The cAMP signaling system regulates LH β gene expression: roles of early growth response protein-1, SP1 and steroidogenic factor-1

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Abstract

Expression of the gonadotropin genes has been shown to be modulated by pharmacological or physiological activators of both the protein kinase C (PKC) and the cAMP second messenger signaling pathways. Over the past few years, a substantial amount of progress has been made in the identification and characterization of the transcription factors and cognate cis-elements which mediate the PKC response in the LH β -subunit (LH β) gene. In contrast, little is known regarding the molecular mechanisms which mediate cAMP-mediated regulation of this gene. Using pituitary cell lines, we now demonstrate that rat LH β gene promoter activity is stimulated following activation of the cAMP system by the adenylate cyclase activating agent, forskolin, or by the peptide, pituitary adenylate cyclase-activating peptide. The forskolin response was eliminated with mutation of a previously identified 3' *cis*-acting element for the early growth response protein-1 (Egr-1) when evaluated in the context of region $-207/+5$ of the LH β gene. Activation of the cAMP system increased Egr-1 gene promoter activity, Egr-1 protein levels and Egr-1 binding to the LH β gene promoter, supporting the role of this transcription factor in mediating the cAMP response. Analysis of a longer LH β promoter construct ($-797/+5$) revealed additional contribution by upstream Sp1 DNA-regulatory regions. Of interest, forskolin-induced stimulation of LH β gene promoter activity was observed to increase synergistically with introduction of the transcription factor, steroidogenic factor-1 (SF-1). Although SF-1 is a critical mediator of the cAMP response in other genes, mutation of the SF-1 DNA-binding sites in the rat LH β gene did not alter the forskolin response nor did forskolin increase SF-1 protein levels in a gonadotrope cell line. In a further set of experiments, it was determined that forskolin-responsiveness was maintained following mutation of the previously defined homeobox-binding element at position -100 . We conclude that both Egr-1 and Sp1 contribute to cAMP-dependent transcription of the rat LH β gene promoter. While SF-1 does not act independently to mediate the cAMP/PKA response, SF-1 is important for magnification of this response.

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Introduction

The pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are critical mediators of sexual development and adult reproductive function. LH and FSH consist of a common α -subunit linked non-covalently to one of two unique β -subunits (LH β and FSH β respectively). As would be predicted by their central role in reproductive physiology, expression of the gonadotropin genes is tightly regulated at both the

biosynthetic and secretory levels through the complex interaction of hypothalamic, pituitary and gonadally derived factors. These factors, in turn, activate an array of intracellular signaling systems. The hypothalamic factor gonadotropin-releasing hormone (GnRH) has been widely recognized to alter gonadotropin biosynthesis via activation of both the protein kinase C (PKC) and calcium pathways (Stojilkovic *et al.* 1994, Garrel *et al.* 1997, Saunders *et al.* 1998, Weck *et al.* 1998). Recent evidence suggests that GnRH may also stimulate

Figure 1 Schematic of identified and putative DNA-regulatory elements in the rat LH β gene promoter. Sp1=Sp1; SF-1=steroidogenic factor-1; Egr-1=early growth response protein-1; Ptx1=pituitary homeobox 1; Otx=orthodenticle-related homeobox (Ingraham *et al.* 1994, Halvorson *et al.* 1996, 1998, Kaiser *et al.* 1998, Tremblay *et al.* 1998, Rosenberg & Mellon 2002).

the cAMP/protein kinase A (PKA) signaling system 3 (Stanislaus *et al.* 1994, Liu *et al.* 2002). Gonadotropin gene expression is further modulated by additional hypothalamic peptides, including pituitary adenylate-cyclase activating peptide (PACAP). PACAP is a member of the vasoactive intestinal peptide/secretin/glucagon family of peptides. As its name suggests, PACAP activates the cAMP/PKA system by binding to specific cell-surface G-protein coupled receptors (Miyata *et al.* 1989, Schomerus *et al.* 1994, Tsujii *et al.* 1994).

A number of studies have pointed towards a role for the cAMP/PKA system in modulating expression of the LH β gene. In early investigations, pharmacological activation of the adenylate cyclase system was shown to increase steady-state LH β mRNA levels (Starzec *et al.* 1986, 1989). This cAMP-induced increase was blocked by the transcriptional inhibitor, actinomycin D, consistent with an effect of this signaling system on LH β gene promoter activity (Park *et al.* 1997). In subsequent experiments, which utilized the somatolactotrope GH $_3$ cell line, LH β gene promoter activity was reported to be increased by cell-permeable cAMP analogues and by the adenylate-cyclase activating agent, forskolin; however, these observations were not confirmed in gonadotrope cells (Clayton *et al.* 1991, Saunders *et al.* 1998). Together, these results strongly suggest the presence of a cAMP-inducible regulatory element(s) in the LH β gene promoter; however, no subsequent progress has been made in identifying this site(s). This lack of progress contrasts with the advances achieved in the characterization of the transcription factors which are critical for basal and GnRH-stimulated expression of the LH β gene in a variety of species (Fig. 1). The DNA-binding sites for three of these transcription factors, steroidogenic factor-1 (SF-1), early growth response protein-1 (Egr-1) and Sp1, form a tripartite response element in the rat LH β gene promoter which confers

GnRH-responsiveness (Kaiser *et al.* 2000). Interestingly, each of these transcription factors has been implicated in mediating cAMP-responsiveness in other genes.

Egr-1, also known as zif/268, Krox-24 and NGFI-A, is a member of the immediate early response gene family whose members are highly regulated by a variety of growth and differentiation factors (Christy *et al.* 1988, Cao *et al.* 1990). Two groups have demonstrated that targeted disruption of the Egr-1 gene in mice results in specific loss of LH β gene expression with maintenance of normal FSH β gene expression (Lee *et al.* 1996, Topilko *et al.* 1997). Experiments by our laboratory and others have demonstrated the ability of Egr-1 to transactivate the LH β gene promoter alone and in synergy with SF-1 via two Egr-1 binding sites, which form DNA-regulatory pairs with the SF-1 *cis*-elements, also known as the gonadotrope-specific elements (GSEs) (Lee *et al.* 1996, Halvorson *et al.* 1998, Wolfe & Call 1999). Egr-1 gene expression is markedly increased by GnRH or phorbol ester treatment of pituitary cell lines, consistent with a role for Egr-1 in mediating PKC-induced stimulation of LH β gene expression (Dorn *et al.* 1999, Halvorson *et al.* 1999, Wolfe & Call 1999). Egr-1 gene expression may also be increased by activation of the cAMP/PKA signaling system as suggested by studies in the ovary, as well as non-reproductive systems (Bernal-Mizrachi *et al.* 2000, Espey *et al.* 2000, Tai *et al.* 2001).

The transcription factor, Sp1, likewise has been implicated in both basal and GnRH-stimulated expression of the LH β gene through action at its two binding sites located 5' to the SF-1 and Egr-1 *cis*-acting elements (Kaiser *et al.* 1998a, Weck *et al.* 2000). Sp1 binds to GC-rich sequences, which are similar to, but distinct from, the consensus Egr-1 *cis*-element (Berg 1992). Constitutively expressed in a wide range of cell types, Sp1 is best known as a

regulator of basal gene transcription; however, cAMP-induced transcription of the *CYP11A* gene (P450 scc) has been attributed to the presence of an Sp1 binding site in addition to a GSE (Begeot *et al.* 1993, Venepally & Waterman 1995, Liu & Simpson 1997). It is not known if Sp1 plays a similar role in mediating the cAMP/PKA response in the LH β gene.

SF-1, also known as NR5A1, also has been shown to be critical for gonadotropin gene expression. An orphan member of the nuclear hormone receptor superfamily, SF-1/NR5A1 is expressed in the gonadotrope subpopulation of the anterior pituitary gland as well as in the gonads and adrenal gland (Ingraham *et al.* 1994, Luo *et al.* 1994). The SF-1 DNA-regulatory region, or GSE, resembles a nuclear hormone receptor half-site and is present as two copies in the LH β gene promoter (Barnhart & Mellon 1994, Halvorson *et al.* 1998, Wolfe 1999). SF-1 has been implicated in mediating cAMP/PKA responses in a wide variety of gonadal and adrenal genes, including the *CYP19* (aromatase), *CYP11A* (P450 scc), *CYP17* and StAR genes (Lynch *et al.* 1993, Clemens *et al.* 1994, Michael *et al.* 1995, Zhang & Mellon 1996, Carlone & Richards 1997, Chau *et al.* 1997, Liu & Simpson 1997, Jacob & Lund 1998, Sandhoff *et al.* 1998). Thus, SF-1, Egr-1 and Sp1 potentially may all play a role in mediating cAMP-induced expression of the LH β gene.

The homeobox *cis*-element located at position -100 in the LH β gene promoter also deserves consideration as a potential modulator of cAMP-induced LH β gene transcription. This DNA-regulatory region has been shown to bind members of the Ptx (pituitary homeobox) and Otx (orthodenticle-related homeobox) families of homeobox proteins (Tremblay *et al.* 1998, Rosenberg & Mellon 2002). Ptx1 is required for normal anterior pituitary development as well as for the expression of a broad array of pituitary-specific genes (Lamonerie *et al.* 1996, Lanctot *et al.* 1997, Tremblay & Drouin 1999, Quirk *et al.* 2001). Ptx1 acts in synergy with Egr-1 and SF-1 to generate GnRH-induced transcriptional activity of the LH β gene; however, this effect is thought to be the result of Ptx1 interaction with modified transcription factors, particularly Egr-1, rather than through alteration of Ptx1 gene expression itself (Tremblay & Drouin 1999). LH β gene expression also has been demonstrated to be regulated by

Otx1 and an Otx-related factor may be required for gonadotrope maturation (Acampora *et al.* 1998, Tremblay *et al.* 1998, 1999, Rosenberg & Mellon 2002). Thus, while clearly critical for gonadotrope function, it is unknown whether these homeobox proteins are able to directly mediate hormonal responsiveness.

In summary, substantial progress has been made in the identification of basal and GnRH/PKC-responsive elements in the LH β gene promoter. Limited data from prior studies have suggested that the cAMP/PKA system may also mediate LH β gene expression; however, this observation has not been evaluated at the molecular level. In the present study, we demonstrate that activation of the cAMP/PKA system through various methods does, in fact, increase rat LH β gene promoter activity in a gonadotrope-derived cell line. We then investigate the transcriptional mechanisms which mediate this response with a focus on the previously identified LH β DNA-regulatory elements for Egr-1, Sp1, SF-1 and Ptx1/Otx.

Materials and methods

Electrophoretic mobility shift assay (EMSA) analysis

The nucleotide sequence of the rat LH β gene promoter is based on sequencing data available at GenBank accession number AF020505, which differs slightly from that of Jameson *et al.* (1984). Probes were created by T4 polynucleotide kinase end-labeling with [γ -³²P]ATP followed by purification over a NICK column (Pharmacia Biotech, Uppsala, Sweden). A double-stranded oligonucleotide corresponding to region -67/-35 of the rat LH β gene promoter was used to detect Egr-1 DNA-binding (Halvorson *et al.* 1998, 1999).

In vitro-translated Egr-1 was generated from 3.2 kb of the mouse Egr-1 cDNA (provided by D Nathans, Johns Hopkins University, Baltimore, MD) using the TNT Coupled Reticulocyte Lysate System (Promega, Madison, WI, USA). The resultant product was determined to be of appropriate size by comparison with [³⁵S]methionine-labeled protein markers using SDS-PAGE. The method of Andrews & Faller (1991) was used to prepare crude nuclear extracts from the mouse gonadotrope-derived cell line,

α T3–1, or the rat somatolactotrope cell line, GH₃, following treatment with vehicle or forskolin for 1 h. Protein samples were incubated with 50 000 c.p.m. of oligonucleotide probe in DNA-binding buffer (20 mM Hepes (pH 7.9), 60 mM KCl, 5 mM MgCl₂, 10 mM phenylmethylsulfonyl-fluoride, 10 mM dithiothreitol, 1 mg/ml BSA and 5% (v/v) glycerol) for 30 min on ice. Where indicated, 1 ml Egr-1 polyclonal antisera (sc-110; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was added 30 min following the addition of probe and the incubation continued for 2 h. Protein–DNA complexes were resolved on a 5% non-denaturing polyacrylamide gel in 0.5 × Tris–borate–EDTA buffer and subjected to autoradiography.

Plasmids used in transfection studies

The largest LH β reporter construct used for these studies contained 794 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 (Nordeen 1988). Deletions in this construct were created by subcloning PCR products containing the LH β promoter sequences into the pXP2 vector using BamHI/HindIII sites which were introduced by the primers (Kaiser *et al.* 1998b). Mutations were introduced into the LH β promoter region using the Transformer Site-Directed Mutagenesis Kit (Clontech Laboratories, Inc., Palo Alto, CA, USA). The SF-1, Egr-1 and Sp1, and Otx/Ptx mutations have been described previously and are known to eliminate DNA binding on EMSA (Kaiser *et al.* 1998a, Halvorson *et al.* 1999, Rosenberg & Mellon 2002).

To create GSE4-GH50, an oligonucleotide was designed containing four copies of the 5′-GSE site flanked by BamHI/BglII restriction sites (sense strand: 5′-GATCCTTTTCTGACCTTGTCTGTCTCGCCTCTGACCTTGTCTGTA-3′ present as a tandem repeat). This oligonucleotide was inserted upstream of the minimal growth hormone promoter, GH50, in the pXP1 luciferase reporter plasmid (Nordeen 1988, Suen *et al.* 1994). All reporter constructs were confirmed by dideoxy-sequencing.

The SF-1 expression vector contains 2.1 kb of the mouse SF-1 cDNA driven by cytomegalovirus (CMV) promoter sequences in the vector, pCMV5 (provided by K L Parker, Southwestern University

School of Medicine, Dallas, TX) (Lala *et al.* 1992). The Δ LBD-SF-1 expression vector lacks the ligand-binding domain of SF-1 and was created by excising the SalI–SalI region of the pCMV5-SF-1 vector. The CMV expression vectors encoding the wild-type and constitutively active G-protein α -subunit (G α S) protein were provided by R Iyengar, Mount Sinai School of Medicine, New York, NY (Chen & Iyengar 1994). The Egr-1 expression vector was created by cloning 3.2 kb of the mouse Egr-1 cDNA into pCMV5 at BamHI and HindIII restriction sites (Egr-1 cDNA provided by D Nathans, Johns Hopkins University, Baltimore, MD) (Christy *et al.* 1988). The Egr-luc reporter construct contains 1.2 kb of the mouse Egr-1 gene promoter sequence cloned into the SalI site of pXP2 (Egr-1 5′-flanking sequence provided by V Sukhatme, Harvard Medical School, Boston, MA) (Sukhatme *et al.* 1987). The SF-1-luc expression vector contains 1885 bp upstream of the mouse SF-1 transcriptional start site in the vector pLKS β -LUC and was kindly supplied by Y Sadosky, Washington University, St Louis, MO (Woodson *et al.* 1997).

Transfection experiments

Rat somatolactotrope GH₃ cells or mouse gonadotrope-derived L β T2 cells were cultured to 50–70% confluence in low glucose (GH₃) or high glucose (L β T2) Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA). The L β T2 cell line was generously provided by P Mellon (University of California, San Diego, CA, USA) (Turgeon *et al.* 1996). For the GH₃ cell line, approximately 5×10^6 cells were suspended in 0.4 ml Dulbecco's PBS plus 5 mM glucose with the DNA to be transfected. The cells received a single electrical pulse of 240 V at a total capacitance of 1000 mF using an Invitrogen Electroporator II apparatus (Invitrogen). GH₃ cells received 1.5 μ g/well of the reporter constructs. As indicated for the GH₃ cell line, cells also received 1 μ g/well of the SF-1 expression vector or an equivalent amount of the appropriate 'empty' expression vector. For the L β T2 cell line, cells growing in 3.5 cm tissue culture wells were transfected with reporter plasmids (1.5 μ g/well) using the calcium phosphate precipitation method. Co-transfection with an RSV- β -galactosidase plasmid (1 μ g/well) allowed

correction for differences in transfection efficiency between wells in all experiments.

Cells were treated with vehicle, forskolin (2.5 μ M) (Sigma, St Louis, MO, USA), or PACAP-38 (10 nM) (Calbiochem, La Jolla, CA, USA), for 4–6 h starting approximately 40 h following transfection. In a subset of experiments, cells were treated with the selective PKA inhibitor, H-89 (10 μ M), or with the PKC inhibitor, GF 109203X (5 μ M), starting 1 h prior to DMSO or forskolin treatment (inhibitors obtained through LC Laboratories, Woburn, MA, USA). Cells were then harvested and the cell extracts analyzed for luciferase and β -galactosidase activity (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 means $\pm$ S.E.M. from three to ten independent experiments.

Western blot analysis

Nuclear extracts were obtained from L β T2 cells as described by Tremblay *et al.* (1998). Protein concentration were determined using the BCA protein assay reagent (Pierce Chemical, Rockford, IL, USA). *In vitro*-translated Egr-1 and SF-1 were generated using the TNT Coupled Reticulocyte Lysate System (Promega) as described for EMSA. Nuclear proteins (5 μ g) were resolved on a 10% SDS-PAGE in 25 mM Tris/250 mM glycine (TG) buffer. The protein was subsequently transferred onto 0.2 μ M Protran nitrocellulose (Schleicher & Schuell, Keene, NH, USA) in TG buffer containing 20% methanol. The membrane was blocked overnight at 4 °C in 5% Carnation non-fat dry milk in TBS (10 mM Tris-HCl, 0.9% NaCl, pH 7.5) and then washed with TBS-T (TBS, 0.05% Tween-20). The blot was incubated overnight at 4 °C in 5% milk-TBS with a polyclonal antibody directed against SF-1 (Upstate Biotechnology, Inc., Lake Placid, NY, USA) or Egr-1 (Santa Cruz Biotechnology), diluted to 1:1000 or 1:8000 respectively. After washing with TBS-T, the blot was then incubated at room temperature for 1 h in 5% milk in TBS-T containing donkey anti-rabbit IgG-horseradish peroxidase diluted 1:1000 (Santa Cruz Biotechnology). The blot was washed with TBS-T and then TBS and developed using Western Blotting Luminol Reagent according

to the manufacturer's instructions (Santa Cruz Biotechnology).

Statistical analysis

ANOVA followed by comparisons with Student's *t*-test was used to assess whether promoter activity was statistically different between the indicated groups. Statistical significance was set at the $P < 0.05$ level.

Results

Activation of the cAMP/PKA signaling system stimulates LH β gene expression in two pituitary cell lines

Transfection experiments were performed in two pituitary cell lines, a gonadotrope-derived cell line (L β T2) and a somatolactotropic cell line (GH $_3$). L β T2 cells were transiently transfected with a reporter construct containing region -794/+5 of the rat LH β gene promoter. Cells were treated with PACAP, a known physiological activator of the cAMP/PKA system in gonadotrope cells. As shown in Fig. 2A, PACAP treatment modestly, but significantly, increased LH β gene promoter activity relative to control wells (2-fold). Although PACAP is best known for activation of the cAMP/PKA system, it also has been shown to stimulate the phospholipase C (PLC) and calcium pathways (Schomerus *et al.* 1994, Hezareh *et al.* 1996, Pisegna & Wank 1996, Bresson-Bepoldin *et al.* 1998). Therefore, in order to more specifically investigate the cAMP system, cells were treated with forskolin, a pharmacological activator of adenylate cyclase. As observed with PACAP treatment, luciferase activity was stimulated with the addition of forskolin (1.8-fold).

The ability of forskolin to augment LH β gene promoter activity was also investigated in GH $_3$ cells. GH $_3$ cells have been used extensively to study gonadotropin gene regulation and have been found to provide qualitatively accurate observations with regards to both basal and hormonally regulated LH β gene expression (Kim *et al.* 1990, Clayton *et al.* 1991, Kuphal *et al.* 1994, Stanislaus *et al.* 1994, Saunders *et al.* 1998, Pinter *et al.* 1999). This cell line lacks endogenous SF-1, thereby permitting independent analysis of the effects of signaling pathways and SF-1 (Kaiser *et al.* 1994, Halvorson *et al.* 1998).

As shown in Fig. 2B, LH β gene promoter activity was significantly increased with forskolin treatment or in the presence of a constitutively active mutant form of the G α S protein (3- and 2-fold respectively). Co-transfection with the CMV-driven SF-1 expression vector resulted in a 15-fold increase in luciferase activity, consistent with previously published results (Halvorson *et al.* 1998). Of interest, LH β promoter activity increased by nearly 50-fold in the presence of SF-1 and either

forskolin or the mutant G α S, demonstrating synergy between SF-1 and cAMP signaling.

We next used pharmacological inhibitors in order to formally test the specificity of forskolin in our system. As shown in Fig. 2C, neither the PKA blocking agent, H-89, nor the PKC inhibitor, GF 109203X, altered basal LH β -driven luciferase activity. However, the forskolin-stimulated increase in LH β promoter activity was significantly blunted by H-89, but not by GF 109203X, consistent with a specific effect of forskolin on the cAMP/PKA activity.

The LH β gene promoter contains two regions which contribute to the cAMP/PKA response

Sequential 5'-deletion constructs of the rat LH β gene promoter were tested for forskolin-responsiveness. This analysis identified two regions which contribute to the forskolin response, the first

Figure 2 The cAMP/PKA system acts both alone and in synergy with SF-1 to increase LH β gene promoter activity. (A) L β T2 gonadotrope cells were transiently transfected with -794/+5 rat LH β gene promoter linked to a luciferase reporter construct, pXP2. Cells were treated for 4–6 h with PACAP-38 (10 nM), forskolin (2.5 μ M) or the appropriate vehicle and harvested 48 h following transfection. Results are shown as the means $\pm$ S.E.M. of eight experiments. * P <0.01 vs control. (B) GH $_3$ cells were transiently transfected with region -207/+5 of the rat LH β gene promoter linked to a luciferase reporter construct, pXP2. A subset of cells also received CMV-driven expression vectors encoding SF-1 and/or a constitutively active G α S protein. Control wells received equivalent amounts of 'empty' expression vector. All cells were co-transfected with an RSV- β -galactosidase vector. Approximately 40 h after transfection, cells were treated for 4–6 h with vehicle or with the adenylate cyclase activating agent, forskolin. Luciferase activity was normalized to β -galactosidase activity and promoter activity was calculated as fold-change over expression in the control wells. Results are shown as the means $\pm$ S.E.M. of four independent experiments. * P <0.01 vs control; ** P <0.05 vs SF-1 alone. (C) A luciferase reporter construct containing region -797/+5 of the rat LH β gene promoter was transiently transfected into GH $_3$ cells, followed by treatment with vehicle or 2.5 μ M forskolin for 4–6 h starting 40 h after transfection. Where indicated, cells received the PKA inhibitor, H-89, or the PKC blocking agent GF 109203X, beginning 1 h prior to the addition of forskolin. Results are shown as the means $\pm$ S.E.M. of three independent experiments. * P <0.001 vs forskolin; NS=not significant, P >0.05 vs control.

Figure 3 Stepwise loss of forskolin-responsiveness with sequential 5'-deletion of the LH β gene promoter. GH $_3$ cells were transiently transfected with varying regions of the rat LH β gene promoter in the luciferase reporter vector, pXP2. Cells were treated with vehicle or 2.5 μ M forskolin for 4–6 h starting 40 h after transfection. Luciferase activity was normalized to RSV-driven β -galactosidase activity and promoter activity was calculated as fold-change over expression in the control wells. Results are shown as the means $\pm$ S.E.M. Each construct was tested in between three and ten experiments with points run in triplicate. * P <0.001.

between positions –797 and –207 and the second between position –134 and the transcriptional start site (Fig. 3).

Loss of the cAMP/PKA response with mutation of the Egr-1 binding sites

Pharmacological or hormonal activation of the cAMP/PKA signaling system has been reported to increase Egr-1 gene expression (mRNA and/or protein levels) in the ovary and in pheochromocytoma and insulinoma cell lines (Bernal-Mizrachi *et al.* 2000, Espey *et al.* 2000, Tai *et al.* 2001). We hypothesized that cAMP-responsiveness of the LH β gene may be mediated likewise by Egr-1 acting through previously identified Egr-1 cis-elements in this gene. As shown in Fig. 4, GH $_3$ cells were transfected with a –207/+5 rat LH β gene reporter construct present as the wild-type construct or with mutations in one or both of the Egr-1 DNA-binding sites. We have previously demonstrated that *in vitro*-translated Egr-1 is unable to bind to oligonucleotide probes harboring analogous mutations (Halvorson *et al.* 1998). Mutation of the 3'-Egr-1 site eliminated the forskolin response,

Figure 4 Loss of the cAMP/PKA response with mutation of the Egr-1 binding sites. Transient transfection was performed in GH $_3$ cells using luciferase reporter constructs containing region –207/+5 of the rat LH β gene promoter present as either the wild-type sequence or with mutations in one or both of the previously characterized Egr-1 DNA cis-elements. Cells were treated as in Fig. 2. The results are presented relative to the forskolin response of the wild-type construct, which has been set at 100%. Data given as the means $\pm$ S.E.M. of three independent experiments. * P <0.01 vs wild-type.

while mutation of the 5' site had no effect. Although mutation of both Egr-1 cis-elements further reduced the response, this result was not statistically different than the response in the 3'-Egr mutant construct.

The forskolin response is not attributable to the presence of SF-1 or its cognate cis-elements (GSEs) in the LHβ gene promoter

The proximal forskolin-responsive region identified in Fig. 3 was also noted to contain previously characterized DNA-regulatory elements for the orphan nuclear receptor, SF-1. It is clear that SF-1 cannot be implicated in the ability of forskolin treatment alone to increase LH β gene promoter activity in the SF-1-deficient GH $_3$ cell line. Nevertheless, as ultimate interest is in the physiological regulation of gonadotrope function, it is critical to determine whether SF-1 and its associated cis-elements are involved in the observed cAMP-dependent transcription of the LH β gene. SF-1 has been demonstrated to be crucial for mediating cAMP/PKA-induced increases in the

transcription a variety of genes encoding enzymes in the steroidogenesis pathway (Lynch *et al.* 1993, Clemens *et al.* 1994, Michael *et al.* 1995, Zhang & Mellon 1996, Carlone & Richards 1997, Chau

et al. 1997, Liu & Simpson 1997, Jacob & Lund 1998, Sandhoff *et al.* 1998). In a number of these studies, alterations in SF-1 transactivation efficiency have been attributed to cAMP-dependent phosphorylation of the AF-2 domain of SF-1 (Crawford *et al.* 1997, Jacob & Lund 1998).

We therefore investigated whether the interaction between forskolin and SF-1 was altered with deletion of the SF-1 ligand-binding domain containing the AF-2 domain. As shown in Fig. 5A, the presence of the carboxy-truncated SF-1 construct (Δ LBD) markedly increased LHβ promoter activity, although to a lesser extent than observed with the native SF-1 (4- vs 9-fold respectively). The addition of forskolin further stimulated LHβ gene expression in the presence of either SF-1 construct (upper panel). The data were recalculated to evaluate whether the magnitude of the forskolin response differed depending on which SF-1 construct was present. As shown in the lower panel, the fold-change in LHβ promoter activity was not significantly different between constructs

Figure 5 Role of SF-1 and associated DNA-regulatory elements (GSEs) in mediating cAMP/PKA-responsiveness of the LHβ gene promoter. GH₃ cells were transiently transfected with various rat LHβ gene promoter reporter constructs. Cells were co-transfected with CMV-driven SF-1 expression vectors or control 'empty' vectors as well as the RSV-β-galactosidase vector. Prior to harvesting, cells received 2.5 μM forskolin or vehicle for 4–6 h starting 40 h after transfection. Results are shown as the means ± s.e.m. of three to eight experiments with each data point tested in triplicate. **P* < 0.001; NS = not significant, *P* > 0.05. (A) Comparison of the forskolin (F) effect in the presence of CMV-driven expression vectors encoding the full-length vs a carboxy-truncated SF-1 mutant lacking the ligand binding domain (Δ LBD). Promoter activity was calculated as fold-change relative to control wells (upper panel). Alternatively, data was expressed as the response to forskolin treatment in the presence of the intact or the truncated SF-1 vs the response to forskolin treatment alone (lower panel). (B) PKA effects in a heterologous LHβ-GSE-promoter construct. GH₃ cells were transiently transfected with a construct containing the growth hormone minimal promoter, GH50, linked to a luciferase reporter construct, pXP1, with or without the addition of four copies of the 5'-GSE site. (C) Maintenance of the forskolin response following mutation of the SF-1 binding sites (GSEs). GH₃ cells were transfected with region -207/+5 of the rat LHβ gene promoter present as either the wild-type sequence or with mutations in both GSE sites.

(approximately 3-fold with either construct), suggesting that the observed synergy between SF-1 and the cAMP system is not dependent on alterations at the carboxy-terminus of the SF-1 molecule.

B

	C	F1	F4	P1
Egr-1	1.0	16	11	70
SF-1	1.0	1.0	1.2	0.7

As an alternative approach, we reasoned that a forskolin response should be observed in the presence of SF-1 DNA-regulatory elements if, in fact, activation of the cAMP/PKA system is directly altering SF-1 functional activity. Four copies of the rat LHβ 5'-GSE sequence were inserted upstream of the growth hormone minimal promoter, GH50, in the pXP1 reporter vector. As seen in Fig. 5B, these sequences conferred an SF-1 response, but failed to confer a response to forskolin (10·5- and 11·8-fold for SF-1 alone and SF-1 plus forskolin respectively).

These results suggest that the cAMP/PKA effect is unlikely to be due to direct effects on the transcription factor, SF-1. This conclusion was confirmed by transfecting cells with a construct containing point mutations in both GSE sites within the context of the native rat LHβ gene promoter. These mutations have been shown to eliminate SF-1 DNA-binding and SF-1-induced transactivation (Halvorson *et al.* 1998). In contrast to the loss of the response to SF-1, forskolin-induced stimulation was conserved in the mutated construct (1·9- vs 1·8-fold in the wild-type construct) (Fig. 5C). These data strongly suggest that SF-1 and its cognate *cis*-element are not required for mediating the cAMP response in the rat LHβ gene promoter.

Figure 6 Activation of the cAMP/PKA system induces Egr-1 gene expression. (A) Cells from the gonadotrope-derived cell line, LβT2, were treated with a vehicle control (lane C), 2·5 μM forskolin for 1 h (lane F1) or 4 h (lane F4), or with 100 ng/ml PMA for 1 h (lane P1). Nuclear proteins (5 μg) or *in vitro*-translated Egr-1 or SF-1 were then separated on 10% SDS-PAGE gels followed by chemiluminescent Western blot analysis. I.V., *in vitro* translated Egr-1. (B) Band intensity from (A) was quantified using a Kodak EDAS 290 Image Analysis station and calculated as fold-change relative to vehicle-treated cell extracts. (C) LβT2 or GH₃ cells were transiently transfected with a luciferase reporter construct containing 1·2 kb of the 5'-flanking region of the Egr-1 gene. An RSV-β-galactosidase vector was co-transfected to control for transfection efficiency. Cells were treated with vehicle or 2·5 μM forskolin for 4 h prior to harvesting at 48 h post-transfection. Following normalization to β-galactosidase activity, promoter activity was calculated as fold-change in luciferase activity in treated vs untreated wells. Results are shown as the means±S.E.M. of five independent experiments. **P*<0·001.

Activation of the cAMP/PKA signaling system induces Egr-1, but not SF-1, gene expression

Results from experiments presented in Figs 4 and 5 suggest that the cAMP/PKA signaling system acts via Egr-1, but not SF-1, to stimulate LHβ gene expression. In order to further evaluate these observations, we asked whether activation of the cAMP system altered Egr-1 and/or SF-1 protein expression in gonadotrope cells. As shown by Western blot analysis in Fig. 6A and B, treatment of gonadotrope LβT2 cells with 2.5 μM forskolin markedly increased Egr-1 protein levels, but did not alter SF-1 protein levels in this protocol. The observed induction of Egr-1 protein expression by the phorbol ester, phorbol 12-myristate 13-acetate (PMA), is consistent with previous reports (Dorn *et al.* 1999).

The observed increase in Egr-1 protein levels may be due to increased Egr-1 biosynthesis and/or post-translational modification, resulting in increased protein stability. In order to determine whether activation of the cAMP pathway system altered Egr-1 biosynthesis, pituitary cell lines were transiently transfected with a luciferase reporter construct containing 1.2 kb of the Egr-1 gene promoter. Forskolin treatment increased Egr-1 promoter activity nearly 3-fold in the LβT2 cell line, with a 4-fold increase in the line GH₃ cells (Fig. 6C). In contrast, SF-1 promoter activity was not regulated by forskolin in the GH₃ cell line (data not shown).

We next used EMSA to determine whether increased Egr1 gene expression results in altered Egr-1 DNA-binding to the rat LHβ gene promoter. Nuclear extracts from untreated GH₃ or gonadotrope αT3-1 cells formed a single dominant band with an oligonucleotide probe spanning the 3'-Egr-1 site (Fig. 7B, lanes 2 and 7). Prior studies utilizing specific antibodies have demonstrated the presence of the transcription factor, Sp1, in this complex (Halvorson *et al.* 1999). The addition of forskolin (2.5 or 25 μM × 1 h) induced a faster migrating complex (lanes 3, 5, 8 and 10) that contained Egr-1 as shown by the addition of an Egr-1-specific antibody (lanes 4, 6, 9 and 11). The similar mobility of *in vitro*-translated Egr-1 (lane 1) was consistent with the presence of Egr-1 in the treated extracts.

Taken together, these results suggest that the cAMP signaling system induces Egr-1 transacti-

Figure 7 Forskolin treatment induces Egr-1 binding to rat LHβ gene promoter sequences. Activation of the cAMP/PKA system induces Egr-1 gene expression. EMSA was performed using *in vitro*-translated Egr-1 (lane 1) or nuclear extracts from GH₃ cells (left panel) or from the gonadotrope-derived αT3-1 cell line (right panel). Nuclear extracts were derived from cells maintained in the absence or presence of forskolin (2.5 or 25 μM for 1 h). Region –67/–35 of the rat LHβ gene promoter containing the 3'-Egr-1-binding site was ³²P labeled and used as a probe. Where indicated, antiserum specific for Egr-1 was added 2 h prior to electrophoresis. NE=nuclear extract; * indicates Egr-1-containing complex.

vation capability, at least in part, via increased biosynthesis of the Egr-1 gene.

Sp1 DNA-binding sites contribute to the full forskolin response of the –797/+5LHβ gene promoter region

We then tested the importance of the Egr-1 *cis*-elements for cAMP/PKA-responsiveness in the context of a longer region of the rat LHβ gene 5'-flanking sequence (–797/+5). As shown in Fig. 8, the forskolin effect was significantly blunted with the introduction of point mutations into the Egr-1 sites. Nevertheless, a substantial response remained in the mutant promoter constructs suggesting the presence of a non-Egr-1, PKA-responsive regulatory region in the more distal LHβ gene promoter.

Figure 8 Both Egr-1 and Sp1 DNA-binding sites contribute to the full cAMP response of region -797/+5 of the LH β gene promoter. GH $_3$ cells were transiently transfected with region -797/+5 of the rat LH β gene promoter present as the wild-type sequence or with mutations in the previously described Egr-1 and Sp1 *cis*-elements. Forskolin treatment and data calculation were as described in Fig. 5B and represent seven independent experiments. The results are presented relative to the forskolin-response of the wild-type construct, which has been set at 100%. * P <0.05 vs wild-type.

Region -451/-386 of the rat LH β gene promoter has been shown to contain two binding sites for the ubiquitous transcription factor, Sp1. These sites have been shown to contribute to both basal and GnRH-mediated LH β gene expression (Kaiser *et al.* 1998a). Mutation in the LH β -Sp1 sites significantly decreased the forskolin response, with further blunting in a construct containing mutations in both the Sp1 and Egr-1 *cis*-elements. These data suggest that both Sp1 and Egr-1 contribute to the full cAMP response of the -797/+5 rat LH β gene promoter.

Mutation of the homeodomain *cis*-element does not alter cAMP-responsiveness in the rat LH β gene

As shown in Fig. 8, a small residual cAMP-response was noted in the -797/+5 rat LH β gene promoter construct despite mutation of both the Sp1 and Egr-1 *cis*-elements. These results suggested a contribution by a third DNA-regulatory region. In an attempt to identify this site, we analyzed a previously identified homeodomain *cis*-element located at position -100 in the rat LH β gene

promoter. Mutation of this site did not alter the magnitude of the forskolin response, arguing against a role for Ptx/Otx-related proteins in mediating this response (data not shown).

Discussion

LH β gene expression is regulated by the complex interaction of hypothalamic, pituitary and gonadal factors, which in turn activate a variety of intracellular signaling systems. GnRH-induced stimulation of the PKC system has been widely recognized and intensively investigated (Kaiser *et al.* 1998b, Halvorson *et al.* 1999, Wolfe & Call 1999, Weck *et al.* 2000, Vasilyev *et al.* 2002). Studies have suggested that gonadotrope function is also mediated by physiological modulators of the cAMP/PKA system. The hypothalamic peptide, PACAP, is perhaps the best studied of these factors. PACAP has been shown to increase cAMP levels in both primary pituitary cells as well as in gonadotrope cell lines (Miyata *et al.* 1989, Schomerus *et al.* 1994). Interestingly, GnRH has recently been demonstrated to activate G $_s$ protein and increase intracellular cAMP levels in the gonadotrope L β T2 cell line, confirming prior studies in GH $_3$ cells stably expressing the GnRH receptor (Stanislaus *et al.* 1994, Liu *et al.* 2002). As reported by Ortmann *et al.* (1999) the gonadal steroid, estradiol, may also modulate adenylate cyclase activity. Furthermore, it is increasingly clear that crosstalk occurs between the PKC and PKA systems in the gonadotrope (McArdle *et al.* 1994, Garrel *et al.* 1997). Collectively, these data strongly support the physiological relevance of the cAMP/PKA pathway in regulating gene expression in gonadotrope cells.

In prior studies, PACAP has been shown to increase gonadotropin secretion and to modulate steady-state α -, LH β - and FSH β -subunit mRNA levels (McArdle *et al.* 1994, Tsujii & Winters 1995, Winters *et al.* 1997, Attardi & Winters 1998, Burrin *et al.* 1998, Fujii *et al.* 2002). We now demonstrate PACAP increases LH β gene promoter activity in the gonadotrope-derived cell line, L β T2 (Fig. 2). These results suggest that increased transcriptional activity contributes to the observed PACAP-induced increase in LH β mRNA levels. As previously noted, PACAP stimulates the PLC and calcium pathways in addition to increasing

intracellular cAMP levels (Schomerus *et al.* 1994, Hezareh *et al.* 1996, Pisegna & Wank 1996, Bresson-Bepoldin *et al.* 1998). In order to focus our studies on the effects of the cAMP/PKA signaling system, we chose to utilize the specific adenylate cyclase activator, forskolin, for the remainder of our experiments.

Forskolin treatment stimulated LH β gene promoter activity in both L β T2 and GH $_3$ cells (Fig. 2). Forskolin increased Egr-1 promoter activity, protein levels, and Egr-1 binding to the LH β gene promoter (Figs 6 and 7). Furthermore, mutation of the 3'-Egr-1 cis-element in the proximal LH β gene promoter was observed to blunt the forskolin response (Fig. 4). Based on these data, we propose that cAMP/PKA-induced stimulation of LH β promoter activity is achieved, in part, through induction of Egr-1 gene expression.

It should be noted that two other groups did not observe a forskolin-mediated increase in steady-state Egr-1 mRNA levels or Egr-1 gene promoter activity using the gonadotrope-derived α T3-1 cell line (Tremblay & Drouin 1999, Duan *et al.* 2002). Interestingly, one of these groups did observe that GnRH-induced increases in Egr-1 transcription and protein levels could be partially abrogated by the PKA blocking agent, H-89, results which support a role for cAMP/PKA in modulating Egr-1 gene function (Duan *et al.* 2002).

Egr-1 is a member of the immediate early gene family whose members are markedly regulated at the biosynthetic and post-translational level by a wide variety of growth and differentiation factors (Christy *et al.* 1988, Cao *et al.* 1990). Forskolin treatment has been shown to increase Egr-1 promoter and protein expression in insulinoma and pheochromocytoma cell lines respectively (Bernal-Mizrachi *et al.* 2000, Tai *et al.* 2001). Richards and co-workers (Espey *et al.* 2000) have shown that Egr-1 mRNA and protein levels are increased by human chorionic gonadotropin (hCG) treatment in ovarian granulosa cells, presumably due to LH receptor-mediated activation of the cAMP pathway. More recently, Yoshino *et al.* (2002) demonstrated that both hCG and 8-Br-cAMP increase Egr-1 mRNA levels in luteinized granulosa cells. Thus, current data now exist to suggest that cAMP modulates Egr-1 gene expression at multiple levels of the reproductive axis.

Mutation of the Sp1 DNA-binding sites further blunted the cAMP response in the context of the

longer region of the rat LH β gene promoter (Fig. 8). Constitutively expressed, Sp1 classically has been thought to act as a basal transcription factor. Nevertheless, recent studies have implicated Sp1 in the hormonal regulation of a variety of genes, including cAMP-induced transcription of the *CYP17* and *CYP11A* (P450 scc) steroidogenic genes (Begeot *et al.* 1993, Venepally & Waterman 1995, Borroni *et al.* 1997, Ahlgren *et al.* 1999). The precise mechanism by which Sp1 mediates the cAMP effect in the rat LH β gene remains to be determined. In preliminary studies, forskolin treatment did not alter the intensity of endogenous Sp1 binding to LH β -Sp1 sites, implying that Sp1 protein levels are not altered (data not shown). Sp1 is known to be modified by *N*- and *O*-linked glycosylation as well as by phosphorylation (Jackson *et al.* 1990). It has been proposed that these post-translational modifications may alter Sp1 interactions with other transcription factors, such as SF-1, and/or with specific co-activators including the cAMP-responsive co-factor, CBP/p300 (Liu & Simpson 1999).

Unlike the highly conserved Egr-1 and SF-1 sites, the Sp1 *cis*-elements are not conserved across species, suggesting that each species may utilize different combinations of transcription factors to achieve cAMP-dependent stimulation of the LH β gene. Of note, a DNA-regulatory site for the CCAAT-binding factor NF-Y has been characterized in the bovine LH β gene promoter in the region of the Sp1 sites of the rat gene (Keri *et al.* 2000). NF-Y has been shown to contribute to cAMP-dependent regulation of both the rat fatty acid synthase and TIMP-2 genes (Roder *et al.* 1997, Zhong *et al.* 2000). It would be of interest to evaluate whether the NF-Y *cis*-acting elements mediate cAMP-responsiveness in the bovine LH β gene.

The orphan nuclear receptor SF-1 is known to alter LH β gene expression independently, as well as through interactions with other transcription factors and with the PKC signaling system (Halvorson *et al.* 1996, 1998, 1999, Keri & Nilson 1996, Dorn *et al.* 1999, Wolfe 1999). We now demonstrate that the presence of SF-1 augments cAMP-induced stimulation of the rat LH β gene promoter activity when tested in SF-1-deficient GH $_3$ cells. A similar observation has been reported for the equine LH β gene in placental JEG3 cells (Heckert 2001).

SF-1 is believed to be directly responsible for conferring cAMP-dependent transcription in a number of genes, although the mechanism by which this effect is achieved remains unclear (Lynch *et al.* 1993, Clemens *et al.* 1994, Michael *et al.* 1995, Zhang & Mellon 1996, Carlone & Richards 1997, Chau *et al.* 1997, Liu & Simpson 1997, Jacob & Lund 1998, Sandhoff *et al.* 1998, Aesoy *et al.* 2002). In a variety of studies in non-pituitary cells, neither hormonal nor pharmacological stimulation of the cAMP/PKA pathway increased steady-state SF-1 mRNA levels (Zhang & Mellon 1996, Chau *et al.* 1997, Aesoy *et al.* 2002). Aesoy *et al.* (2002) have shown that prolonged forskolin treatment (10 μ M $\times$ 24 h) increases SF-1 protein levels by decreasing SF-1 degradation in adrenocortical cells. We did not observe a forskolin-mediated increase in gonadotrope-derived SF-1 protein (Fig. 7); however, we cannot rule out the possibility that a higher dose and/or longer treatment regimen would increase pituitary SF-1. Nevertheless, this response would not explain the ability of forskolin to induce LH β gene transcription in the 4 h time course demonstrated in our studies. Furthermore, the persistent cAMP response with mutation of the LH β -GSEs and the inability of a GSE-multimer construct to confer cAMP-responsiveness argue against a direct role for SF-1 or its *cis*-element (Fig. 5). Our results strongly suggest that cAMP-induced activation of the LH β gene does not require SF-1 or its *cis*-elements; however, the presence of SF-1 does amplify the cAMP response, probably through known functional interactions with Egr-1 and Sp1 (Liu & Simpson 1997, Halvorson *et al.* 1998, Kaiser *et al.* 2000).

The studies reported here clearly indicate the complexity of the molecular mechanisms which mediate cAMP-stimulated LH β gene expression. Based on our data, the Egr-1 and Sp1 sites contribute nearly 75% of cAMP-responsiveness to the LH β gene promoter; however, despite mutation of the only other identified *cis*-element in this gene (the Ptx/Otx element), we have been unable to account for the residual response. By sequence analysis, the LH β gene lacks a classic cAMP-response element. Furthermore, mutation of a putative AP-2-like site at position -84 does not alter cAMP-mediated LH β promoter activity (data not shown). It will be of interest to attempt to identify any additional cAMP-responsive *cis*-elements in future studies.

In conclusion, our results demonstrate that activation of the cAMP system by PACAP or forskolin increases LH β gene promoter activity. The forskolin response is mediated via Egr-1 and Sp1 acting at their cognate DNA-binding sites in the rat LH β gene. Although not required for cAMP-responsiveness, SF-1 markedly augments cAMP-induced LH β gene transcription. Of note, this mechanism is similar to a model proposed for GnRH/PKC-induced activation of the rat LH β gene (Kaiser *et al.* 2000). We, therefore, propose that Sp1, Egr-1 and SF-1 play critical roles in integrating the transcriptional response of the LH β -subunit gene to a variety of hormonal stimuli.

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References

- Acampora D, Mazan S, Tuorto F, Avantaggiato V, Tremblay JJ, Lazzaro D, di Carlo A, Mariano A, Macchia PE, Corte G *et al.* 1998 Transient dwarfism and hypogonadism in mice lacking Otx1 reveal prepubescent stage-specific control of pituitary levels of GH, FSH and LH. *Development* **125** 1229–1239.
- Aesoy R, Mellgren G, Morohashi K & Lund J 2002 Activation of cAMP-dependent protein kinase increases the protein level of steroidogenic factor-1. *Endocrinology* **143** 295–303.
- Ahlgren R, Suske G, Waterman MR & Lund J 1999 Role of Sp1 in cAMP-dependent transcriptional regulation of the bovine CYP11A gene. *Journal of Biological Chemistry* **274** 19422–19428.
- Andrews NC & Faller DV 1991 A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Research* **19** 2499.
- Attardi B & Winters SJ 1998 Transcriptional regulation of the glycoprotein hormone alpha-subunit gene by pituitary adenylate cyclase-activating polypeptide (PACAP) in alphaT3-1 cells. *Molecular and Cellular Endocrinology* **137** 97–107.
- Barnhart KM & Mellon PL 1994 The orphan nuclear receptor, steroidogenic factor-1, regulates the glycoprotein hormone α -subunit gene in pituitary gonadotropes. *Molecular Endocrinology* **8** 878–885.
- Begeot M, Shetty U, Kilgore M, Waterman M & Simpson E 1993 Regulation of expression of the CYP11A (P450 scc) gene in bovine ovarian luteal cells by forskolin and phorbol esters. *Journal of Biological Chemistry* **268** 17317–17325.
- Berg JM 1992 Sp1 and the subfamily of zinc finger proteins with guanine-rich binding sites. *PNAS* **89** 11109–11110.
- Bernal-Mizrachi E, Wice B, Inoue H & Permutt MA 2000 Activation of serum response factor in the depolarization induction of Egr-1 transcription in pancreatic islet beta-cells. *Journal of Biological Chemistry* **275** 25681–25689.
- Borroni R, Liu Z, Simpson ER & Hinshelwood MM 1997 A putative binding site for Sp1 is involved in transcriptional regulation of CYP17 gene expression in bovine ovary. *Endocrinology* **138** 2011–2020.

- Bresson-Bepoldin L, Jacquot MC, Schlegel W & Rawlings SR 1998 Multiple splice variants of the pituitary adenylate cyclase-activating polypeptide type 1 receptor detected by RT-PCR in single rat pituitary cells. *Journal of Molecular Endocrinology* **21** 109–120.
- Burrin JM, Aylwin SJ, Holdstock JG & Sahye U 1998 Mechanism of action of pituitary adenylate cyclase-activating polypeptide on human glycoprotein hormone α -subunit transcription in α T3-1 gonadotropes. *Endocrinology* **139** 1731–1737.
- Cao XM, Koski RA, Gashler A, McKiernan M, Morris CF, Gaffney R, Hay RV & Sukhatme VP 1990 Identification and characterization of the Egr-1 gene product, a DNA-binding zinc finger protein induced by differentiation and growth signals. *Molecular and Cellular Biology* **10** 1931–1939.
- Carlone DL & Richards JS 1997 Functional interactions, phosphorylation, and levels of 3',5'-cyclic adenosine monophosphate-regulatory element binding protein and steroidogenic factor-1 mediate hormone-regulated and constitutive expression of aromatase in gonadal cells. *Molecular Endocrinology* **11** 292–304.
- Chau YM, Crawford PA, Woodson KG, Polish JA, Olson LM & Sadovsky Y 1997 Role of steroidogenic-factor 1 in basal and 3',5'-cyclic adenosine monophosphate-mediated regulation of cytochrome P450 side-chain cleavage enzyme in the mouse. *Biology of Reproduction* **57** 765–771.
- Chen J & Iyengar R 1994 Suppression of ras-induced transformation of NIH 3T3 cells by activated Gas. *Science* **263** 1278–1281.
- Christy BA, Lau LF & Nathans D 1988 A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with 'zinc finger' sequences. *PNAS* **85** 7857–7861.
- Clayton RN, Lalloz MRA, Salton SRJ & Roberts JL 1991 Expression of luteinising hormone- β subunit chloramphenicol acetyltransferase (LH- β -CAT) fusion gene in rat pituitary cells: induction by cyclic 3'-adenosine monophosphate (cAMP). *Molecular and Cellular Endocrinology* **80** 193–202.
- Clemens JW, Lala DS, Parker KL & Richards JS 1994 Steroidogenic factor-1 binding and transcriptional activity of the cholesterol side-chain cleavage promoter in rat granulosa cells. *Endocrinology* **134** 1499–1508.
- Crawford PA, Polish JA, Ganpule G & Sadovsky Y 1997 The activation function-2 hexamer of steroidogenic factor-1 is required, but not sufficient for potentiation by SRC-1. *Molecular Endocrinology* **11** 1626–1635.
- deWet JR, Wood KV, DeLuca M, Helinski DR & Subramani S 1987 Firefly luciferase gene: structure and expression in mammalian cells. *Molecular and Cellular Biology* **7** 725–737.
- Dorn C, Ou Q, Svaren J, Crawford PA & Sadovsky Y 1999 Activation of luteinizing hormone beta gene by gonadotropin-releasing hormone requires the synergy of early growth response-1 and steroidogenic factor-1. *Journal of Biological Chemistry* **274** 13870–13876.
- Duan WR, Ito M, Park Y, Maizels ET, Hunzicker-Dunn M & Jameson JL 2002 GnRH regulates early growth response protein 1 transcription through multiple promoter elements. *Molecular Endocrinology* **16** 221–233.
- Edlund T, Walker MD, Barr PJ & Rutter WJ 1985 Cell specific expression of the rat insulin gene: evidence for a role of two distinct 5' flanking elements. *Science* **230** 912–916.
- Espey LL, Ujioka T, Russell DL, Skelsey M, Vladu B, Robker RL, Okamura H & Richards JS 2000 Induction of early growth response protein-1 gene expression in the rat ovary in response to an ovulatory dose of human chorionic gonadotropin. *Endocrinology* **141** 2385–2391.
- Fujii Y, Okada Y, Moore JP Jr, Dalkin AC & Winters SJ 2002 Evidence that PACAP and GnRH down-regulate follicle-stimulating hormone-beta mRNA levels by stimulating follistatin gene expression: effects on folliculostellate cells, gonadotrophs and LbetaT2 gonadotroph cells. *Molecular and Cellular Endocrinology* **192** 55–64.
- Garrel G, McArdle CA, Hemmings BA & Counis R 1997 Gonadotropin-releasing hormone and pituitary adenylate cyclase-activating polypeptide affect levels of cyclic adenosine 3',5'-monophosphate-dependent protein kinase A (PKA) subunits in the clonal gonadotrope α T3-1 cells: evidence for cross-talk between PKA and protein kinase C pathways. *Endocrinology* **138** 2259–2266.
- Halvorson LM, Kaiser UB & Chin WW 1996 Stimulation of luteinizing hormone β gene promoter activity by the orphan nuclear receptor, steroidogenic factor-1. *Journal of Biological Chemistry* **271** 6645–6650.
- Halvorson LM, Ito M, Jameson JL & Chin WW 1998 Steroidogenic factor-1 and early growth response protein 1 act through two composite DNA binding sites to regulate luteinizing hormone β -subunit gene expression. *Journal of Biological Chemistry* **273** 14712–14720.
- Halvorson LM, Kaiser UB & Chin WW 1999 The protein kinase C system acts through early growth response protein 1 to increase LH β gene expression in synergy with steroidogenic factor-1. *Molecular Endocrinology* **13** 106–116.
- Heckert LL 2001 Activation of the rat follicle-stimulating hormone receptor promoter by steroidogenic factor 1 is blocked by protein kinase A and requires upstream stimulatory factor binding to a proximal E box element. *Molecular Endocrinology* **15** 704–715.
- Hezareh M, Journot L, Bepoldin L, Schlegel W & Rawlings SR 1996 PACAP/VIP receptor subtypes, signal transducers, and effectors in pituitary cells. *Annals of the New York Academy of Sciences* **805** 315–327 [discussion 327–328].
- Ingraham HA, Lala DS, Ikeda Y, Luo X, Shen WH, Nachtigal MW, Abbud R, Nilson JH & Parker KL 1994 The nuclear receptor steroidogenic factor 1 acts at multiple levels of the reproductive axis. *Genes and Development* **8** 2302–2312.
- Jackson SP, MacDonald JJ, Lees-Miller S & Tjian R 1990 GC box binding induces phosphorylation of Sp1 by a DNA-dependent protein kinase. *Cell* **63** 155–165.
- Jacob AL & Lund J 1998 Mutations in the activation function-2 core domain of steroidogenic factor-1 dominantly suppresses PKA-dependent transactivation of the bovine CYP17 gene. *Journal of Biological Chemistry* **273** 13391–13394.
- Jameson JL, Chin WW, Hollenberg AN, Chang AS & Habener JF 1984 The gene encoding the β -subunit of rat luteinizing hormone. *Journal of Biological Chemistry* **259** 15474–15480.
- Kaiser UB, Katzenellenbogen RA, Conn PM & Chin WW 1994 Evidence that signalling pathways by which thyrotropin-releasing hormone and gonadotropin-releasing hormone act are both common and distinct. *Molecular Endocrinology* **8** 1038–1048.
- Kaiser UB, Sabbagh E, Chen MT, Chin WW & Saunders BD 1998a Sp1 binds to the rat LH β gene promoter and mediates GnRH-stimulated expression of the LH β subunit gene. *Journal of Biological Chemistry* **273** 12943–12951.
- Kaiser UB, Sabbagh E, Saunders BD & Chin WW 1998b Identification of cis-acting deoxyribonucleic acid elements that mediate gonadotropin-releasing hormone stimulation of the rat luteinizing hormone β -subunit gene. *Endocrinology* **139** 2443–2451.
- Kaiser UB, Halvorson LM & Chen MT 2000 Sp1, steroidogenic factor 1 (SF-1), and early growth response protein 1 (Egr-1) binding sites form a tripartite gonadotropin-releasing hormone response element in the rat luteinizing hormone- β gene promoter: an integral role for SF-1. *Molecular Endocrinology* **14** 1235–1245.
- Keri RA & Nilson JH 1996 A steroidogenic factor-1 binding site is required for activity of the luteinizing hormone β subunit promoter in gonadotropes of transgenic mice. *Journal of Biological Chemistry* **271** 10782–10785.

- Keri RA, Bachmann DJ, Behrooz A, Herr BD, Ameduri RK, Quirk CC & Nilson JH 2000 An NF-Y binding site is important for basal, but not gonadotropin-releasing hormone-stimulated, expression of the luteinizing hormone β subunit gene. *Journal of Biological Chemistry* **275** 13082–13088.
- Kim KE, Day KH, Howard P, Salton SRJ, Roberts JL & Maurer RA 1990 DNA sequences required for expression of the LH β promoter in primary cultures of rat pituitary cells. *Molecular and Cellular Endocrinology* **74** 101–107.
- Kuphal D, Janovick JA, Kaiser UB, Chin WW & Conn PM 1994 Stable transfection of GH $_3$ cells with rat gonadotropin-releasing hormone receptor complementary deoxyribonucleic acid results in expression of a receptor coupled to cyclic adenosine 3',5'-monophosphate-dependent prolactin release via a G-protein. *Endocrinology* **135** 315–320.
- Lala DS, Rice DA & Parker KL 1992 Steroidogenic factor 1, a key regulator of steroidogenic enzyme expression, is the mouse homolog of Fushi tarazu-factor 1. *Molecular Endocrinology* **6** 1249–1258.
- Lamonerie T, Tremblay JJ, Lancot C, Therrien M, Gauthier Y & Drouin J 1996 Ptx1, a bicoid-related homeo box transcription factor involved in transcription of the pro-opiomelanocortin gene. *Genes and Development* **10** 1284–1295.
- Lancot C, Lamolet B & Drouin J 1997 The bicoid-related homeoprotein Ptx1 defines the most anterior domain of the embryo and differentiates posterior from anterior lateral mesoderm. *Development* **124** 2807–2817.
- Lee SL, Sadovsky Y, Swirnoff AH, Polish JA, Goda P, Gavrilina G & Milbrandt J 1996 Luteinizing hormone deficiency and female infertility in mice lacking the transcription factor NGFI-A (Egr-1). *Science* **273** 1219–1221.
- Liu F, Usui I, Evans LG, Austin DA, Mellon PL, Olefsky JM & Webster NJ 2002 Involvement of both G(q/11) and G(s) proteins in gonadotropin-releasing hormone receptor-mediated signaling in L beta T2 cells. *Journal of Biological Chemistry* **277** 32099–32108.
- Liu Z & Simpson ER 1997 Steroidogenic factor 1 (SF-1) and Sp1 are required for regulation of bovine CYP11A gene expression in bovine luteal cells and adrenal Y1 cells. *Molecular Endocrinology* **11** 127–137.
- Liu Z & Simpson ER 1999 Molecular mechanism for cooperation between Sp1 and steroidogenic factor-1 (SF-1) to regulate bovine CYP11A gene expression. *Molecular and Cellular Endocrinology* **153** 183–196.
- Luo X, Ikeda Y & Parker KL 1994 A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. *Cell* **77** 481–490.
- Lynch JP, Lala DS, Peluso JJ, Luo W, Parker KL & White BA 1993 Steroidogenic factor 1, an orphan nuclear receptor, regulates the expression of the rat aromatase gene in gonadal tissues. *Molecular Endocrinology* **7** 776–786.
- McArdle CA, Poch A, Schomerus E & Kratzmeier M 1994 Pituitary adenylate cyclase-activating polypeptide effects in pituitary cells; modulation by gonadotropin-releasing hormone in alpha T3-1 cells. *Endocrinology* **134** 2599–2605.
- Michael MD, Kilgore MW, Morohashi K & Simpson ER 1995 Ad4 BP/SF-1 regulates cyclic AMP-induced transcription from the proximal promoter (P1) of the human aromatase P450 (CYP19) gene in the ovary. *Journal of Biological Chemistry* **270** 13561–13566.
- Miyata A, Arimura A, Dahl RR, Minamino N, Uehara A, Jiang L, Culle DH & Coy DH 1989 Isolation of a novel 38 residue hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochemical and Biophysical Research Communications* **164** 567–574.
- Nordeen S 1988 Luciferase reporter gene vectors for analysis of promoters and enhancers. *BioTechniques* **6** 454–458.
- Ortmann O, Asmus W, Diedrich K, Schulz K-D & Emons G 1999 Interactions of ovarian steroids with pituitary adenylate cyclase-activating polypeptide and GnRH in anterior pituitary cells. *European Journal of Endocrinology* **140** 207–214.
- Park D, Kim C, Cheon M, Kim K & Ryu K 1997 cAMP and protein kinase C elevate LH beta mRNA levels by activating transcription rather than stabilizing mRNA in rat pituitary cells. *Molecules and Cells* **7** 98–103.
- Pinter JH, Janovick JA & Conn PM 1999 Gonadotropin-releasing hormone receptor concentration differentially regulates intracellular signaling pathways in GH $_3$ cells. *Pituitary* **2** 181–190.
- Pisegna JR & Wank SA 1996 Cloning and characterization of the signal transduction of four splice variants of the human pituitary adenylate cyclase activating polypeptide receptor. Evidence for dual coupling to adenylate cyclase and phospholipase C. *Journal of Biological Chemistry* **271** 17267–17274.
- Quirk CC, Lozada KL, Keri RA & Nilson JH 2001 A single Pitx1 binding site is essential for activity of the LH β promoter in transgenic mice. *Molecular Endocrinology* **15** 734–746.
- Roder K, Wolf SS, Beck KF, Sickinger S & Schweizer M 1997 NF-Y binds to the inverted CCAAT box, an essential element for cAMP-dependent regulation of the rat fatty acid synthase (FAS) gene. *Gene* **184** 21–26.
- Rosenberg SB & Mellon PL 2002 An Otx-related homeodomain protein binds an LH β promoter element important for activation during gonadotrope maturation. *Molecular Endocrinology* **16** 1280–1298.
- Sandhoff TW, Hales DB, Hales KH & McLean MP 1998 Transcriptional regulation of the rat steroidogenic acute regulatory protein gene by steroidogenic factor 1. *Endocrinology* **139** 4820–4831.
- Saunders BD, Sabbagh E, Chin WW & Kaiser UB 1998 Differential use of signal transduction pathways in the gonadotropin-releasing hormone-mediated regulation of gonadotropin subunit gene expression. *Endocrinology* **139** 1835–1843.
- Schomerus E, Poch A, Bunting R, Mason WT & McArdle CA 1994 Effects of pituitary adenylate cyclase-activating polypeptide in the pituitary: activation of two signal transduction pathways in the gonadotrope-derived α T3-1 cell line. *Endocrinology* **134** 315–323.
- Stanislaus D, Janovick JA, Jennes L, Kaiser UB, Chin WW & Conn PM 1994 Functional and morphological characterization of four cell lines derived from GH $_3$ cells stably transfected with gonadotropin-releasing hormone receptor complementary deoxyribonucleic acid. *Endocrinology* **135** 2220–2227.
- Starzec A, Counis R & Jutisz M 1986 Gonadotropin-releasing hormone stimulates the synthesis of the polypeptide chains of luteinizing hormone. *Endocrinology* **119** 561–565.
- Starzec A, Jutisz M & Counis R 1989 Cyclic adenosine monophosphate and phorbol ester, like gonadotropin-releasing hormone, stimulate the biosynthesis of luteinizing hormone polypeptide chains in a nonadditive manner. *Molecular Endocrinology* **3** 618–624.
- Stojilkovic SS, Reinhart J & Catt KJ 1994 Gonadotropin-releasing hormone receptors: structure and signal transduction pathways. *Endocrine Reviews* **15** 462–499.
- Suen C-S, Yen PM & Chin WW 1994 *In vitro* transcriptional studies of the roles of the thyroid hormone (T3) response elements and minimal promoters in T3-stimulated gene transcription. *Journal of Biological Chemistry* **269** 1314–1322.
- Sukhatme VP, Kartha S, Toback FG, Taub R, Hoover RG & Tsai-Morris C 1987 A novel early growth response gene rapidly induced by fibroblast epithelial cell and lymphocyte mitogens. *Oncogene Research* **1** 343–355.
- Tai TC, Morita K & Wong DL 2001 Role of Egr-1 in cAMP-dependent protein kinase regulation of the phenylethanolamine N-methyltransferase gene. *Journal of Neurochemistry* **76** 1851–1859.
- Topilko P, Schneider-Maunoury S, Levi G, Trembleau A, Gourdji D, Driancourt M-A, Rao CV & Charnay P 1997 Multiple

- pituitary and ovarian defects in Krox-24 (NGFI-A, Egr-1)-targeted mice. *Molecular Endocrinology* **12** 107–122.
- Tremblay JJ & Drouin J 1999 Egr-1 is a downstream effector of GnRH and synergizes by direct interaction with Ptx1 and SF-1 to enhance luteinizing hormone beta gene transcription. *Molecular and Cellular Biology* **19** 2567–2576.
- Tremblay JJ, Lanctot C & Drouin J 1998 The pan-pituitary activator of transcription, Ptx1 (pituitary homeobox 1), acts in synergy with SF-1 and Pit1 and is an upstream regulatory of the Lim-homeodomain gene Lim3/Lhx3. *Molecular Endocrinology* **12** 428–441.
- Tremblay JJ, Marcil A, Gauthier Y & Drouin J 1999 Ptx1 regulates SF-1 activity by an interaction that mimics the role of the ligand-binding domain. *EMBO Journal* **18** 3431–3441.
- Tsujii T & Winters SJ 1995 Effects of pulsatile pituitary adenylate cyclase activating polypeptide (PACAP) on gonadotropin secretion and subunit mRNA levels in perfused rat pituitary cells. *Life Sciences* **56** 1103–1111.
- Tsujii T, Ishizaka K & Winters SJ 1994 Effects of pituitary adenylate cyclase activating polypeptide (PACAP) on gonadotropin secretion and subunit mRNAs in perfused rat pituitary cells. *Endocrinology* **135** 826–833.
- Turgeon JL, Kimura Y, Waring DW & Mellon PL 1996 Steroid and pulsatile gonadotropin-releasing hormone (GnRH) regulation of luteinizing hormone and GnRH receptor in a novel gonadotrope cell line. *Molecular Endocrinology* **10** 439–450.
- Vasilyev VV, Lawson MA, Dipaolo D, Webster NJ & Mellon PL 2002 Different signaling pathways control acute induction versus long-term repression of LH β transcription by GnRH. *Endocrinology* **143** 3414–3426.
- Venepally P & Waterman MR 1995 Two Sp1-binding sites mediate cAMP-induced transcription of the bovine CYP11A gene through the protein kinase A signaling pathway. *Journal of Biological Chemistry* **270** 25402–25410.
- Weck J, Fallest PC, Pitt LK & Shupnik MA 1998 Differential gonadotropin-releasing hormone stimulation of rat luteinizing hormone subunit gene transcription by calcium influx and mitogen-activated protein kinase-signaling pathways. *Molecular Endocrinology* **12** 451–457.
- Weck J, Anderson AC, Jenkins S, Fallest PC & Shupnik MA 2000 Divergent and composite gonadotropin-releasing hormone-responsive elements in the rat luteinizing hormone subunit genes. *Molecular Endocrinology* **14** 472–485.
- Winters SJ, Dalkin AC & Tsujii T 1997 Evidence that pituitary adenylate cyclase activating polypeptide suppresses follicle-stimulating hormone-beta messenger ribonucleic acid levels by stimulating follistatin gene transcription. *Endocrinology* **138** 4324–4329.
- Wolfe MW 1999 The equine luteinizing hormone beta-subunit promoter contains two functional steroidogenic factor-1 response elements. *Molecular Endocrinology* **13** 1497–1510.
- Wolfe MW & Call GB 1999 Early growth response protein 1 binds to the luteinizing hormone-beta promoter and mediates gonadotropin-releasing hormone-stimulated gene expression. *Molecular Endocrinology* **13** 752–763.
- Woodson KG, Crawford PA, Sadovsky Y & Milbrandt J 1997 Characterization of the promoter of SF-1, an orphan nuclear receptor required for adrenal and gonadal development. *Molecular Endocrinology* **11** 117–126.
- Yoshino M, Mizutani T, Yamada K, Tsuchiya M, Minegishi T, Yazawa T, Kawata H, Sekiguchi T, Kajitani T & Miyamoto K 2002 Early growth response gene-1 regulates the expression of the rat luteinizing hormone receptor gene. *Biology of Reproduction* **66** 1813–1819.
- Zhang P & Mellon SH 1996 The orphan nuclear receptor steroidogenic factor-1 regulates the cyclic adenosine 3',5'-monophosphate-mediated transcriptional activation of rat cytochrome P450c17 (17 α -hydroxylase/c17–20 lyase). *Molecular Endocrinology* **10** 147–158.
- Zhong ZD, Hammani K, Bae WS & DeClerck YA 2000 NF-Y and Sp1 cooperate for the transcriptional activation and cAMP response of human tissue inhibitor of metalloproteinases-2. *Journal of Biological Chemistry* **275** 18602–18610.

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