, we now present data that identify the presence of a second functional Pitx1 *cis*-element in this gene.

Our data confirmed the ability of SF-1 and Pitx1 to act independently and in synergy to stimulate LHβ transcription in both gonadotrope (LβT2) and fibroblast (CV-1) cell lines (Fig. 1). Furthermore, we were able to demonstrate the ability of endogenous Pitx1 from gonadotrope cell lines (αT3–1 and LβT2) to bind to the previously defined Pitx1 site (Fig. 3). However, we were intrigued to observe a significant residual Pitx1 response following mutation or deletion of the −101 (5′Pitx1) *cis*-element (Fig. 2). This response did not appear to be due to a cryptic site within the reporter vector, pXP2, as it persisted in a second reporter vector, pGL3. We therefore analyzed the LHβ gene promoter sequence and identified three potential Pitx1 *cis*-elements (Fig. 4). One of these sites, which we call the 3′Pitx1B *cis*-element, was determined to bind Pitx1 on EMSA (Figs. 5 and 6). In transfection analysis, mutation of the 3′Pitx1B site significantly blunted Pitx1 responsiveness and, in conjunction with mutation of the 5′Pitx1 site, eliminated the Pitx1 response (Fig. 7A). Our data also suggested a possible role of site A within the 3′ region, based on 5′ deletion studies, although EMSA results were less conclusive. Nevertheless, it is possible that both sequences in the 3′ region act cooperatively to confer Pitx1 responses.

We also analyzed the functional interaction between SF-1 and Pitx1. Mutation of the 5′Pitx1 site markedly blunted synergistic effects by these transcription factors (Figs. 7B and C). Mutation of the 3′Pitx1B site alone did not impact the degree to which Pitx1 was able to augment the SF-1 response although mutation of this site further decreased the residual Pitx1–SF-1 synergy observed with an isolated 5′Pitx1 mutation. On EMSA, following the addition of Pitx1 and SF-1 to the 5′Pitx1–SF-1 region, we were able to detect formation of a larger complex consistent with simultaneous binding by both of these factors (Fig. 9A). In contrast, this larger complex was not detectable on the 3′ region probe (Fig. 9C). We propose that Pitx1 competes with SF-1 for binding to the 3′ region, with the proportion of SF-1 to Pitx1 binding dependent on relative expression levels, relative affinity, or possibly activation by ligand in the case of SF-1. Pitx1 bound to the 3′ region could potentially interact with SF-1 bound to the 5′ SF-1 *cis*-element, but our data suggest that the 3′Pitx1 site plays a relatively small role in providing synergy with SF-1 (Figs. 7B and C). Based on these functional and binding data, we conclude that Pitx1–SF-1 synergy is dependent on Pitx1 DNA binding in the rat LHβ gene.

It should be noted that we have been unable to definitively detect binding by endogenous Pitx1 from LβT2 nuclear extracts on the 3′ region despite the use of a variety of nuclear extraction and EMSA protocols. While we were able to detect binding to the 5′ site (Fig. 3), these data required relatively large amounts of nuclear extract and prolonged exposure times. As the 3′ region has lower affinity than the 5′ site (Fig. 5), we believe that our inability to detect endogenous Pitx1 binding to the 3′ region is due to a lack of assay sensitivity, rather than a reflection of inability of Pitx1 to bind to this region. There is precedent for the presence of low affinity, but functionally important, Pitx1 *cis*-elements in other gonadotrope-specific genes, including the GnRH receptor gene as reported by Jeong and coworkers (2004).

In our studies of the rat LHβ gene promoter, the 3′Pitx1 region contributed little to the synergistic response to Pitx1–SF-1 (Fig. 7B); however, functional interaction was lost with mutation of both putative Pitx1 regions. In contrast, mutation of the 3′ region clearly blunted the Pitx1–Egr-1 interaction, although neither site was absolutely required for synergy between these two factors (Fig. 10). Mutation of the 5′Pitx1 *cis*-element alone blunted the response to either pair of transcription factors. Thus, our data clearly suggest that the 5′ and 3′ Pitx1 regions differ in terms of Pitx1 binding affinity as well as importance for interaction with other transcription factors.

Tremblay and colleagues have evaluated Pitx1 effects on the bovine LHβ gene promoter (Tremblay *et al.* 1998, 1999). They reported that synergy between Pitx1 and SF-1 was maintained, although diminished, despite mutation in the 5′Pitx1 site. Of note, the bovine gene lacks an obvious Pitx1 consensus site in the 3′ region identified in the rat promoter. As a result, this persistent response cannot be attributed to an analogous secondary Pitx1 site in this species. Tremblay *et al.* next demonstrated direct protein–protein interaction between the C-terminus of Pitx1 and the N-terminus of SF-1 and postulated that physical interaction between these two proteins unmasks SF-1 activity by mimicking the effect of a still unidentified SF-1 ligand. They concluded that Pitx1–SF-1 synergy can occur in the absence of Pitx1 DNA binding in the bovine promoter, a molecular mechanism which does not appear to exist for Pitx1–SF-1 in the rat gene. Zakaria *et al.* (2002) have described a similar activating pathway that is independent of Pitx1 DNA binding in the rat FSHβ gene.

Precedent exists for species-specific regulation of the LHβ gene. For example, the bovine LHβ gene 5′ flanking region contains an NF-Y *cis*-element that is critical for mediating basal expression in this species, but is absent in the rat promoter. Conversely, the rat LHβ promoter sequence contains an Sp1 region which is lacking in the corresponding bovine sequence (Keri *et al.* 2000).

The presence of multiple Pitx1 *cis*-elements appears to be a common theme. For example, the salmon LHβ gene promoter contains two Pitx1 DNA-regulatory regions (neither clearly analogous to the mammalian regions): (1) a proximal *cis*-element which interacts with SF-1 and the estrogen receptor and confers a small degree of GnRH-responsiveness, and (2) a complex distal region with at least four Pitx1 sites which contribute to basal and GnRH-induced expression (Melamed *et al.* 2002). Of interest, Pitx1 is not required for GnRH expression in either the rat or bovine LHβ genes (Quirk *et al.* 2001 and data not shown).

The importance of Pitx1 for gonadotrope development and gene expression has been underscored by the generation of both Pitx1 transgenic and Pitx1-null mouse models (Szeto *et al.* 1999, Quirk *et al.* 2001). In animals null for Pitx1 expression, analysis from embryonic day 15·5 through postpartum day 0 demonstrated markedly blunted expression of LHβ, FSHβ, TSHβ and α-subunit due to both a decrease in the number of gonadotropes and thyrotropes as well as a decrease in transcript levels per cell. Quirk *et al.* (2001) generated mice harboring the bovine LHβ gene promoter upstream of a chloramphenicol acetyltransferase (CAT) reporter. CAT activity was undetectable in all lines containing a mutation in the bovine LHβ-5′Pitx1 site. These data do not formally eliminate the possibility of a second Pitx1 site; however, they clearly suggest that activation of additional DNA-regulatory elements is unable to compensate for loss of this site. As discussed above, Pitx1-mediated stimulation of LHβ gene expression also may differ between species. Therefore, it would ultimately be of interest to generate transgenic mice containing rat LHβ promoter sequences.

Elimination of Pitx1 protein expression in the knockout animals was less disruptive on pituitary function than mutation of the LHβ 5′Pitx1 site (Szeto *et al.* 1999, Quirk *et al.* 2001). These results suggest that other transcription factors may be assuming the role of Pitx1 on this promoter. As one possibility, Rosenberg & Mellon (2002) have demonstrated the presence of an as-yet-unidentified Otx-related protein present in immortalized gonadotrope cells which binds to the 5′Pitx1 region and directs expression to mature gonadotropes. If identified, it would be important to test this factor for functional activity on the rat LHβ 3′Pitx1 *cis*-element.

In conclusion, a wide array of *in vivo* and *in vitro* data point to a critical role for Pitx1 in pituitary development and gene expression. We have identified a second functional Pitx1 *cis*-element in the rat LHβ gene promoter which contributes to Pitx1 responsiveness. These results further our understanding of the molecular

mechanisms which regulate expression of this critical reproductive gene.

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