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Gonadotroph-specific expression of the human follicle stimulating hormone β gene in transgenic mice

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Abstract

A paucity of in vitro models has hampered studies of molecular mechanisms of FSH subunit gene expression. Consequently, we used an in vivo transgenic strategy to map the location of regulatory elements in the cloned 10 kb human FSHβ gene. Analyses of transgenic mouse lines revealed that successive 5′ truncations of the hFSHβ promoter region to −350 bp relative to the transcriptional initiation site retained gonadotroph-specific expression and the sexually dimorphic pattern of male greater than female FSHβ mRNA levels found normally in rodent pituitary. Truncation of the 3′ flanking sequences from positions +3142 to +2138 bp relative to the translational stop codon in exon 3 resulted in a complete loss of transgene expression, suggesting the presence of critical regulatory elements mapping to the 1 kb genomic segment downstream of position +2138, in addition to the proximal 5′ promoter elements. In silico phylogenetic comparisons of mammalian FSHβ genes revealed five islands of highly conserved sequence homology corresponding precisely to the proximal 5′ promoter region, exon 2, the 5′ translated region of exon 3, and two regions at the 3′ untranslated end of exon 3 that include putative polyadenylation and transcriptional termination signals. Sequence analyses of the 5′ proximal promoter revealed the presence of several putative homeodomain binding sites as well as GATA, SMAD, AP-1, NF-1, NF-Y and steroid hormone transcription factor binding sites within the highly conserved −350 bp promoter region. Notably absent from these 5′ sequences, however, are consensus binding sites for either Egr-1 or Lim-2 transcription factors known to be critical for the gonadotroph-specific expression of the LHβ gene. These findings support the hypothesis that one of the mechanisms underlying the differential regulation of the LHβ, FSHβ, and common α-gonadotropin subunits within pituitary gonadotrophs may be differences in sequence-specific binding requirements for distinct combinations of transcription factors.

Keywords: Pituitary; Gonadotroph; Gene expression; SV40 T-antigen

1. Introduction

Normal reproductive function requires the coordinated regulation of the LHβ, FSHβ and common α-glycoprotein subunit genes. The mechanisms underlying the coordination of these subunits are poorly understood, but clearly involve differences in both gene expression and ultimately secretion of each of the heterodimeric gonadotropin hormones. Both LHβ and FSHβ are highly regulated by pulsatile GnRH via multiple signal transduction pathways, gonadal steroids and gonadal peptides (Shupnik, 1996; Vasilyev et al., 2002), however, there are significant differences between the two genes in terms of their responses to each of these regulatory factors (Ishizaka et al., 1992; Kaiser et al., 1995; Lee et al., 1996). Developmental onset of LHβ and FSHβ gene expression also occurs on distinct days in mouse embryonic development (Japón et al., 1994). The common α-subunit appears to be less stringently regulated and is thought to be present in excess relative to the β-subunits (Gharib et al., 1990). The α-subunit gene expression is not affected by gonadal peptides (Attardi and Miklos, 1990) and is stimulated independently of GnRH pulse frequency (Shupnik and Weck, 1998). Despite these observations of less stringent regulation, the inter-

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actions among regulatory elements and transcription factors for the α-subunit gene is complex and has been extensively reviewed (Albanese et al., 1996; Jorgensen et al., 2004).

However, our understanding of the molecular mechanisms underlying regulation of the hormone-specific β-subunit genes remains relatively less detailed, as reviewed recently for LHβ (Jorgensen et al., 2004). The structure of the human FSHβ (hFSHβ) gene was first described in detail by Jameson et al. (1988) and is comprised of three exons, the first of which contains no translated sequence but does contain an alternate splicing donor site. There are multiple consensus polyadenylation signals (AAUAAA), one proximal coinciding with the stop codon in exon 3 and a cluster of three additional sites about 1–1.2 kb downstream of the stop codon resulting in an unusually long 3′ untranslated region (UTR). Analyses of mRNA size by Northern blots, alignments of genomic and expressed sequence tag (EST) sequences, and phylogenetic comparisons of multiple mammalian FSHβ 3′ UTRs suggest that the most distal AAUAAA site at nucleotides +1218 to +1223 is the predominantly utilized signal (Jameson et al., 1988; Brockman et al., 2005; Manjithaya and Dighe, 2004).

In large part, the lack of further details regarding transcriptional regulation of this gene and the other gonadotropin β-subunits is due to the lack of a homologous in vitro expression system. Expression of FSH in primary pituitary cultures has been disappointing (Maurer and Kim, 1990). Until recently, the only established cell line to express FSH was the RC-4B line (Berault et al., 1990). However, because of its heterogeneity and multiple cell phenotypes, it was never established as a useful model of gonadotrophs. More recently the LβT2 cell line has been shown to express the mouse LHβ (Turgeon et al., 1996) and mouse FSHβ (Graham et al., 1999; Pernasetti et al., 2001) subunit genes. In addition, there are reports of expression of rat, ovine and porcine FSHβ reporter constructs in cell lines, although we have been unable to demonstrate expression of transfected human genomic FSHβ constructs in a fashion necessary for more detailed analysis of promoter structure. Because of these limitations, we have pursued further identification of potential transcriptional regulatory elements of the hFSHβ gene utilizing a transgenic expression strategy. We previously reported transgenic expression of a 10 kb hFSHβ genomic clone which recapitulated gonadotroph-specific and appropriate hormonal regulation in the mouse pituitary (Kumar et al., 1992; Kumar and Low, 1993, 1995). We report here a detailed transgenic analysis that characterizes the minimal regulatory elements required for the normal pattern of sexually dimorphic, gonadotroph-specific regulation of the hFSHβ gene in vivo and compare putative conserved regulatory elements in the minimal proximal 5′ flanking region across mammalian species.

2. Materials and methods

2.1. Design and generation of transgene constructs

Transgene constructs were based on the 10 kb genomic hFSHβ sequences described previously (Kumar et al., 1992; Kumar and Low, 1993, 1995) and generated by standard subcloning techniques. DNA sequences of the cloned exon three 3′ UTR and adjacent 3′ flanking region were confirmed to be identical to those in the public human genome data base and are designated by the number of nucleotides following the translational stop codon in exon 3. The −600/+3142 transgene was generated by digestion of the original 10 kb genomic clone with *Bgl*II and *Sph*I. In addition to preparation for microinjection, this 6.7 kb fragment was subcloned into pSL1180 (Pharmacia LKG Biotechnologies, Piscataway, NJ). The −350/+3142 transgene was obtained by partial digestion of the *Bgl*II–*Sph*I–pSL1180 plasmid with *Nsi*I and *Sph*I and isolation of the linear 6.5 kb fragment. The −350/+2138 transgene was generated by complete *Nsi*I digestion of the *Bgl*II–*Sph*I–pSL1180 plasmid and isolation of the linear 5.5 kb fragment. The −350/+1262 transgene was obtained by double digestion of the *Bgl*II–*Sph*I–pSL1180 plasmid with *Nsi*I and *Bsr*GI and isolation of the linear 4.7 kb fragment.

The −600/+1227 transgene was isolated as a linear 4.9 kb fragment by digestion of an intermediate plasmid with *Sal*I and *Nae*I at restriction sites present in the multiple cloning site. The intermediate plasmid was first generated by a three-way ligation of *Bgl*II–*Kpn*I (−600 to the second intron) and *Kpn*I–*Nhe*I (second intron to +1227 of 3′ UTR/flank) fragments from the 10 kb FSHβ genomic clone into a pGEM2 vector linearized with *Bam*HI and *Nhe*I (Promega, Madison, WI).

The −350/+1227SV40 polyA transgene was generated as a linear 5.6 kb fragment by restriction digestion of an intermediate plasmid with *Eco*RI and *Sph*I. The intermediate plasmid was generated by first subcloning the *Nsi*I–*Nsi*I 5.5 kb fragment (−350/+2138 of hFSHβ described above) into the *Pst*I polylinker site of pGEM3Zf(+). The resulting plasmid was then linearized at the internal *Nhe*I site at position +1227 of hFSHβ, blunt-ended by a fill-in reaction with the Klenow fragment of DNA polymerase, and further digested with *Sph*I in the polylinker site. Finally a 0.9 kb *Sma*I–*Sph*I fragment (*Mbo*I–*Mbo*I representing nucleotides 4710–4100 comprising the intron of small T-antigen ligated to *Bcl*I–*Bam*HI, nucleotides 2770–2533 containing the polyadenylation signals of SV40 TAg early mRNAs previously subcloned into *Sma*I and *Bam*HI sites of pSL1180) was ligated into the blunted *Nhe*I–*Sph*I vector.

Assembly of the SV40 tsTAg transgene construct has been described previously (Kumar et al., 1998). Briefly, the −4000 bp of 5′ flanking sequences, the first exon, the first intron, and the 5′ end of exon 2 up to a PCR-generated *Eco*RV site immediately upstream of the methionine start codon for FSHβ was ligated to a 2.7 kb *Stu*I–*Bam*HI fragment including the large T coding region and polyadenylation signals of the SV40 temperature sensitive T-antigen A58 (tsTAg A58) and 2.1 kb of 3′ flanking sequences starting from a *Bam*HI site at +1031 in the 3′ UTR of hFSHβ.

2.2. Transgenic mouse lines

Development of transgenic lines expressing the 10 kb hFSHβ clone and the hFSHβ-SV40 tsTAgA58 construct have been described previously (Kumar et al., 1992; Kumar and Low, 1993, 1995). Subsequent transgenic lines were produced from DNA fragments generated as indicated above and purified and microinjected into fertilized oocytes as described previously (Low, 1992). Founders and subsequent transgenic progeny from outbreeding to CD-1 or SW-1 mice were identified by Southern blotting or by DNA dot blot analysis as previously described utilizing [^{32}P]-dCTP random primer labeled probes (Boehringer Mannheim, Indianapolis, IN) (Kumar et al., 1992; Kumar and Low, 1993, 1995). For all constructs containing hFSHβ coding sequences, the probes were directed towards either a 450 bp *Pst*I–*Bam*HI segment in the 3′ UT region or a 1.4 kb *Pst*I–*Pst*I segment comprising part of the second intron, the third exon and a portion of the 3′ UT region. In addition, the integrity of 5′ sequences was verified in the −350/+2138 transgenic pedigrees by Southern blotting utilizing a 1.1 kb probe directed towards the *Bgl*II–*Asp*I segment comprising a portion of the 5′ flanking sequences and the first exon and intron. SV40 tsTAgA58 transgenic mice were identified utilizing a probe directed towards a 1.55 kb *Pst*I–*Bst*XI segment in the SV40 tsTAgA58 sequence. All animal studies were approved by the OHSU Institutional Animal Care and Use Committee and conducted in accord with the Public Health Service Guide for the Care and Use of Laboratory Animals.

2.3. Expression analysis

Evaluation of transgenic expression of the 10 kb hFSHβ genomic clone has been described in detail (Kumar et al., 1992; Kumar and Low, 1993, 1995) utilizing Northern blotting, RNA dot blotting, dual-label immunofluorescence

and radioimmunoassay of serum and pituitary extracts. Subsequent transgenic lines containing coding regions of the hFSHβ gene were screened for expression by RNase protection assay (RPA) described below and/or dual-label immunofluorescence.

2.4. RNase protection assay

Pituitary glands were collected and triturated ten times through a 25 g needle in 225 μl 0.01 M Tris–HCl pH 7.0, 0.15 M NaCl, 0.002 M $MgCl_2$, 1% NP-40 (Sigma, St. Louis, MO) with 10 units RNasin, then added to an equal volume of 0.01 M Tris–HCl, pH 7.6, 0.15 M NaCl, 0.005 M EDTA and 1% SDS. Nuclei were pelleted by centrifugation (14,000 rpm at 4 °C for 10 min). Cytosolic fractions were collected and extracted with 25:24:1 phenol–chloroform–isoamyl alcohol using 10 μg tRNA as a carrier. Total RNA was precipitated with two volumes of ethanol at −20 °C overnight and was collected by centrifugation (14,000 rpm at 4 °C for 30 min). Riboprobes were prepared using SP6 or T7 RNA polymerases (Promega, Madison, WI) and full-length probes isolated by electrophoresis on a 5% denaturing polyacrylamide gel and eluted overnight in Probe Elution Buffer (Ambion, Austin, TX). The mFSHβ riboprobe corresponded to nucleotides 2790–3115 in exon 3 (Graham et al., 1999) resulting in a 396 nt full-length probe and a 325 nt protected band. The hFSHβ riboprobe spanned the *Xba*I–*Sac*I sites, nucleotides +401 to +673 after the end of the translated sequence in the 3′ UT region (Graham et al., 1999) and resulted in a 356 nt full-length probe and a 277 nt protected band. The β-actin full-length probe was 300 nt and protected a 250 nt fragment. RPA was performed using the RPA II kit (Ambion, Austin, TX). RNA pellets were resuspended in hybridization solution containing 80,000 cpm each of [^{32}P]-rUTP-labeled antisense β-actin, mFSHβ- and/or hFSHβ-specific riboprobes and hybridized overnight at 37 °C. Unprotected fragments were digested with a mixture of RNase A and T1. Digested RNA was precipitated according to the manufacturer's protocol and resuspended in a denaturing loading buffer. Protected bands were separated on a 5% denaturing polyacrylamide gel run at 25 mA for 2–3 h and imaged using a phosphorimaging system (Molecular Dynamics, Sunnyvale, CA). Statistical analysis of relative band intensities was performed using StatView 5.0.1 Power PC version (SAS Institute Inc., Cary, NC).

2.5. Pituitary hormone immunofluorescence

Control and transgenic mice were anesthetized and perfused transcardially with 4% paraformaldehyde in phosphate-buffered saline, pH 7.2. Pituitary glands were post-fixed and freeze-protected in the same fixative with 10% sucrose. Ten to 20 μm cryostat sections were mounted on gelatin-coated slides for immunofluorescence.

Co-localization of hFSH and other pituitary hormones was evaluated with the following primary antisera: mouse monoclonal IgG anti-hFSH F-217-06 (Medix Biotech, Inc., Foster City, CA) at 1 μg/ml; anti-rLHβ S-10, 1:5000; anti-rTSHβ S-5, 1:5000; anti-hGH S-2, 1:1000; anti-rPRL S-9, 1:25; anti-hACTH Kendall 699, 1:1000 (provided by the National Hormone Pituitary Program, NIDDK and Dr. A.F. Parlow). The specificity of the hFSH monoclonal antibody has been previously demonstrated, specifically with no cross-reactivity seen with LH or mouse FSH. Binding was detected with either goat anti-rabbit IgG-fluorescein (FITC) or -rhodamine isothiocyanate (RITC, 1:50; Tago, Inc., Burlingame, CA) or goat anti-mouse IgG–rhodamine isothiocyanate (1:50; American-Qualex, LaMirada, CA). Pituitary sections from nontransgenic and mice expressing the 10 kb hFSHβ transgene were included on each slide as negative and positive controls, respectively (Kumar et al., 1992).

2.6. SV40 T-antigen detection

Detection of the hFSHβ-SV40 tsTAgA58 transgene has been previously described in detail by in situ hybridization (Grewal et al., 1999) and demonstration of T-antigen-induced gonadotroph adenomas in transgenic animals (Kumar et al., 1998).

2.7. Analysis of the genomic sequence

Sequence determination of the minimal regions of the hFSHβ gene demonstrated to be required for gonadotroph-specific expression was performed by automated sequencing using AutoAssembler (Applied Biosystems, Branchburg, NJ) and was identical to sequence publicly available from the NCBI human genome database. Phylogenetic comparisons of mammalian FSHβ genes were performed using software tools available in Ensembl V30 Sanger Institute (http://www.ensembl.org). The specific genome compilations used were: Human NCBI 35 April 2005; Mouse NCBI m33 April 2005; Rat RGSC 3.4 April 2005; Dog BROADD1 April 2005. The multicontig alignment feature in Ensembl uses BLASTz to identify regions of conserved sequence between species' pairs. The highly conserved tracks are then produced by re-scoring the BLASTz alignments using the 'subsetAxt' program (University of California, Santa Cruz) with a much stricter 'tight' nucleotide scoring matrix, a gap open penalty of 2000, a gap extension penalty of 50 and a minimum score threshold of 3400. Alignment of proximal FSHβ promoter sequences from multiple species was then performed with ClustalW program (http://www.ch.embnet.org/software/ClustalW.html). Inspection of sequences for potential transcription factor binding sites was performed by searching the TRANSFAC database using MatInspector (http://www.thr.cit.nih.gov/molbio/signal). In addition, a manual search was performed for Pit-1, SF-1, LHX-3, Pitx2c, Pbx1/Prep1, Ptx1 and CArG sites, androgen response elements, activin response elements and SMAD protein binding sites, and areas homologous for PGBE, URE, GnRHE and JRE sequences. The 3′ UTR and flanking regions of FSHβ genes from various species were analyzed using the Polyadenylation Cleavage Database (PACdb) (Brockman et al., 2005) at http://www.harlequin.jax.org/pacdb/ and multiple sequence alignments were performed with the combination of CHAOS and DIALIGN (Brudno et al., 2003) at http://www.dialign.gobics.de/chaos-dialign-submission.

3. Results

3.1. Detection of hFSHβ mRNA by RNase protection assay

Expression of hFSHβ mRNA relative to β-actin in the pituitary glands of transgenic mice was detected by RNase protection assay as shown in Fig. 1A. Human FSHβ mRNA was easily detected in transgenic pedigree #678 carrying multiple copies of the 10 kb −4000/+3142 genomic clone (Kumar et al., 1992) and was clearly distinguishable from the protected mFSHβ and β-actin bands. Generally, the levels of hFSHβ mRNA were similar to or less than endogenous mFSHβ mRNA. No protection of the hFSHβ probe was demonstrated in liver or non-transgenic pituitary glands. Expression of the transgene was retained with progressive truncations to −350 bp of 5′ flanking sequences, but not with truncations of 3′ flanking sequences. Pituitary expression of hFSHβ mRNA from the most truncated construct retaining sequences from −350 to +3142 is shown in Fig. 1B for male and female F1 mice derived from three independent lines. Levels of expression were variable among lines but consistent between mice within the same line. This pattern was also observed in the 10 kb −4000/+3142 transgenic lines and was independent of transgene copy number as previously reported (Kumar et al., 1992). Sexually dimorphic expression was consistently observed in all transgenic lines in which expression was detected, with significantly stronger expression demonstrated in male mice than in females (ratio ∼ 2:1) (two-factor ANOVA for band density of hFSHβ mRNA signal normalized to actin mRNA from Fig. 1B: $F_{1,6} = 21.9$, $P < 0.005$, main effect of gender; $F_{2,6} = 0.1$, $P = 0.95$, not significant, gender by line interaction).

3.2. Demonstration of hFSHβ expression by immunofluorescence in transgenic pituitary glands

Detection of the hFSHβ-mouse α-subunit protein heterodimer was performed by immunofluorescence with a hFSH-

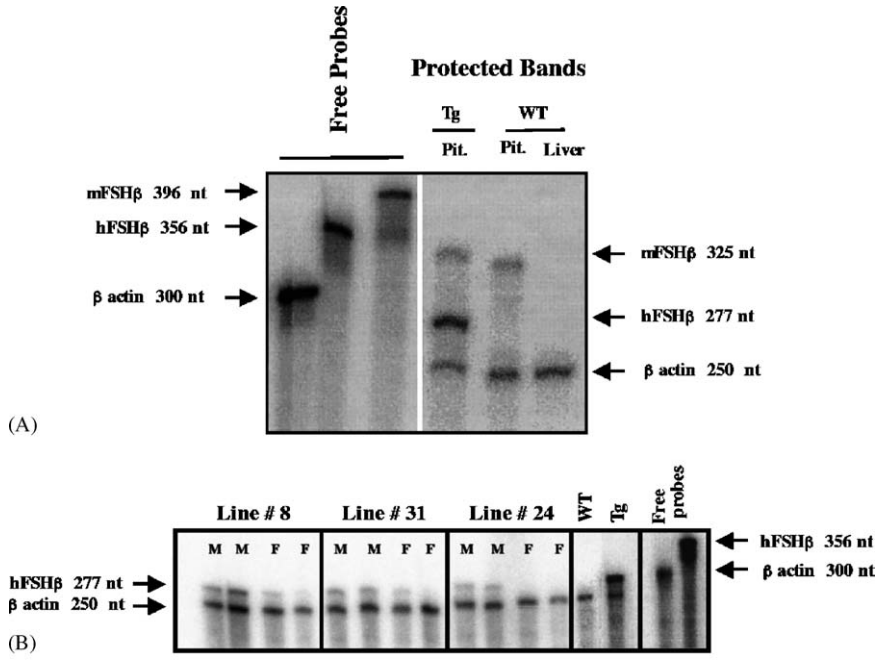

Fig. 1. Detection of hFSHβ mRNA in transgenic and wild-type mouse pituitary glands by RNase protection assay. (A) Protected RNA fragments (right) from pituitary glands demonstrating expression of the mFSHβ (325 nt) and hFSHβ (277 nt) genes as well as the control gene, β-actin (250 nt) in the pituitary gland of a male mouse carrying the 10 kb transgene (Tg) and protection of only the mFSHβ (325 nt) and β-actin (250 nt) bands in the pituitary of a wild-type (WT) mouse. β-actin (250 nt) expression only is detected in a liver RNA sample. The position of full-length free probes (left) is indicated (left): mFSHβ (396 nt), hFSHβ (356 nt) and β-actin (300 nt). (B) Demonstration of protected hFSHβ and β-actin mRNA fragments in male and female mice from three independent lines carrying the −350/+3142 transgene. There is stronger expression of the transgene in the male compared to female siblings. M: male; F: female.

specific monoclonal antibody and representative data are shown for male transgenic mice of lines from the highly expressing −350/+3142 transgene construct in Fig. 2A. The specificity of this antibody and lack of cross-reactivity for LH or mFSH was previously demonstrated (Kumar et al., 1992) and is again illustrated by the lack of fluorescence signal in a non-transgenic male pituitary gland (Fig. 2B). Expression of the transgene is clearly demonstrated in gonadotrophs by co-localization with a polyclonal rat LHβ antibody (Fig. 2C). A minority of cells demonstrating expression appear to be monohormonal gonadotrophs; LH-only gonadotroph (upper arrow) is shown in Fig. 2C and D and FSH-only gonadotroph (lower arrow) in Fig. 2C and E. No co-localization of hFSH with any other pituitary hormone including prolactin (Fig. 2F), TSH (Fig. 2G), GH (Fig. 2H) or ACTH (data not shown) was demonstrated.

Immunofluorescence for hFSH was qualitatively greater in male than in female transgenic mice, consistent with their respective hFSHβ mRNA levels. The number of immunopositive hFSH cells and intensity of immunofluorescence varied among lines, but was consistent between mice derived from any given line (compare line #31 in Fig. 2A to line #24 in Fig. 2C). Qualitatively, the total number of gonadotrophs as well as the pattern of immunoreactive gonadotroph cells evaluated by endogenous LHβ immunoreactivity and the distribution of other pituitary hormone cell types was similar to wild-type mice in all positively expressing transgenic lines from both the −600/+3142 and −350/+3142 constructs.

3.3. Localization of hFSHβ regulatory elements

The expression of all hFSHβ transgenes as evaluated by combinations of RNase protection assay, dual-label immunofluorescence, and SV40 tsTAg expression is summarized in Fig. 3. Truncation of 5′ genomic flanking sequences to either −600 or −350 bp of promoter relative to the transcriptional start site, in the context of intact 3′ flanking sequences extending to position +3142 relative to the translational stop codon, retained sexually dimorphic, gonadotroph-specific transgene expression. Deletion and replacement of exon 2, intron 2, and most of exon 3 by SV40 tsTAgA58 coding and polyadenylation signal sequences also resulted in the high penetrance of gonadotroph-specific transgene expression. Notably, very similar fractions of positively expressing lines/total lines generated were evident for each of the four transgenes exhibiting gonadotroph-specific expression, indicating that the penetrance and transcriptional strength of the retained hFSHβ regulatory elements were roughly equivalent and consistent with an equal probability of permissive integration sites.

In contrast, none of the hFSHβ transgenes that contained truncations of the 3′ genomic flanking sequences to either positions +2138, +1262, or +1227 demonstrated expression despite the inclusion of necessary 5′ promoter elements (either −600 or −350 bp). It has been shown that mRNA stability is related to the length of the polyA tail and specific sequences in untranslated regions (Wahle and Keller, 1992). Given the presence

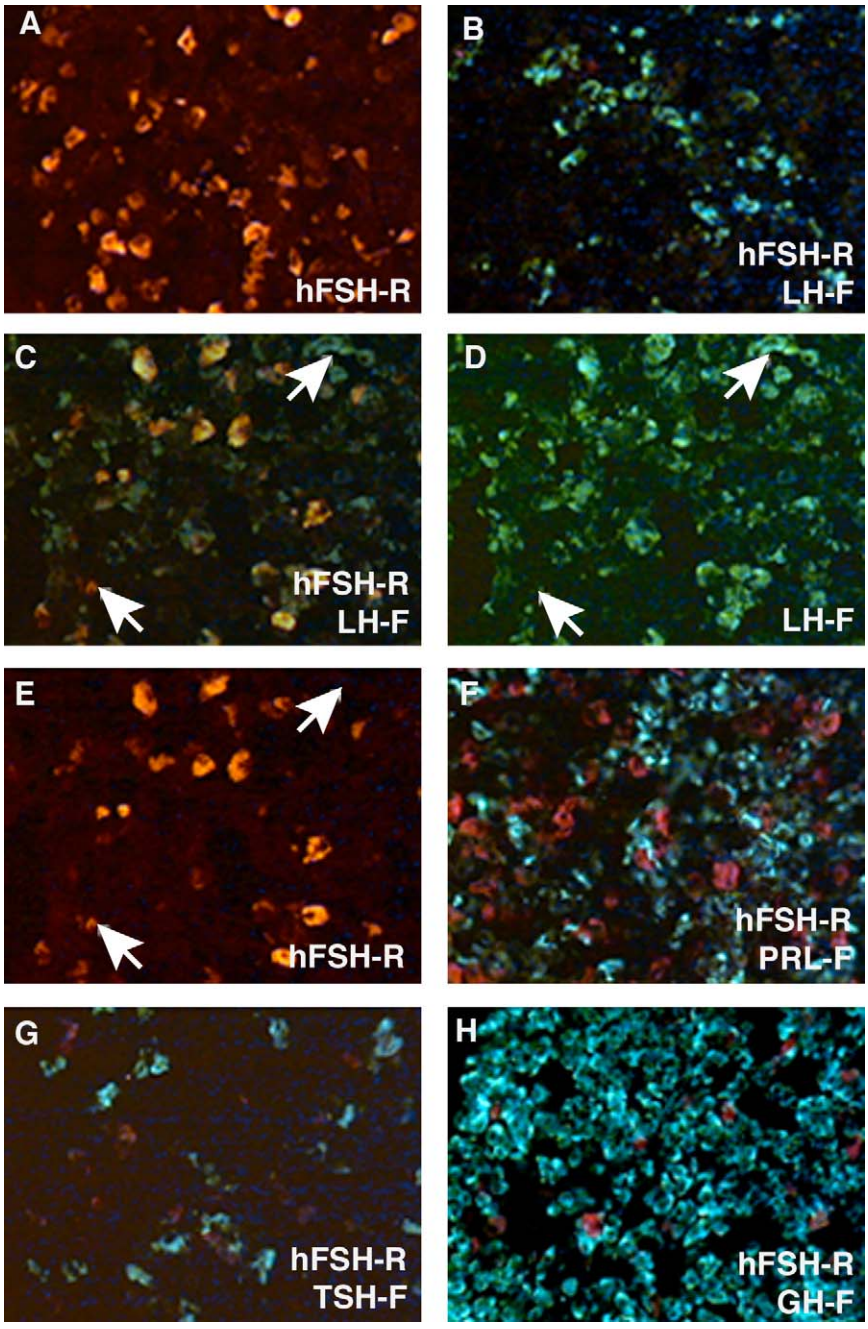

Fig. 2. Immunofluorescence detection of hFSH expression in pituitary glands from wild-type mice and transgenic mice expressing the −350/+3142 hFSHβ construct. (A) Detection of hFSHβ-mouse α-subunit heterodimers in a male pituitary gland with a hFSH-specific monoclonal primary antibody (Ab), RITC secondary Ab. (B) Specificity of the hFSH monoclonal Ab with no RITC staining seen in a wild-type male pituitary gland (also stained with LH primary Ab, FITC secondary Ab). (C) Co-localization of hFSH immunoreactivity (RITC second Ab) with rLH (FITC second Ab) in gonadotrophs. Note the presence of monohormonal gonadotrophs shown by the arrows (upper arrow, LH-only; lower arrow, FSH-only). (D and E) Monohormonal gonadotrophs are demonstrated more clearly in single filter images of the dual-labeled section. The LH-only cells are shown in (D), FSH-only in (E). (F) Lack of co-localization with lactotrophs. (G) Lack of co-localization with thyrotrophs. (H) Lack of co-localization with somatotrophs.

of multiple polyadenylation signals in the hFSHβ gene and location of the preferentially utilized site at position +1218 to +1223 at the end of the long 3′ UTR, mRNA misprocessing or instability was a potential explanation for the requirement of 3′ flanking sequences for high-level expression, particularly with the +1262 and +1227 3′ truncations. Therefore, we tested the ability of heterologous sequences that include strong termination and polyadenylation signals from the SV40 TAg gene to compensate for the lack of hFSHβ 3′ flanking sequences. Inclusion of these SV40 regulatory sequences in a construct containing −350 bp of 5′ promoter failed to recover detectable pituitary expression in any individual founder transgenic mouse out of 13 independent animals generated and tested.

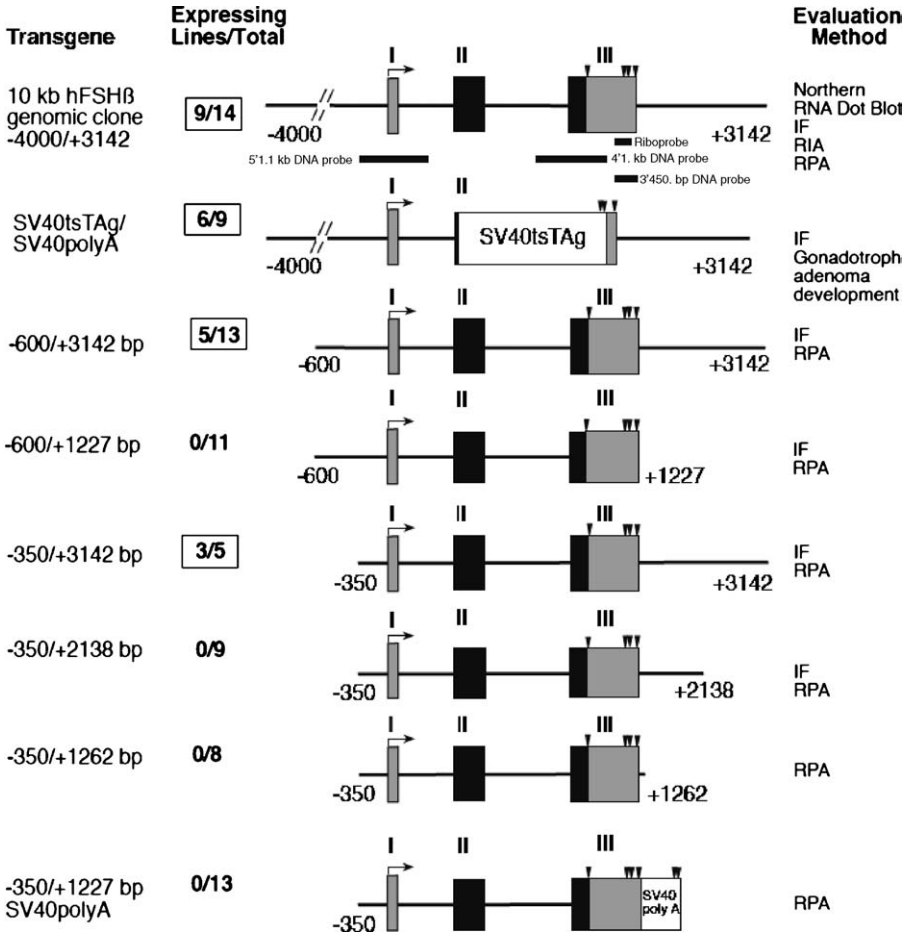

Fig. 3. Structure of the original 10 kb hFSHβ genomic clone and the transgene constructs. Negative numbers indicate positions 5′ to the transcriptional start, which is indicated by the arrow above exon I. Exons (I, II, and III) are indicated by rectangles with untranslated regions shaded gray and translated regions black. Polyadenylation signals (AAUAAA) are indicated by inverted triangles. Positive numbers refer to positions 3′ to the translational stop codon in exon III. The location of DNA probes used in genotyping and the riboprobe used in the RNase protection assay (RPA) to evaluate hFSHβ expression are shown below the 10 kb genomic clone. Expressing transgenes are indicated by boxed ratios of expressing lines/total. Nucleotide positions of natural restriction endonuclease sites used in subcloning or isolation of the hFSHβ transgene fragments are: *Bgl*II, −600; *Nsi*I, −350; *Nhe*I, +1227; *Bsr*GI, +1262; *Nsi*I, +2138; *Sph*I, +3142. Immunofluorescence, IF; radioimmunoassay, RIA. Drawings are not to scale.

3.4. Phylogenetic footprinting and analysis of the sequence of potential regulatory elements in the FSHβ gene

A multicontig comparison of mammalian FSHβ gene loci was performed with the Ensembl web server to align regions of phylogenetically conserved DNA sequences and compare their location within the 10 kb cloned hFSHβ gene and to the functional expression data obtained from the series of transgene constructions. This analysis revealed five highly conserved islands of sequence among human, dog, rat, and mouse that correspond precisely to the 5′ proximal promoter region, exon 2, the 5′ translated end of exon 3, the distal 3′ UTR of exon 3, and the most proximal portion of the 3′ genomic flanking sequence just downstream of exon 3 (Fig. 4). The latter two sequence islands encompass the most distal putative polyadenylation signal (AAUAAA) and transcriptional termination signals. In addition, there was a sixth highly conserved sequence island towards the 3′ end of intron 2 that was shared only between human and rat FSHβ genes. Less highly conserved regions of DNA sequence among the four mammalian FSHβ genes were present in the distal 5′ flanking regions corresponding to positions between −4000 and −350 and throughout the remainder of the transcriptional unit. Interestingly, an additional distal 3′ area of sequence conservation lying between positions +2138 and +3142 of hFSHβ was present among human, dog, and rat but absent from the mouse FSHβ gene.

Currently only the human FSHβ gene is annotated in the PACdb and the limited EST-genomic comparison identified only the proximal polyA signal sequence that is coincident with the STOP codon in exon 3 and results in the minor proportion of 700 nt FSHβ mRNA transcripts in human pituitary. However, using the CHAOS/DIALIGN program, >90% nucleotide sequence identity was found among nine mammalian species of FSHβ 3′ UTRs for a 40 bp A/T rich region from positions +1192 to +1231 surrounding the AAUAAA signal (data not shown). This small region of the 3′ UTRs was clearly the most highly conserved phylogenetically and is consistent with an assignment of the most distal AAUAAA sequence as the preferential polyadenylation and transcriptional termination signal for mammalian FSHβ genes.

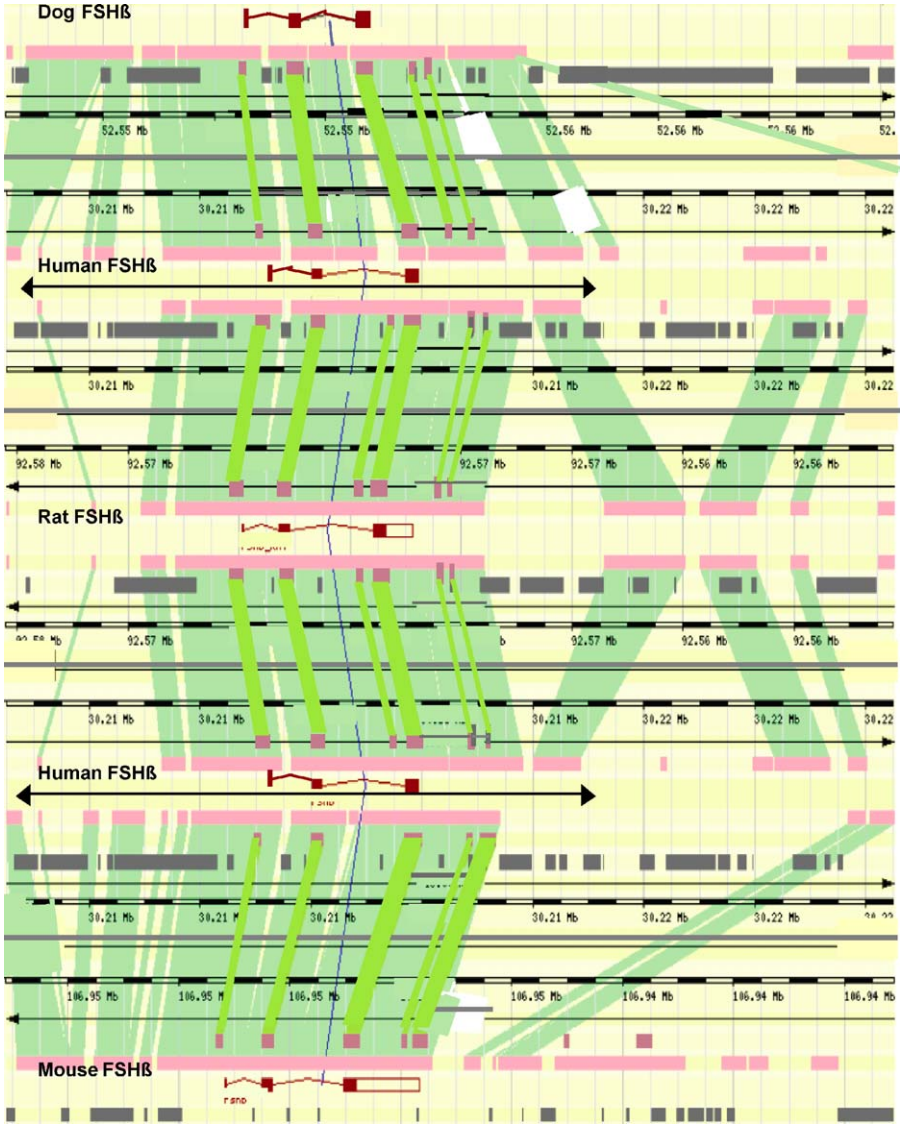

Fig. 4. Multicontig alignment of mammalian FSHβ genomic sequences. Genomic sequences from dog chromosome 21 (+strand), rat chromosome 3 (−strand), and mouse chromosome 2 (−strand) were aligned to human chromosome 11 (+strand) using the multicontig feature of the Ensembl genome web server. The black double-headed arrow indicates the location of the 10 kb cloned human genomic fragment used to generate transgene constructions. The three exons separated by two introns and long 3′ untranslated sequence of exon 3 for each species' primary FSHβ transcript are depicted by dark red solid boxes and open red boxes, respectively. The alignments of conserved sequences (light pink boxes) and highly conserved sequences (dark pink boxes) between species′ pairs of genomic sequences are indicated by light green and dark green bands, respectively. Repetitive DNA sequences of all classes for each species are depicted by gray boxes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

A more comprehensive sequence alignment and analysis of potential transcription factor binding sites among seven mammalian species was performed for the highly conserved −350 bp of proximal 5′ flanking genomic DNA containing the basal promoter and functionally important enhancer region for FSHβ gene transcription in pituitary gonadotrophs (Fig. 5). Consensus binding sites for a number of transcription factors were identified in −350 bp of FSHβ promoter region of human as well as in various other species using a combination of TRANSFAC analysis and manual inspection based on published sequences (Table 1). Multiple sex steroid hormone binding sites are conserved, as are sites for tissue-specific transcription factors including NF-1 and GATA-1, and more ubiquitously expressed factors including AP-1, SMADs, SP-1 and CCAAT-binding factor (CBF, or NF-Y). Notably absent are consensus binding sites for Egr-1, Lim-2 or CArG, required elements for LHβ transcriptional activation or any other factors (besides SF-1, LHX3 and Ptx1) required for basal or GnRH stimulated expression of the α-subunit.

4. Discussion

The identification of genetic elements involved in the molecular mechanism of the regulation of the gonadotropin β-subunits has been significantly hampered by the paucity of homologous in vitro expression systems. Although the LβT2 cell line has recently emerged as an extremely useful model system, rigorous analysis of the promoters and 3′ flanking regulatory elements

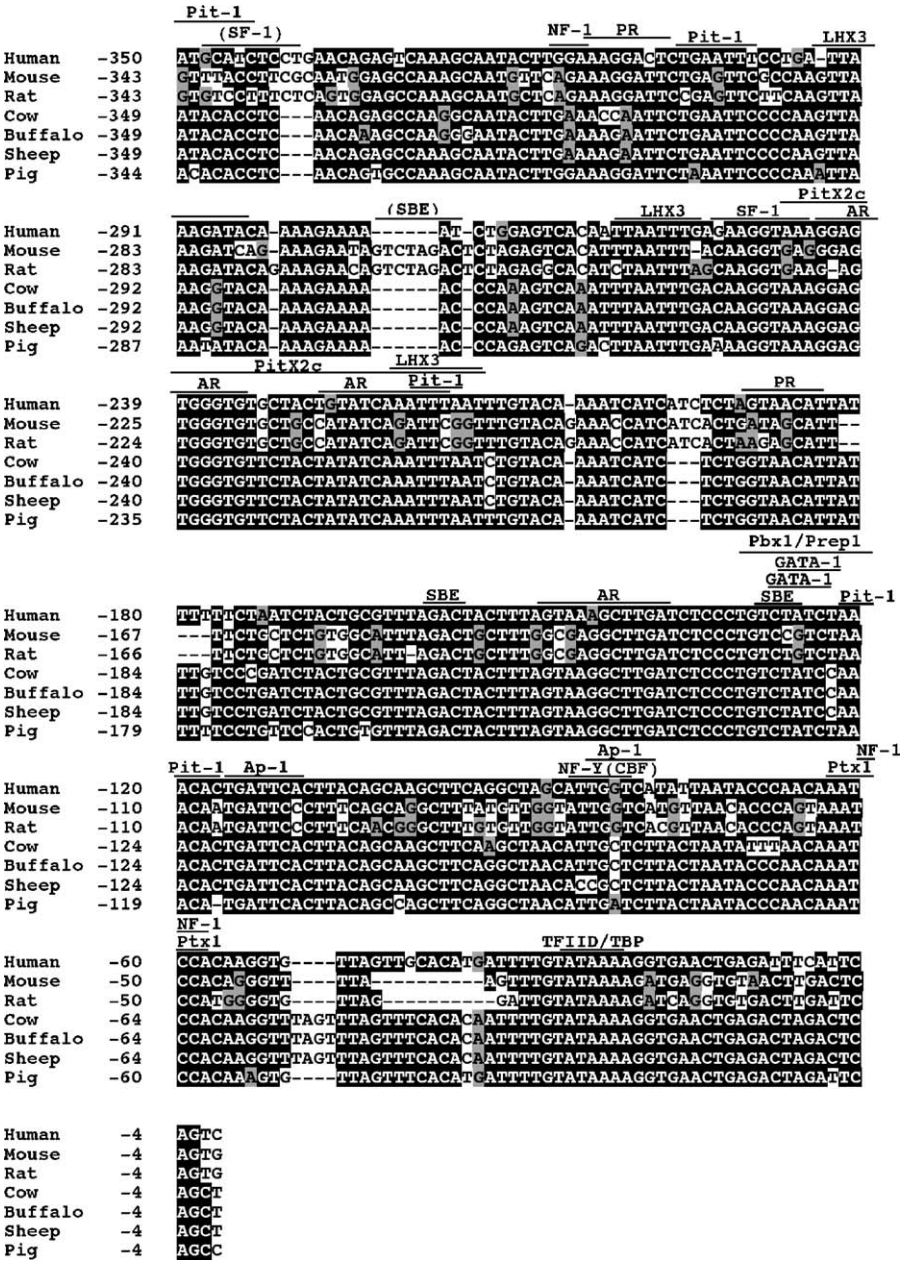

Fig. 5. Sequence alignment of the proximal 5′ FSHβ gene promoter from seven mammalian species and location of putative transcription factor binding sites. The nucleotide sequence ATGCAT at the 5′ end of the human sequence (+1) corresponds to the *Nsi*I restriction endonuclease site at position −350 bp relative to the transcriptional start site in the transgene constructions. The corresponding promoter regions of six additional species including mouse, rat, cow, buffalo, sheep and pig were downloaded from NCBI BLAST search of the human promoter sequence and used in a ClustalW multiple sequence alignment program as described in the Methods and Results section. The conserved nucleotides across species are indicated in shaded backgrounds.

of the FSHβ subunits has only been partially achieved. In contrast to its utility for evaluation of ovine, mouse and rat FSHβ subunit genes, the pattern of transgenic expression of human genomic FSHβ constructs has not been recapitulated by reporter constructs in this cell line (our unpublished data). Therefore, we report here transgenic mapping of the hFSHβ gene which reveals a minimum sequence requirement of −350 bp of 5′ flanking region and an unusual importance of +3142 bp following the translational stop codon in exon 3 to confer gonadotroph-specific, and sexually dimorphic transgene expression. This −350 bp proximal 5′ promoter region contains numerous transcription factor consensus sequences and areas of homology to sequences in other species that have been demonstrated in several in vitro studies to be important for hormonally regulated expression of the FSHβ subunit gene in different species. Specifically, although there were no Lim-2 sites (important for LHβ expression), areas homologous to LHX3 (Lim-3) binding sites in the pFSHβ gene (West et al., 2004) are present at −295, −261 and −221 in the hFSHβ promoter. LHX3 also induces expression of −5663 bp and −1622 bp hFSHβ reporter constructs and the α-subunit gene in LβT2 and heterologous cells (West et al., 2004) and thus is poised along with SF-1 and Ptx1 to be one

Table 1
Potential transcription factor binding sites in the −350 bp promoter region of the hFSHβ gene that are also conserved across multiple mammalian species

Transcription factor	Location	Strand	Sequence
Pit-1	−350	(−)	GATGCAT
	−307	(−)	AATTCAG
	−219	(−)	TAAAT
	−123	(+)	TAAACAC
SF-1	−253	(−)	TTACCTTCT
NF-1	−318	(−)	TCCA
	−61	(+)	TCCA
PR	−315	(−)	AGTCCTTT
	−191	(−)	TGTTACT
LHX3 (LIM-3, p-LIM)	−295	(+)	A-TTAAAGATA
	−261	(+)	TTAATTT
	−221	(+)	AAATTTAAT
Pitx2c	−247		(Exact binding sequence within 34 bp not established)
SMAD2/4, SMAD3/4 (SBE)	−159	(−)	AGAC
	−130	(+)	GTCT
Pbx1/Prep1	−132	(+)	CTGTCTATCTAA
AR	−244	(+)	AGGAGTGGGTG
	−227	(+)	TGTATCAAATTT
	−149	(+)	AGTAAAGCTTGA
GATA-1	−129	(+)	TCTATC
	−129	(−)	GATAGA
	−128	(+)	CTATCT
	−128	(−)	AGATAG
AP-1	−116	(−)	TGAATCA
	−85 (1/2 site)	(+)	TTGGTCA
CCAAT-binding factor (CBF, NF-Y)	−86	(+)	ATTGG
Ptx1	−56	(+)	AAATCCA
Sp-1	−38	(+)	TGCAC
TFIID/TBP	−31	(+)	TATAAA

of the mediators of coordinated LHβ and FSHβ gonadotropin subunit expression.

LHβ expression involves combinatorial transcriptional activation by the zinc finger proteins Egr-1 and SF-1, as well as the homeodomain Ptx1 (Tremblay and Drouin, 1999). Also absent from the minimal 5′ proximal promoter region of hFSHβ are any sites for Egr-1 binding. However, in the mFSHβ gene, SF-1 interacts with NF-Y (CCAAT binding factor, CBF) and has been proposed to be a component of a similar combinatorial mechanism of transcriptional activation. The second of the two functional SF-1 sites is conserved across species, including hFSHβ at −253 (the distal site at −348 is conserved only in mouse and rat) and the NF-Y site is also conserved at −86 (Jacobs et al., 2003). Further, Ptx1 is involved in basal and GnRH stimulated expression of the rFSHβ gene and the DNA sequences involved in that binding are also conserved at −56 in the hFSHβ promoter (Zakaria et al., 2002). Thus, there is conservation of binding sites for the full spectrum of appropriate types of transcription factors sufficient to allow a similar mechanism of combinatorial transcriptional activation as that demonstrated for the LHβ gene.

Consistent with the involvement of these factors in FSHβ regulation are the differential effects on FSH and LH observed in mice with SF-1 and Egr-1 gene deletions. Specifically, the *FTZ*-F1 mouse, in which SF-1 was deleted, demonstrates a lack of both pituitary LH and FSH, due to failure of pituitary gonadotrophs to develop (Ingraham et al., 1994). Evaluation of the Egr-1 knockout mouse revealed male and female sterility due to specific failure to synthesize pituitary LHβ and ovarian LH receptor, but normal FSH expression (Lee et al., 1996; Topilko et al., 1998). The lack of consensus paired Egr-1 sites in the functionally relevant and conserved inter-species FSHβ gene sequences analyzed here suggests that this transcriptional regulatory pathway is one of the key differences between the molecular mechanisms resulting in gonadotroph-specific expression of the LHβ and FSHβ genes.

Another significant difference between the regulation of the two gonadotropin β-subunits is the critical importance of the activin system in FSHβ regulation (Bailey et al., 2004; Graham et al., 1999; Pernasetti et al., 2001; Weiss et al., 1993, 1995). One of the major transcriptional mediators of the activin system is the Smad signaling pathway, with Smad-2/Smad-3 or Smad-3/Smad-4 complexes binding at Smad-binding elements (SBEs), which contain the short 4 bp sequence CAGA or GTCT. This sequence is commonly found, estimated to occur randomly every 256 bp, and binding is generally low affinity, requiring transcription factor partners for full activity. Therefore, demonstrating functionality of each identified putative element is critical. An elegant mapping of functional Smad sites in the oFSHβ gene was recently done by Bailey et al. (2004) and evaluated in rFSHβ by Gregory et al. (2005). They identified multiple pairs of SBEs including three within the proximal 300 bp of 5′ FSHβ promoters. Of those, the site at −268 in the rat promoter binds Smad-3 (Gregory et al., 2005) that interacts with the bicoid-related homeodomain factor Pitx2c, but this SBE is notably missing from all species except rat and mouse (Suszko et al., 2003). The Pitx2c site appears conserved at −247 in the hFSHβ promoter, however, leading to the possibility of interaction of Pitx2c with Smads bound at other SBEs. There is conservation of the SBE at −159, however Bailey and coworkers demonstrated that the ovine sequence does not bind Smads (Bailey et al., 2004). The site at −130 is conserved across multiple species, binds Smads and confers activin responsiveness to the ovine and mouse promoters in association with the TALE homeodomain proteins Pbx1 and Prep1 which bind an adjacent region that is also conserved. This body of evidence confirms activin responsiveness as a second major difference between LHβ and FSHβ regulation. The sequence homology observed in the hFSHβ promoter suggests that the activin system is a critical regulator for this gene as well.

In addition to gonadal peptides, gonadal steroids are known to regulate the FSHβ gene (Burger et al., 2004). Estrogen is thought to mediate its repressive effects indirectly via modulation of GnRH secretion. Androgen administration results in species-specific effects on direct pituitary regulation

of FSHβ transcription. Consistent with this model, no ERE was identified in the −350 bp proximal promoter, despite this region conferring sexually dimorphic expression. In contrast, conserved androgen response elements were identified, and those corresponding to −244 and −149 are functional in the oFSHβ promoter (Spady et al., 2004).

Equally complex is the role of AP-1 sites in the regulation of FSHβ genes. Multiple AP-1 sites have been reported in various species, and have been evaluated extensively in the ovine FSHβ gene (Huang et al., 2001a, 2001b; Strahl et al., 1997, 1998). Of the four sites present within 200 bp of proximal oFSHβ promoter, only those at −120 and −83 demonstrate binding and are functional in activating the promoter in vitro. The −120 site differs by one nucleotide in the mFSHβ gene and is nonfunctional, however is conserved identically at −116 in the hFSHβ gene. The −83 site is not conserved in the hFSHβ promoter. Additionally, the hFSHβ promoter shows conservation of an AP-1 half-site at −76 (distinct from the −83 ovine site) corresponding to a site in the mFSHβ promoter that was demonstrated to be functional and to interact with NF-Y (CBF) in binding the adjacent CCAAT box. Thus, it is difficult to predict whether the hFSHβ gene will mimic the ovine or mouse model, but transcriptional activation by AP-1 binding is likely to be a contributing factor to high-level expression.

Several promoter elements were identified that are of lower likelihood to represent true functional elements, despite the high level of conservation of DNA sequence observed. Pit-1 is a POU homeodomain protein important in the development of the thyrotroph, somatotroph and lactotroph lineages with transcriptional activity at the growth hormone, prolactin, TSHβ and Pit-1 genes (Zhu et al., 2005). Actions of Pit-1 at the gonadotroph have not been described. In the tripartite model of LHβ activation, paired Sp-1 sites in the distal promoter facilitate interaction of tissue-specific transcription factors in the proximal promoter (Kaiser et al., 2000). The presence of a single Sp-1 site in the proximal FSH promoter is of questionable significance. Although GATA-2 and GATA-3 transcription factors are known to be involved in α-subunit expression (Steger et al., 1994), GATA-1 is not known to be involved in other gonadotropin subunit expression, and is typically expressed solely in hematopoietic tissues. Further, this short sequence has high frequency of random appearance in the genome; a similar caveat exists for NF-1 sequences. For all of these potential promoter elements, functional characterization will be even more critical.

The requirement of a small portion of proximal 5′ flanking sequences for high-level expression of FSHβ is not surprising, as critical promoter and enhancer elements are typically present in this location. Further, our phylogenetic analysis revealed areas of high homology only in the proximal portion of the 5′ flanking region, suggesting that the other areas of moderate homology in the 5′ flanking region between −4000 and −350 bp might contain modifying but not critical regulatory elements. However, the importance of elements in the 3′ flanking region demonstrated by our transgenic mapping is more unusual, and may be unique to hFSHβ among the gonadotropin subunit genes. Reporter constructs for both the α-subunit and LHβ subunit genes, containing only 5′ promoter elements, demonstrate pituitary expression in transgenic mice (Albanese et al., 1996; Keri et al., 1994). In addition, pituitary-specific expression has been obtained with 5′ sequences alone for both the bovine FSHβ (Markkula et al., 1993) and ovine FSHβ (Webster et al., 1995) genes in transgenic mice. Further, the importance of 3′ elements may be specific to transgenic expression of the hFSHβ gene, as we and others have found that 5′ hFSHβ reporter constructs expressed in LβT2 cells (West et al., 2004), but did not recapitulate the same pattern of expression found in our transgenic experiments (unpublished data).

The hFSHβ gene has an unusually long 3′ UTR. In addition, the gene contains multiple polyadenylation signals with the location of the preferred site utilized in human pituitary gonadotrophs at nucleotide positions +1218 to +1223 in this 3′ UTR based on analyses of mRNA species (Jameson et al., 1988). This assignment is also supported by the >90% nucleotide sequence identity among 9 mammalian species of FSHβ 3′ UTRs for a 40 bp region from +1192 to +1231 surrounding the AAUAAA signal (data not shown). Therefore, a potential role of mRNA instability or inefficient transcription termination as a reason for the lack of expression of the 3′ truncated constructs was intriguing. Evaluation of an hFSHβ-SV40 polyA transgene, which contains the strong termination sequence and polyadenylation signals of SV40 early mRNAs, also revealed poor expression and argues against deficient mRNA processing as an explanation for the lack of transgene expression. However, it is a formal possibility that an inefficient utilization of SV40 pA signals in the gonadotrophs may also result in lack of expression of the above transgene. For example, studies in gene targeted mutant mice with intronic insertions of a neomycin selection marker followed by SV40 pA showed that in some cell populations of the mouse, the SV40 pA is not used efficiently (Kakoki et al., 2004; Liang et al., 2004; Riviere et al., 1998). Similarly, in some cases, transgene constructs in which the SV40 polyA replaced the polyA of the endogenous gene gave rise to variable expression when expressed in mice (Guo et al., 1998). Nevertheless, we and others have demonstrated that SV40 pA sequences are sufficient for transgene expression in the pituitary (Low et al., 1993) and specifically in gonadotrophs (Alarid et al., 1996; Turgeon et al., 1996; Windle et al., 1990; Pernasetti et al., 2003).

The need of the 3′ UTR in the hFSHβ expressed in TG mice might not exactly recapitulate what occurs in human cells. This might be due lack or gain of different transcription factors in either organism. An alternate explanation for the lack of expression of the 3′ truncated hFSHβ transgenes could be mRNA instability. In a recent systematic study, it has been demonstrated that the expression of genes can be altered in ES cells or in mice by modifying their 3′ UTRs while retaining chromosomal location, promoters and introns (Kakoki et al., 2004). To distinguish between transcriptional regulation and mRNA stability, additional transgenic mice will need to be produced in the future that express constructs containing −350 of 5′ flanking region followed by exon I-SV40 TAg-SV40 polyA and +3142 of 3′ flanking sequences and/or −350 of 5′ flanking region followed by exons I, II and III and SV40 polyA +3142 of 3′ flanking sequences.

The 3′ flanking region in various genes has been shown to be important for efficient expression in several cell types. Human activin-β A subunit gene requires 3′ flanking sequences for its expression in HT 1080 fibrosarcoma cells (Tanimoto et al., 1993) and a calcium-inducible, epidermal-specific regulatory element in the 3′ flanking region of the human keratin 1 gene (Rothnagel et al., 1993) has been previously identified. Although functional 3′ elements are relatively unusual, distant enhancers are not unique to the glycoprotein family of genes. High-level expression of the α-subunit gene requires an as yet unidentified enhancer located 3.7–4.6 kb upstream of the transcriptional start site (Brinkmeier et al., 1998). Further localization of the critical element(s) within positions +2138 and +3142 of the hFSHβ gene and functional characterization of potential transcriptional enhancer properties (including position and orientation independence) will require additional transgenic analyses. It is intriguing that in our phylogenetic analysis, this region of sequence appears to be absent from the mouse, but moderately conserved in rat and cow FSHβ genes (data not shown). These observations suggest that there are species-specific differences in regulation of the FSHβ genes.

Similar to most transgenic analyses of gene regulation, expression was not observed in all independent lines established for any given transgene construct, and levels of expression were variable among lines that did express (Brinkmeier et al., 1998; Kendall et al., 1994; Kumar et al., 1992). We have previously demonstrated for the transgene comprised of the full 10 kb genomic clone that the variability in expression among lines was not solely dependent on transgene copy number. We postulate that more or less favorable chromosomal integration sites play a role in this variability. A small number of monohormonal hFSH cells were present in all the expressing transgenic lines consistent with reports of monohormonal gonadotrophs in various conditions (Childs and Unabia, 1997). We cannot completely exclude the possibility that hFSH expression in these cells was ectopic, however, the lack of co-localization with any of the classical pituitary hormones argues against it.

In summary, our transgenic studies map the location of critical regulatory elements for gonadotroph-specific expression to −350 bp of the proximal 5′ promoter. Phylogenetic analysis demonstrates regions of highly conserved sequence corresponding to this location. Sequence analysis of this region reveals that it contains putative binding sites for tissue-specific transcription factors that could serve to coordinate transcription of all three gonadotropin subunits, specifically by LHX3, SF-1 and Ptx1. Although there are distinct differences identified between putative sites in the FSHβ and LHβ genes, the spectrum of transcription factors identified is promising for a model of combinatorial transcriptional activation similar to that observed for the LHβ gene. The finding of high sequence conservation across multiple species strongly support the recent analyses of FSHβ gene transcription in transfected cell lines by other laboratories (Bailey et al., 2004; Bernard, 2004; Coss et al., 2004; Gregory et al., 2005; Jacobs et al., 2003; Pernasetti et al., 2001; West et al., 2004; Zakaria et al., 2002). The essential agreement of the in vivo and in vitro data provide further impetus to concentrate future analyses of FSHβ gene regulation on these proximal promoter sequences until the unusual relationship of the distal 3′ flanking sequences to hFSHβ transgene expression in vivo is better understood.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mce.2005.12.006.

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