Liver receptor homologue-1 regulates gonadotrope function

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Abstract

Over the past decade, substantial advances have been made in our understanding of the transcription factors which regulate gene expression in gonadotropes. One of the most important of these factors, steroidogenic factor-1 (SF-1; NR5A1) is critical for gonadotropin and GnRH-receptor expression. Interestingly, a closely related nuclear hormone receptor, liver receptor homologue-1 (LRH-1; NR5A2) has recently been detected in the anterior pituitary gland; however, its functional significance in this tissue has not been investigated. For the experiments reported here, we hypothesized that LRH-1 plays a previously unrecognized role in gonadotrope physiology. Towards this end, we first demonstrate LRH-1 mRNA and protein expression in both primary pituitary cells and gonadotrope-derived cell lines. We next show that LRH-1 stimulates promoter activity of the GnRH-receptor and gonadotropin subunit genes. Within the *LH β* gene, this response appears to be mediated by DNA-binding and transactivation through previously characterized SF-1 *cis*-elements. To our knowledge, this is the first report demonstrating a functional role for LRH-1 in the gonadotrope population of the anterior pituitary gland.

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Introduction

Normal reproductive function requires precise quantitative and temporal regulation of gonadotropin biosynthesis by the gonadotrope subpopulation of the anterior pituitary gland. Over the past decade, studies have identified a complex array of transcription factors and associated DNA-regulatory elements which mediate basal, tissue-specific, and hormonally responsive expression of the gonadotropin subunits and the gonadotropin-releasing hormone (GnRH)-receptor gene (Barnhart & Mellon 1994, Haisenleder *et al.* 1996, Lee *et al.* 1996, Halvorson *et al.* 1998, Tremblay & Drouin 1999, Kaiser *et al.* 2000, Keri *et al.* 2000, Huang *et al.* 2001, Quirk *et al.* 2001, Norwitz *et al.* 2002, Jacobs *et al.* 2003, Jeong *et al.* 2004).

One of these transcription factors, the orphan nuclear receptor steroidogenic factor-1 (SF-1; NR5A1), has been implicated in the regulation of a wide variety of genes which play a role in steroidogenesis, sexual differentiation, and adult reproductive function (Luo *et al.* 1994, Sadovsky *et al.* 1995). Within the pituitary gland, SF-1 is selectively expressed in the gonadotrope subpopulation of the pituitary gland, as well as in the ventromedial hypothalamus, gonads, and adrenal gland (Ikeda *et al.* 1995). SF-1 binds as a monomer to a consensus sequence, known as an Ad4 or gonadotrope-specific element (GSE), which resembles a nuclear receptor half-site (Horn *et al.* 1992). Functionally important GSEs have been identified in the common α -subunit, luteinizing hormone

(LH) β -subunit and GnRH-receptor (GnRH-R) gene promoters (Halvorson *et al.* 1996, Keri & Nilson 1996, Pincas *et al.* 2001, Fowkes *et al.* 2003).

Liver receptor homologue-1 (LRH-1; NR5A2) – also known as α -fetoprotein transcription factor (FTF), CYP7A (cholesterol 7 α -hydroxylase) promoter binding factor (CPF), and human B-1 binding factor (hB1F) – is closely related to SF-1 based on similarities in amino acid sequence and protein structure. As suggested by its name, LRH-1 was initially discovered in the mouse liver and subsequently has been described as a key regulator of bile acid metabolism and cholesterol homeostasis (Goodwin *et al.* 2000, Lu *et al.* 2000). More recent studies have revealed LRH-1 expression in the adrenal gland, adipocytes, pancreas, and gut (Sirianni *et al.* 2002, Clyne *et al.* 2004). Especially, high levels of LRH-1 mRNA are present in the ovary and testis (Falender *et al.* 2003, Hinshelwood *et al.* 2003, Pezzi *et al.* 2004, Lambard *et al.* 2005). LRH-1, like SF-1, has been demonstrated to stimulate expression of genes which encode steroidogenic enzymes, including the StAR, CYP11A1 and the aromatase genes (Sirianni *et al.* 2002, Clyne *et al.* 2004). The predominant form of LRH-1 protein is approximately 64 kDa in size; however, multiple transcriptional and post-transcriptional variants have been described which result in products spanning 54–64 kDa. The expression of these variants shows both species and tissue specificity (Galarneau *et al.* 1996, Li *et al.* 1998, Goodwin *et al.* 2000, Wang *et al.* 2001, Falender *et al.* 2003, Peng *et al.* 2003, Kudo & Sutou 2006). In a recent report, LRH-1 mRNA

expression was detected in the pituitary gland; however, its function in this tissue has not been elucidated (Falender *et al.* 2003). In the studies reported here, we present evidence that LRH-1 plays a role in gonadotrope physiology, with a focus on the LH β gonadotropin gene.

Materials and methods

Reverse transcription PCR (RT-PCR)

Total RNA was isolated from cell lines, rat pituitary tissue or mouse liver using the Qiagen RNeasy Mini Kit according to the manufacturer's instructions (Qiagen). The immortalized mouse gonadotrope cell lines, L β T2 and α T3-1, were generously provided by P L Mellon (University of California, San Diego, CA, USA). Pituitary tissue samples were homogenized by trituration first through an 18-gauge and then through a 21-gauge syringe needle in RLT lysis buffer containing 10 μ l/ml β -mercaptoethanol (Sigma-Aldrich Corp.). Tissue culture cell-samples were washed with PBS and harvested by scraping in RLT lysis buffer. Cell and tissue samples were further homogenized by centrifugation through Qias shredder spin columns (Qiagen) and the resulting samples were then subjected to the RNA isolation process. The concentration of total RNA in the final samples was determined by measurement on a Bio-Rad SmartSpec 3000 spectrophotometer (Bio-Rad Laboratories).

Total RNA (1 μ g) was reverse transcribed at 37 °C for 1 h in the presence of 500 ng of random hexamers (Invitrogen) and 10 units AMV Reverse Transcriptase (Promega) in buffer containing 5 mM MgCl₂, 1 $\times$ PCR buffer, 1 mM dNTP, and 20 units RNase Out (Invitrogen). PCR was performed on 5 μ l of the resulting cDNA sample in the presence of 2 mM MgCl₂ and 2.5 units Taq polymerase (Promega). PCR conditions were as follows: 95 °C $\times$ 3 min, 35 cycles $\times$ (95 °C, 30 s; 55 °C, 30 s; 72 °C, 60 s), and 72 °C $\times$ 5 min. The product was resolved by 1% agarose gel electrophoresis. Primer pairs for mouse LRH-1 samples were 5'-GAAGCTGCTTCAAACTGC-3' (sense) and 5'-CCGTTACAGTGCTTAGTAC-3' (antisense). The rat LRH-1 primer sequences were 5'-TGAAGCTGCTTCAGAACTGC-3' (sense) and 5'-CCATTACAGTGCTTGTAGTAC-3' (antisense). These primers produce a predicted product size of 447 bp (mouse) or 448 bp (rat) which spans the ligand-binding domain.

Western blot analysis

Nuclear extracts were obtained using the NE-PER extraction reagents supplemented with protease and phosphatase inhibitors and quantified by BCA assay

(Pierce Biotechnology, Inc., Rockford, IL, USA). Proteins were separated using 12% SDS-PAGE and transferred to PVDF membrane. After overnight blocking, the membranes were incubated with the primary antibody followed by a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody. After washing, the bound secondary antibody was visualized using enhanced chemiluminescent (ECL) western blot detection reagents (Amersham Biosciences). The SF-1 antibody was obtained through Upstate Biotechnologies (Charlottesville, VA, USA). Two LRH-1/FTF antibodies were kindly provided by L Belanger (Laval University Cancer Research Center, Quebec, Canada; Galarneau *et al.* 1996). The antibody designated LRH-1 Ab1 was raised in rabbit against rat amino acid #142–156. A second LRH-1/FTF antiserum, LRH-1 Ab2 was directed against rat amino acid positions #242–560 and was unsuccessful on Western but was effective on electrophoretic mobility shift assay (EMSA). Both antibodies are cross reactive with mouse.

Transient transfection of cell lines

Green monkey kidney fibroblast (CV-1) and mouse gonadotrope-derived cells (L β T2) were maintained in monolayer culture in 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% CO₂/95% air. Cells were transfected at approximately 50–80% confluence in 12-well plates using the calcium phosphate precipitation method (CV-1 cells) or Lipofectamine Reagent (L β T2 cells; Invitrogen). CV-1 cells received 0.4 μ g/well of reporter vector and 0.1 μ g/well of expression vector or as indicated in the dose-response experiments. L β T2 cells received 0.2 μ g/well of reporter vector and 0.3 μ g/well of expression vector. Cotransfection with a pSV- 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 as previously described and for β -galactosidase activity using the Galacto-Light assay system (Applied Biosystems, Foster City, CA, USA; de Wet *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 $\pm$ S.E.M. from 3 to 7 independent experiments with each point tested in triplicate.

Plasmids used in transfection studies

The rat –207/+5 LH β , rat –2000/+1709 FSH β , and mouse –1164/+62 GnRH-R constructs consist of the

corresponding cDNA subcloned into the luciferase reporter gene, pXP2 (Nordeen 1988; constructs kindly provided by U B Kaiser, Brigham and Women's Hospital, Boston, MA, USA). The α -luciferase construct spans region $-846/+44$ of the human glycoprotein α -subunit inserted into pA3-luc luciferase expression vector (gift of J L Jameson, Northwestern University School of Medicine, Chicago, IL, USA). The sequence of the mutated LH β gene promoter constructs can be found in a previous report by Halvorson *et al.* (1998).

The LRH-1 expression vector contains 1.7 kb of the mouse LRH-1 cDNA subcloned into pcDNA3.1/Amp (Invitrogen; cDNA provided by D J Mangelsdorf, University of Texas Southwestern Medical Center, Dallas, TX, USA). The SF-1 expression vectors contain 2.1 kb of the mouse SF-1 cDNA driven by the cytomegalovirus promoter in the pcDNA3.1 expression vector (SF-1 cDNA provided by K L Parker, University of Texas Southwestern Medical Center, Dallas, TX, USA).

Electrophoretic mobility shift assay (EMSA)

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). 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 3'/GSE and 5'/GSE regions were respectively 5'-TGCTTAGTGGCCTTGCCACCCCA-3' and 5'-TCCTTTCTGACCTTGTCTGTCT-3'.

Nuclear proteins were isolated as described for Western blot analysis. Where indicated, 1 μ l of antisera was added to the protein samples 20 min prior to the addition of labeled probe (see section describing Western Blot for description of antibodies). Protein-DNA complexes were resolved on a 5% non-denaturing polyacrylamide gel in 0.5X Tris-borate-EDTA buffer and subjected to autoradiography. *In vitro* translated mouse SF-1 and LRH-1 were generated using the TNT Coupled Reticulocyte Lysate System (Promega).

Statistical analysis

Statistical calculations were performed using the SigmaStat statistical software package (SPSS Science, Chicago, IL, USA). Data were analyzed for normality followed by calculation of ANOVA or the Kruskal-Wallis ANOVA on ranks for non-parametric data. The Tukey method was utilized for *post hoc* comparison, except for experiments with different sample sizes in which case the Dunn's test was employed. Statistical significance was set at $P < 0.05$.

Results

LRH-1 mRNA is expressed in the pituitary gland and in gonadotrope cell lines

Reverse-transcription PCR was used to determine the presence of LRH-1 transcripts (Fig. 1). Bands of the appropriate size were detected in both the anterior and the posterior pituitary glands from rats (Fig. 1A), as well as the two mouse gonadotrope cell lines, α T3-1 and L β T2 (Fig. 1B). A plasmid encoding LRH-1 and mouse liver were used as positive controls (Fig. 1B). Products obtained from primary pituitary cells and the α T3 and L β T2 cell lines were confirmed to be LRH-1 by sequence analysis.

LRH-1 protein is expressed in the anterior pituitary gland and in gonadotrope cell lines

LRH-1 protein was detected in gonadotrope cell lines and the pituitary gland using the LRH-1 Ab1 antibody in Western analysis (Fig. 2). As shown in Fig. 2A, *in vitro* translated LRH-1 produced a band of the predicted size (approximately 64 kDa; lane 2). No distinct band was

Figure 1 Expression of LRH-1 mRNA in the pituitary gland and in gonadotrope cell lines as analyzed by reverse transcription polymerase chain reaction (RT-PCR). (A) Rat anterior pituitary (Ant. Pit.) and posterior pituitary (Post. Pit.) were tested in the presence (+) or absence (–) of reverse transcriptase (RT). (B) LRH-1 plasmid, mouse liver, α T3-1 cells and L β T2 cells were tested in the presence (+) or absence (–) of reverse transcriptase. M, size markers.

Figure 2 Expression of LRH-1 protein in the anterior pituitary gland and in gonadotrope cell lines by Western blot analysis. (A) LRH-1 antibody was used to detect LRH-1 protein in a reticulocyte lysate (RL) preparation of *in vitro* translated LRH-1 (lanes 1–3). Detection of *in vitro* translated SF-1 was done by an SF-1-specific antibody (lanes 4–6). LRH-1 has a predicted size of approximately 64 kDa and SF-1 has a predicted size of 54 kDa. Note the ability of the antibodies to distinguish between LRH-1 and SF-1. (B) LRH-1 immunoreactivity in the gonadotrope-derived cell lines LβT2 and αT3-1 (lanes 3 and 4) as well as in primary mouse anterior pituitary cells (mPit; lane 5).

detected in the reticulocyte lysate (RL) alone or *in vitro* translated SF-1 lanes (lanes 1 and 3), demonstrating the desired specificity of this antibody. In the right panel, an SF-1 antibody was used to demonstrate that SF-1 runs as a slightly smaller product (54 kDa) on Western blot, as would be predicted. Appropriately, this antibody detects SF-1 in an *in vitro* translated SF-1 preparation (lane 6) but does not detect any proteins in an *in vitro* LRH-1 preparation (lane 5).

The presence of endogenous LRH-1 in mouse pituitary cells and gonadotrope cell lines is shown in Fig. 2B. In order to allow clear visualization of the endogenous protein, this Western gel was exposed for a longer time than in 2A. The multiple proteins observed in the RL and LRH-1 lanes (lanes 1 and 2) were also present in Fig. 2A. The band at approximately 54 kDa in the lane 2 likely represents a post-transcriptional splice variant as previously described by other investigators. The slightly greater mobility observed for endogenous LRH-1 relative to the *in vitro* translated product is also consistent with the multiple splice variants reported for LRH-1 (Galarneau *et al.* 1996, Kudo & Sutou 2006).

LRH-1 stimulates gonadotrope-specific gene expression

Having demonstrated the presence of LRH-1 transcripts and protein in gonadotropes, we next

wished to determine whether LRH-1 modulates expression of the gonadotropin subunit genes or the *GnRH-R* gene (Fig. 3A). For these experiments, we utilized a transient transfection approach. When introduced into the fibroblast cell line, CV-1, LRH-1 significantly increased the promoter activity of the common α-, LHβ, FSHβ, and GnRH-R genes with the largest response observed with the LHβ construct (15-fold; $P < 0.001$). LRH-1 did not significantly alter expression of the corresponding empty expression vectors (data not shown).

As SF-1, which is closely related to LRH-1, is known to be an important regulator of LHβ gene expression, we compared the effects of LRH-1 and SF-1 on this gene promoter (Fig. 3B). In an attempt to provide relatively equal expression of the two proteins, we utilized SF-1 and LRH-1 cDNAs in the same expression construct (pcDNA 3.1). The addition of LRH-1 induced an 18-fold increase in LHβ gene promoter activity, while SF-1 stimulated expression by 37-fold ($P < 0.001$ for LRH-1 and SF-1 vs control; $P < 0.001$ for LRH-1 vs SF-1).

Prior studies by our group and others have identified two regions in the LHβ gene, which are critical for the ability of SF-1 to increase promoter activity of this gene. These sites have been designated gonadotrope-specific elements (5'-GSE and 3'-GSE; Halvorson *et al.* 1998). As LRH-1 has been shown to have a similar consensus DNA-binding sequence, we postulated that LRH-1 stimulation of the LHβ gene promoter may also be mediated via one or both of the GSEs (Solomon *et al.* 2005). Mutations in either of these previously described GSE sites markedly diminished LRH-1-responsiveness (Fig. 3C). Mutation of both GSE sites eliminated LRH-1-mediated stimulation to the level of the empty expression vector, pXP2. These data support a role for each of the GSE sites in mediating the LRH-1 response.

The ability of LRH-1 to stimulate LHβ promoter gene expression was confirmed in the LβT2 gonadotrope cell line (Fig. 3D). As these cells express both LRH-1 and SF-1, overexpression studies routinely demonstrate markedly diminished responses. Nevertheless, the addition of either LRH-1 or SF-1 increased LHβ gene promoter activity. The small LRH-1 effect (1.4-fold) was highly reproducible and significant at the $P < 0.001$ level. Taken as a whole, these results strongly imply that LRH-1 plays a role in mediating gonadotrope-specific gene expression.

Dose-response of LRH-1 vs SF-1 on rat LHβ gene promoter activity

A dose-response experiment was performed in CV-1 cells in order to further assess the effectiveness of LRH-1 relative to SF-1 in the stimulation of rat LHβ gene promoter activity. LRH-1 achieved a maximal 30-fold increase in promoter-reporter expression at

10–30 ng/well of plasmid (Fig. 4A), while the SF-1 vector stimulated the LHβ promoter up to 200-fold at 3 ng/well (Fig. 4B). These results suggest that LRH-1 is an effective, but less robust, activator of LHβ gene promoter activity as compared with SF-1, at least as measured in this *in vitro* system.

Combined effect of LRH-1 and SF-1 on LHβ gene promoter activity

Cotransfection experiments were conducted to determine the effect of addition of both LRH-1 and SF-1 on LHβ promoter activity. As shown in Fig. 4C, the combined stimulatory effect of SF-1 and LRH-1 on the LHβ promoter activity was generally higher than the effect of either SF-1 or LRH-1 alone. When larger amounts of LRH-1 plasmid were transfected, their combined effect increased significantly. These data suggest an additive character of SF-1 and LRH-1 effect on LHβ promoter activity.

LRH-1 binds to the LHβ gene promoter

As demonstrated in Fig. 3C, the ability of LRH-1 to functionally increase LHβ gene promoter activity was conferred by both of the GSE sites. We next wished to determine whether LRH-1 bound directly to these DNA-regulatory regions. EMSA was used to analyze the ability of *in vitro* translated LRH-1 to bind to a nucleotide probe containing the 3'GSE (Fig. 5A) or 5'GSE (Fig. 5B) elements.

As shown in Fig. 5A (lane 2), *in vitro* translated LRH-1 bound to the 3'GSE-LHβ gene promoter region. The multiple band pattern observed is consistent with reports by other investigators studying LRH-1 action in non-gonadotrope genes (Galarneau *et al.* 1996, Li *et al.* 1998, Goodwin *et al.* 2000, Wang *et al.* 2001, Peng *et al.* 2003). Of note, the major complex migrated more

Figure 3 Transcriptional regulation of gonadotrope-specific genes by LRH-1. (A) CV-1 cells were transfected with a CMV-driven LRH-1 expression vector and the gonadotrope α-subunit, LHβ-subunit, FSHβ-subunit or GnRH-R promoters linked to a luciferase reporter vector. (B) CV-1 cells were transfected with region –207/+5 of the rat LHβ gene promoter and co-transfected with expression vectors encoding either LRH-1 or SF-1. (C) CV-1 cells were transfected with the LRH-1 expression vector and reporter constructs containing the rat LHβ gene promoter as wild type or with mutations in the 3'GSE and/or 5'GSE *cis*-elements. (D) LβT2 cells were transfected with region –207/+5 of the rat LHβ gene promoter and co-transfected with expression vectors encoding either LRH-1 or SF-1. In all experiments, cells received a pSV-β-galactosidase vector to control for potential differences in transfection efficiency. Luciferase activity was corrected to β-galactosidase activity and the data expressed relative to expression in cells receiving the empty expression vector. Data represent the means ± S.E.M. of a minimum of three independent experiments. **P* < 0.001. #*P* < 0.05 versus control.

Figure 4 LRH-1 and SF-1 dose-response curves and combined effects. CV-1 cells were transfected with region $-207/+5$ of the rat LH β gene promoter and co-transfected with the indicated amount of expression vectors encoding either LRH-1 (A), SF-1 (B), or both transcription factors (C). For the experiments shown in (A) and (B), the response to the expression vector was calculated relative to the response to an equivalent amount of the empty vector alone. In (C), both treatment and control wells received a total of 33 ng plasmid, with the amount of the plasmid encoding the transcription factors indicated in the figure. Addition of an increased amount of total vector blunted the fold-response to SF-1 or LRH-1 alone, but maintained a dose-response relationship. Bars in the same group with different letters differ significantly.

slowly than the SF-1 complex (lane 6), consistent with its larger predicted size based on sequence analysis and Western data (Fig. 2). The LRH-1 specific antisera were able to block formation of these complexes (lanes 4 and 5), while the SF-1 antibody had no effect (lane 3).

As previously reported, *in vitro* translated SF-1 also bound to the 3'GSE oligonucleotide region (lane 6), producing a complex, which was supershifted by the SF-1-specific antibody (lane 7), but not by either of the LRH-1 antibodies (lanes 8 and 9). An essentially identical result was obtained using the 5'GSE region

as the probe (Fig. 5B). These results demonstrate that LRH-1 is able to bind specifically to the LH β gene promoter and that this binding is distinguishable from SF-1 binding by both migratory pattern and response to the addition of protein-specific antibodies.

LRH-1 DNA-binding competition by unlabeled oligonucleotides

In order to further confirm specificity of the LRH-1 interaction with the LH β gene promoter, unlabeled oligonucleotides containing either the wild type or a mutated 3'GSE LH β sequence were added to the EMSA mixture prior to electrophoresis (Fig. 6). Addition of excess wild type oligonucleotide nearly eliminated DNA-binding by LRH-1 (lane 2 vs lane 3), while the mutated oligonucleotide had no effect (lane 4). Similarly, only the wild type oligonucleotide blocked binding by SF-1 (lanes 6–8).

Identification of nucleotides required for LRH-1 binding to the 3' region

In order to more precisely localize the nucleotides required for LRH-1 binding to the LH β gene promoter, we generated a series of scanning mutations in the region of the 3'GSE site (Fig. 7). The sequence of these oligonucleotides is depicted in Fig. 7A. These oligonucleotides were then used to generate 32 P-labeled probes for analysis by EMSA. As shown in Fig. 7B, mutation of oligonucleotides at positions $-58/-57$ and $-56/-55$ completely eliminated LRH-1 DNA-binding, consistent with prior determination that these nucleotides form the 'core' sequence for interaction with a number of nuclear hormone receptors. Mutation at positions $-52/-51$ substantially blunted the ability of LRH-1 to bind to the oligonucleotide probe. In contrast, mutations outside of these regions did not alter intensity of the LRH-1-DNA complex. In Fig. 7C, these oligonucleotide probes were tested for ability to bind to *in vitro* translated SF-1. The intensity of complex formation mirrored that observed for LRH-1, suggesting identical target sequences for both members of the NR5A nuclear receptor family.

Endogenous LRH-1 binds to the LH β gene promoter

RT-PCR and Western analysis demonstrated the presence of LRH-1 transcripts and protein in pituitary cells and in gonadotrope cell lines (Figs 1 and 2). We next wanted to demonstrate that endogenous gonadotrope LRH-1 protein could bind to the rat LH β gene promoter (Fig. 8). EMSA was performed using 32 P-labeled oligonucleotide probes that span either the 3'GSE (lanes 1–4) or 5'GSE (lanes 5–8) LH β gene

promoter sequences. *In vitro* translated LRH-1 (lanes 2 and 6) produced three complexes as observed in earlier experiments (Figs 5–7). LβT2 nuclear extracts generated two complexes. Addition of an LRH-1 antibody blocked production of the upper band (lanes 4 and 8), demonstrating the presence of LRH-1 in this complex. The size of this complex is similar to the middle-sized

product present in *in vitro* translated LRH-1 and may represent the same splice variant.

Mutually exclusive interactions of LRH-1 and SF-1 on the LHβ gene promoter

Both LRH-1 and SF-1 are classically believed to bind DNA as monomers. Nevertheless, both of these transcription factors are also known to interact with both co-activators and co-repressors (Crawford *et al.* 1997, 1998, Suzuki *et al.* 2002, Xu *et al.* 2003, 2004, Weck & Mayo 2006). In addition, SF-1 has been shown to develop protein–protein interactions with another nuclear hormone receptor, the androgen receptor (Jorgensen & Nilson 2001). We, therefore, wanted to determine whether there was evidence for direct interaction between LRH-1 and SF-1 on the LHβ gene promoter.

EMSA was performed using a ³²P-labeled oligonucleotide probe that spans the 3′GSE-LHβ gene promoter sequence with addition of *in vitro* translated SF-1 and/or LRH-1 with total protein content adjusted by the addition of unprogrammed reticulolysate (Fig. 9). As observed previously, SF-1 produced a single complex, while LRH-1 produced three bands with the lower two bands overlapping the SF-1 complex. Addition of both LRH-1 and SF-1 generated an additive pattern; however, a higher order complex was not observed even with overexposure of the autoradiogram. Thus, LRH-1 and SF-1 appear to bind independently to the 3′GSE-LHβ *cis*-element. The same results were obtained with the oligonucleotide probe spanning the 5′GSE (data not shown).

Discussion

The orphan nuclear receptor, SF-1, has been clearly shown to be critical for the development and function of the hypothalamus, anterior pituitary gland, ovary, testes, and adrenal gland. More recent investigations have demonstrated co-expression of the closely related transcription factor, LRH-1, in the gonads and adrenal gland (Bookout *et al.* 2006). The results presented here strongly suggest that LRH-1 also co-exists with SF-1 in the gonadotrope cells of the anterior pituitary gland. Specifically, our data confirm LRH-1 mRNA expression in primary anterior pituitary cells as well as gonadotrope-derived cell lines and demonstrate, for the first time, the presence of LRH-1 protein in these cells. To our knowledge, our results are also the first to demonstrate that LRH-1 activates gonadotrope-specific gene promoter activity. In the rat *LHβ* gene, this response is achieved, at least in part, via binding to and activation of the previously characterized SF-1 *cis*-elements (GSEs).

Figure 5 LRH-1 binds to the rat LHβ gene promoter. (A) *In vitro* translated LRH-1 (lanes 2–5) and *in vitro* translated SF-1 (lanes 6–9) were added to a ³²P-labeled oligonucleotide probe that spans nucleotide region –66 to –43 of the rat LHβ gene promoter containing the 3′GSE. An SF-1 antiserum (lanes 3 and 7), LRH-1 antibody 1 (lanes 4 and 8) or LRH-1 antibody 2 (lanes 5 and 9) were added where indicated. In lanes 10–12, antibody was added to probe in the absence of protein. (B) Addition of proteins and/or antibodies to an oligonucleotide probe that spans region –134 to –113 of the rat LHβ gene promoter containing the 5′GSE. Lanes organized as in Fig. 5A. RL, reticulocyte lysate.

Figure 6 Specificity of LRH-1 binding to the wild type LHβ gene promoter sequence. *In vitro* translated LRH-1 (lanes 2–4) and SF-1 (lanes 6–8) were added to a ³²P-labeled oligonucleotide probe that contains the 3'GSE-LHβ gene promoter sequence. Excess wild type (lanes 3 and 7) or mutated (M; lanes 4 and 8) oligonucleotide was added as indicated. RL, reticulocyte lysate.

Our current study focuses on LRH-1 expression in primary anterior pituitary cells and in gonadotrope-derived cell lines. We have also detected LRH-1 mRNA expression in the somatolactotrope GH3 cell line (data not shown). As somatotropes and lactotropes comprise 50–80% and gonadotropes comprise at least 10% of the total secretory cells in the anterior pituitary, we predict that the majority of anterior pituitary cells express this protein. LRH-1 mRNA has also been identified in the posterior pituitary (Fig. 1A). It will be of interest in the future to perform co-localization studies in the rodent pituitary in order to fully define the cell types that express LRH-1.

LRH-1 transcript variants have been observed in both humans and mice, with both tissue-specific and developmentally stage-specific expression (Nitta *et al.* 1999, Gao *et al.* 2006). In the studies reported here, *in vitro* translated LRH-1 demonstrated a major product of approximately 64 kDa on Western blot, consistent with the presence of a full-length product (Fig. 2). In contrast, a faster mobility product was detected from pituitary cells and gonadotrope cell

lines, suggesting the presence of a truncation product in the pituitary.

Gao *et al.* (2006) have recently identified a truncated transcript of mouse LRH-1, termed mLRH-1v2, which may encode a protein of 57 kDa compared with the full-length protein at 64 kDa. Expressed in embryonic cells and a subset of adult tissues, this truncated transcript is directed by a promoter located downstream of the originally identified promoter (Gao *et al.* 2006). Based on this report, we designed several new primer pairs to evaluate the presence of the LRH-1 mRNA variants in the pituitary and in gonadotrope cell lines. The full-length mouse LRH-1 mRNA (mLRH-1v1) was detected in the mouse liver (positive control), but not in the mouse primary pituitary, LβT2, or αT3-1 cells (data not shown). These results indicate that the full length LRH-1 protein is probably not expressed in the pituitary, consistent with the lack of full length LRH-1 protein observed by Western blot and EMSA analyses.

We also analyzed the presence of the short form variant described by Gao *et al.* (2006) as well as the expression of the common portion which contains the

C-terminus found in both variants. The short form variant, which contains deletions in the N-terminus, was not detected in pituitary cells. In contrast, the mRNA encoding the common portion was isolated from all cell types, indicating that the C-terminus is likely translated in the pituitary.

This conclusion is supported by the antibody experiments using antibodies that were directed against the common DNA binding domain (Ab1) or the hinge region to the C-terminus (Ab2). Using pituitary cell extracts, these antibodies were able to detect protein on Western blot and supershift DNA-protein complexes from pituitary cells, supporting the presence of these regions in the pituitary-derived LRH-1 variant.

Overall, we believe that these results imply that the anterior pituitary expresses a novel LRH-1 variant that differs from the mLRH-1v2 N-terminal mRNA sequence. This proposed pituitary variant would affect the integrity of the A/B domain but not the DNA-binding or ligand-binding domains. We would predict similar, although not necessarily identical, function of this pituitary LRH-1 variant, as Nitta and coworkers have reported that human CPF (a homologue of mouse LRH-1) and CPF variant 1, which differ in only the A/B region, induced *CYP7A* promoter activity to the same magnitude, suggesting that the hinge region and ligand-binding domain of CPF is important for its function (Nitta *et al.* 1999).

The co-expression of LRH-1 and SF-1 in a single cell type suggests that these closely related factors may exert distinct physiologic actions. Knock-out models for LRH-1 and SF-1 support the concept that these factors have non-redundant functions. Unlike the SF-1 knockout animals, which are liveborn, mice lacking LRH-1 expression die at embryonic days 6.5–7.5, demonstrating a critical role for LRH-1 in embryogenesis (Pare *et al.* 2004). Conversely, the gonads and adrenal glands are absent in SF-1 knockout mice, despite the presence of LRH-1 in these tissues (Luo *et al.* 1994, Sadovsky *et al.* 1995). Of note, transgenic mice containing a mutant GSE in the bovine LHβ gene promoter fail to express LHβ subunit (Keri & Nilson 1996). Furthermore, initial characterization of SF-1 null animals did not detect gonadotropin expression. These results suggested that SF-1 and the GSE are both necessary and sufficient for gonadotropin β-subunit gene expression. However, it

Figure 7 Identification of nucleotides required for LRH-1 DNA-binding. (A) Oligonucleotide sequences used as probes for EMSA experiments. Mutated oligonucleotides are indicated by bold and underlining. (B) LRH-1 complex formation in the presence of wild type or mutated (M) oligonucleotide probes. (C) SF-1 complex formation in the presence of wild type or mutated oligonucleotide probes. In lane 3 of both B and C, the appropriate antibody was added to the reaction mixture to confirm the identity of the proteins present in the complexes.

Figure 8 Endogenous LRH-1 binds to the LHβ gene promoter. EMSA was performed using ³²P-labeled oligonucleotide probes that span either the 3' GSE (left panel) or 5' GSE (right panel) LHβ gene promoter sequences. The proteins added to this probe were unprogrammed reticulocyte lysate (RL; lanes 1 and 5), *in vitro* translated LRH-1 (lanes 2 and 6), or nuclear extracts from the gonadotrope-derived LβT2 cell line (lanes 3, 4, 7, and 8). The asterisks indicate loss of complex formation with addition of a specific LRH-1 antibody (lanes 4 and 8).

Figure 9 LRH-1 and SF-1 bind independently to the LHβ gene promoter. EMSA was performed using a ³²P-labeled oligonucleotide probe that spans the 3' GSE-LHβ gene promoter sequence. Complex formation was analyzed in the presence of reticulocyte lysate (RL; lane 1), SF-1 (lane 2), LRH-1 (lane 3), or both LRH-1 and SF-1 (lanes 4–7). An SF-1 antibody (lane 5), LRH-1 antibody (lane 6) or both antibodies (lane 7) were added to the reaction mixture as indicated.

was subsequently reported that GnRH treatment of SF-1 disrupted mice restored gonadotropin expression in 4 of 5 animals tested (Ikeda *et al.* 1995, Shinoda *et al.* 1995). These observations suggest that another factor, perhaps LRH-1, is able to support gonadotropin gene expression in the absence of SF-1 and this function is dependent on an intact GSE element, now known to bind LRH-1 in addition to SF-1.

LRH-1-mediated transcriptional activation appears to occur via previously identified SF-1 binding sites in the genes which have been studied thus far, including our studies of the gonadotropin LHβ-subunit gene (Wang *et al.* 2001, Clyne *et al.* 2002, Peng *et al.* 2003, Weck & Mayo 2006). Therefore, differential function by these two transcription factors must be achieved by mechanisms other than use of alternative *cis*-elements. Multiple possible mechanisms may be postulated. For example, LRH-1 and SF-1 may be differentially regulated by distinct DNA-regulatory elements within their own promoters or by differential binding to co-activators and co-repressors. Alternatively, *LRH-1* and *SF-1* gene expression and functional activity may differ depending on hormonal milieu, activation of intracellular signaling systems, or differences in ligand-binding.

Transcriptional regulation of LRH-1 and SF-1 gene expression has been shown to be mediated via differing *cis*-elements. LRH-1 is regulated via three GATA elements, as well as DNA-regulatory regions recognized by pancreatic-duodenal homeobox 1 and Nkx homeo-domain proteins (Pare *et al.* 2001, Annicotte *et al.* 2003). In contrast, the SF-1 gene promoter contains a functional E box, CCAAT box and Sp1/Sp3 sites (Woodson *et al.* 1997, Scherrer *et al.* 2002). This disparity in regulatory elements within the LRH-1 and SF-1 gene promoters may allow for both tissue-specific expression as well as differing levels of expression within single cells.

LRH-1 and SF-1 transcriptional activity may be differentially affected by the presence of various coactivators and corepressors within the nucleus. The anterior pituitary, for example, expresses the inhibitory cofactors DAX-1 (dosage-sensitive sex reversal adrenal hypoplasia congenita critical region on the X, gene 1) and small heterodimer partner (SHP; Ikeda *et al.* 1996). SHP mRNA has been detected in anterior pituitary samples (data not shown). DAX-1 inhibits both SF-1 and LRH-1 transcriptional activity, while SHP is believed to be specific to LRH-1, thus providing another mechanism for divergent function.

SF-1 has been shown to activate LHβ gene promoter activity alone and in synergy with other transcription factors, including pituitary homeobox 1 (Pitx1) and early growth response protein 1 (Egr-1; Halvorson *et al.* 1998, Tremblay & Drouin 1999, Kaiser *et al.* 2000, Quirk *et al.* 2001). Pitx1 is likely to be important to pituitary

development, while Egr-1 is highly regulated by GnRH and by both the protein kinase C and protein kinase A second messenger signaling systems. Currently, it is not known whether LRH-1 interacts with additional transcription factors within the pituitary, although this will be an important line of study for future investigations.

Although both SF-1 and LRH-1 have been categorized classically as 'orphan' nuclear receptors, it has recently been demonstrated that phosphatidyl inositols are likely ligands for mouse and human LRH-1 (Krylova *et al.* 2005). Further, characterization of the specific biochemical subtypes which bind to these receptors is lacking. Interestingly, perhaps due to a mutation at amino acid position 440, mouse LRH-1 does not require ligand for activity. Thus, subtle differences in ligand or in the requirement for ligand could allow for yet another level of differential function.

Phosphorylation of either LRH-1 or SF-1 alters the functional activity of these nuclear receptors. SF-1 has been shown to be phosphorylated by both the mitogen activated protein (MAP) kinase and protein kinase A systems (Hammer *et al.* 1999, Desclozeaux *et al.* 2002, Fowkes *et al.* 2003). Lee *et al.* (2006) likewise demonstrated phosphorylation of LRH-1 by MAP kinase. Interestingly, they also observed the presence of extracellular signal-regulated kinases (ERK) phosphorylation sites in LRH-1 that were conserved across species but lacking in SF-1 gene sequence.

Studies in the ovary have demonstrated divergent hormonal regulation of LRH-1 and SF-1. SF-1 mRNA levels in granulosa cells are markedly induced by treatment with either estradiol or FSH. In contrast, LRH-1 is responsive only to FSH treatment (Falender *et al.* 2003).

In a recent report, Weck & Mayo (2006) provide perhaps the strongest indication that LRH-1 and SF-1 can have distinct functions despite interaction with a common *cis*-element. Their data suggest that hormonal or pharmacologic activation of intracellular signaling pathways differentially alter both DNA-binding affinity and transactivation efficacy of LRH-1 and SF-1. Specifically, they demonstrate that forskolin treatment leads to replacement of SF-1 by LRH-1 on the inhibin α -subunit gene in granulosa cells. Thus, the forskolin-mediated increase in α -inhibin promoter activity is achieved through a switch in NR5A DNA-binding in conjunction with recruitment of cAMP response element binding protein. It is possible that variations in physiologic status result in alternative recruitment of LRH-1 or SF-1 to the GSE sites in the *LH β* gene.

Based on our data, SF-1 is likely more potent than LRH-1 in activating basal *LH β* gene promoter activity (Fig. 3D). As these two factors bind the promoter independently as monomers (Fig. 9), we had predicted that the co-expression of high levels of LRH-1, the weaker factor, might blunt the SF-1 response.

Unexpectedly, the addition of both factors was mildly additive (Fig. 4C). This result suggests the presence of a complex recruitment pattern for LRH-1 relative to SF-1 on the *LH β* gene promoter, as described for the inhibin- α subunit.

In summary, our data demonstrate the presence of *LRH-1* gene expression in the gonadotrope subpopulation of the anterior pituitary gland. Furthermore, our results localize LRH-1-mediated stimulation of *LH β* gene promoter activity to the previously described SF-1 *cis*-elements. These data strongly support a role for LRH-1 in the regulation of gonadotrope function. We propose that LRH-1 and SF-1 may provide both overlapping and distinct functions in the anterior pituitary gland.

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