Published in final edited form as:
Mol Endocrinol. 2004 May ; 18(5): 1158–1170. doi:10.1210/me.2003-0442.

Activin Regulation of the Follicle-Stimulating Hormone *β*-Subunit Gene Involves Smads and the TALE Homeodomain Proteins Pbx1 and Prep1

JANICE S. BAILEY, **NAAMA RAVE-HAREL**, **SHAUNA M. McGILLIVRAY**, **DJURDJICA COSS**, and **PAMELA L. MELLON**
Department of Reproductive Medicine, University of California San Diego, La Jolla, California 92093

Abstract

FSH is critical for normal reproductive function in both males and females. Activin, a member of the TGF*β* family of growth factors, is an important regulator of FSH expression, but little is known about the molecular mechanisms through which it acts. We used transient transfections into the immortalized gonadotrope cell line L*β*T2 to identify three regions (at −973/−962, −167, and −134) of the ovine FSH *β*-subunit gene that are required for full activin response. All three regions contain homology to consensus binding sites for Smad proteins, the intracellular mediators of TGF*β* family signaling. Mutation of the distal site reduces activin responsiveness, whereas mutation of either proximal site profoundly disrupts activin regulation of the FSH*β* gene. These sites specifically bind L*β*T2 nuclear proteins in EMSAs, and the −973/−962 site binds Smad4 protein. Interestingly, the protein complex binding to the −134 site contains Smad4 in association with the homeodomain proteins Pbx1 and Prep1. Using glutathione *S*-transferase interaction assays, we demonstrate that Pbx1 and Prep1 interact with Smads 2 and 3 as well. The two proximal activin response elements are well conserved across species, and Pbx1 and Prep1 proteins bind to the mouse gene *in vivo*. Furthermore, mutation of either proximal site abrogates activin responsiveness of a mouse FSH*β* reporter gene as well, confirming their functional conservation. Our studies provide a basis for understanding activin regulation of FSH*β* gene expression and identify Pbx1 and Prep1 as Smad partners and novel mediators of activin action.

FSH IS SECRETED by the anterior pituitary gonadotrope and acts on the gonad to regulate ovulation and spermatogenesis. This glycoprotein hormone is a member of the family that also includes LH, TSH, and chorionic gonadotropin. Each of these hormones is a heterodimer, comprised of a common *α*-subunit and a unique *β*-subunit that confers biological specificity. The peptide hormone activin, a TGF*β* family member that is produced in the gonad and the anterior pituitary as well as other tissues, is a key regulator of FSH that activates transcription of the FSH *β*-subunit gene and stimulates FSH secretion (1-3). Activin's essential role in regulating FSH is demonstrated by targeted disruption of activin signaling in the mouse, which suppresses FSH levels and causes defects in spermatogenesis and decreased testis size in males and impaired ovarian follicle development leading to infertility in females (4). Like all TGF*β* family members, activin signals through receptor serine/threonine kinases, which, in turn, phosphorylate intracellular receptor-specific Smad proteins, in the case of activin, either Smad2 or 3 (5). Upon phosphorylation, these Smad proteins associate with a common partner,

Address all correspondence and requests for reprints to: Pamela L. Mellon, Ph.D., Department of Reproductive Medicine, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0674. pmellon@ucsd.edu..
N.R.-H. and S.M.M. contributed equally to this work and should both be considered second authors.

Smad4, and translocate to the nucleus. Consensus DNA-binding sequences for Smad2/4 and Smad3/4 complexes have been identified, which contain the palindrome GTCTAGAC, half-sites of this sequence, or CAGA motifs (6-9). Smad3/4 complexes are capable of binding DNA alone, but do so with low affinity, and their interaction with additional transcription factors, including AP-1 family members c-Jun/c-Fos (8-11), and the helix-loop-helix protein TFE3 (12), is required for target gene regulation. Smad2/4 complexes require interaction with other transcription factors, including the forkhead/winged helix family members Fast-1 (13) and Fast-3 (14), and the paired-like homeodomain proteins Mixer and Milk (15), to bind to DNA.

Although the importance of activin regulation of FSH *β*-subunit gene expression is well established, little is known about the molecular mechanisms by which it occurs. Until recently, investigation of the transcriptional regulation of FSH*β* was hampered by the lack of an FSH-expressing gonadotrope cell culture model. Studies were instead performed in ovine mixed pituitary cultures, heterologous cell culture models, or in transgenic mice carrying the 5′-regulatory region of the ovine (o)FSH*β* gene targeting luciferase reporter gene expression to the gonadotrope. These experiments identified *cis*-acting elements that mediate responsiveness of the ovine gene to other important regulators of FSH*β* expression, including gonadal steroid hormones (16) and hypothalamic GnRH (17). However, the region(s) of the oFSH-*β* gene important for activin responsiveness and the proteins that mediate this response have not been identified. Recently, a Smad-binding element (SBE) was identified in the rat FSH *β*-subunit gene that is required for full activin responsiveness (18). Interestingly, whereas this site is conserved in the mouse gene, it is not present in the ovine, bovine, porcine, or human genes, suggesting that important species-specific differences exist in activin regulation of FSH*β* gene expression. Because this regulation is crucial for normal FSH expression, gaining insight into the molecular mechanisms governing this process is central to our understanding of reproductive function.

We have previously shown that FSH is expressed endogenously in the immortalized mouse gonadotrope cell line L*β*T2, and that its expression is stimulated by activin treatment, making this cell line an appropriate model system in which to study activin regulation of FSH*β* gene transcription (19). In this report, we used transient transfections in L*β*T2 cells to identify activin-responsive regions in the oFSH*β* gene. We find that three regions are required for full activin response, with consensus SBEs present in all three. Further, we find that one of these elements is bound by a complex containing the TALE homeodomain proteins, Pbx1 and Prep1, along with Smad4. These studies extend our understanding of activin regulation of the FSH*β* gene and identify Pbx1 and Prep1 as Smad partners and important mediators of activin action.

RESULTS

Full Activin Responsiveness of the FSH*β* Reporter Gene Requires Two Regions between −985 and −108

To identify the regions of the oFSH*β* gene that mediate activin responsiveness, transient transfections were performed in the immortalized gonadotrope L*β*T2 cell line. Like pituitary gonadotropes *in vivo*, these cells not only express FSH, but activin, activin receptors, inhibin, and follistatin as well, forming an autocrine activin loop within the cell culture (19). To reduce the level of endogenous activin, cells were placed in serum-free media after transfection for 16 h before addition of activin. The oFSH*β*-Luc reporter plasmid, which contains the −4741 to +750 regulatory region (relative to the start site of transcription) of the oFSH*β* gene controlling luciferase reporter gene expression, responds to 24-h activin stimulation, yielding luciferase expression 2.5- to 4-fold higher than that of vehicle-treated controls (Fig. 1A). Deletion of the gene-regulatory region from −4741 to −985 bp has no statistically significant effect on activin responsiveness of the reporter gene, indicating that the elements necessary for mediating activin action reside within the −985 to +750 bp region of the gene.

Progressive 5′-deletions were created to identify activin response elements within this region. Truncation of the region from −985 to −751 results in a 22% decrease in activin responsiveness, whereas further truncation of the region from −751 to −401 has no statistically significant effect on activin responsiveness of the reporter gene (Fig. 1B). The remaining activin response is lost upon deletion of the promoter region between −401 and −108. Thus, full activin responsiveness is retained within the −985/+750 region of the oFSH*β* gene and requires one upstream region between −985 and −751 and a promoter proximal region between −401 and −108.

Mutation of Putative SBEs within the −985 Region Disrupts Activin Responsiveness of the FSH*β* Gene

Sequence analysis of the −985 to −108 region identified several putative SBEs based on homology to the previously reported consensus (Fig. 2A). Two consensus AGAC sequences are present at −973 and −962. Another exists at −453, with an adjacent site present at −442, and another at −421. Further downstream, a 7 of 8 bp match to the 8-bp consensus, with an intact AGAC sequence, is present at −167, and a 6 of 8 bp match, including a GTCT sequence, exists at −134.

To determine whether any of these putative SBEs contribute to activin responsiveness of the FSH*β* gene, mutations were made in these sites in the context of the −985-Luc reporter plasmid. These mutant plasmids were then tested for activin responsiveness in transient transfections in L*β*T2 cells. Double mutation of the adjacent sites at −973 and −962 causes an approximate 20% decrease in fold induction by activin as compared with the −985-Luc reporter, whereas mutation of the Smad-homology regions at −453/−442 or −421 has no statistically significant effect on activin response (Fig. 2B). A reporter plasmid containing mutations in all of these distal sites exhibits a decrease in activin responsiveness equivalent to that of the M973/962 mutant (data not shown).

Mutation of either of the two putative SBEs closest to the transcriptional start site profoundly disrupts activin responsiveness of the −985 promoter region. Mutation of the −167 site abrogates activin regulation of the reporter entirely, whereas mutation of the −134 site results in an approximate 75% decrease in activin response, implicating these two promoter proximal sites as critical mediators of activin regulation of the FSH*β* gene. These results are consistent with the reduction in activin responsiveness observed upon deletion of the gene regions containing these sites in the previous experiment. Mutation of either of the two proximal elements results in a modest decrease in basal expression of the FSH*β* reporter gene; however, this decrease is not statistically significant (data not shown). These data demonstrate that activin regulation of the oFSH*β* gene requires several distinct 5′-regulatory regions containing homology to consensus SBEs, and that the two promoter proximal sites, at −167 and −134, are particularly important for activin stimulation of the FSH*β* gene.

Smad4 Binds to the −973/−962 and −453/−442 Regions of the oFSH*β* Gene

L*β*T2 cells express components of the activin signaling system including Smad2, −3, −4, and −7 (20). To determine whether nuclear proteins from L*β*T2 cells, and in particular Smad proteins, bind to the putative SBEs identified in the oFSH*β* gene, EMSAs were performed. An oligonucleotide encompassing the −973/−962 region of the gene was end labeled with ^{32}P and used in EMSA with nuclear extracts from L*β*T2 cells. Specific protein complexes bind to the labeled oligonucleotides (Fig. 3A, *arrows*). Detection of these complexes requires an autoradiographic exposure time of 1 wk, relatively long compared with the overnight exposures that we usually find sufficient for the detection of protein complexes in EMSA. Binding of these complexes is competed by the addition of excess unlabeled oligonucleotide (lane 2), but not by competitor containing the same 2-bp mutation (lane 3) in the putative SBEs that disrupts activin responsiveness in transient transfection experiments (Table 1). Inclusion of an antibody

directed against Smad4 results in a marked supershift (*arrowhead*, lane 4), whereas inclusion of a normal IgG negative control has no effect. Radiolabeled mutant oligonucleotide is not capable of binding these specific protein complexes (lane 6), and inclusion of excess unlabeled mutant competitor in the reaction has no effect (lane 7).

Mutation of the −453/−442 and −421 sites decreases activin responsiveness of the −985 oFSH*β*-Luc reporter gene slightly, but this decrease is not statistically significant (Fig. 2). However, because two other TGF*β* family members, bone morphogenetic protein 7 (BMP-7) and BMP-15, also activate expression of the FSH*β* gene (21,22), these SBE homology regions were also tested for their ability to bind Smad protein. EMSA using an oligonucleotide probe that encompasses the −421 site did not detect any specific protein binding to that region under any conditions tested (data not shown). In contrast, L*β*T2 nuclear proteins do bind to an oligonucleotide probe that encompasses the −453/−442 region (Fig. 3B, *arrows*). Binding of these proteins is specific and is competed by excess unlabeled wild-type oligonucleotide (lane 2) but not by excess mutant oligonucleotide. Inclusion of an antibody to Smad4 again results in a marked supershift of these complexes (lane 4, *arrowhead*), whereas the control IgG has no effect (lane 5). Labeled mutant oligonucleotide is not capable of binding these specific protein complexes (lane 6), and inclusion of excess unlabeled mutant competitor in the reaction has no effect (lane 7). As with the −973/−962 oligonucleotide probe, detection of these complexes requires relatively long autoradiographic exposures. Although Smad2, Smad3, and Smad4 are all expressed in L*β*T2 cells (20), antibodies directed against Smads 2 and 3 had no effect on protein binding to any of the EMSA oligonucleotide probes tested (data not shown). However, these commercial antibodies also had no effect on proteins binding to Smad2/4 and Smad3/4 consensus oligonucleotides obtained from the antibody manufacturer. The question of which of these activin receptor-specific Smads is partnered with Smad4 in the complexes binding to the −973/−962 and −453/−442 oligonucleotides remains unresolved. Taken together, these data indicate that the regions at −973/−962 and −453/−442 are true SBEs, and that the SBEs at −973/−962 are required for full activin response of the FSH*β* reporter gene.

Specific L*β*T2 Nuclear Proteins Bind to the −167 Region of the FSH*β* Promoter

When an oligonucleotide encompassing the −167 region of the oFSH*β* promoter is used in EMSA, specific protein complexes bind to the probe (Fig. 4, *arrows*). Inclusion of excess unlabeled oligonucleotide successfully competes with the labeled probe for protein binding (lane 2), whereas excess oligonucleotide containing the 2-bp mutation efficiently competes only the lower band (lane 3). Neither an antibody directed against Smad4 protein nor normal IgG has any effect on these protein complexes. Labeled mutant oligonucleotide (Table 1) is not capable of binding these proteins (lane 6), and inclusion of excess unlabeled mutant competitor with the labeled mutant probe in the reaction has no effect (lane 7). No additional slower mobility complexes similar to the ones observed using the −973/−962 and −453/−442 regions as probes were observed with the −167 oligonucleotide on longer autoradiographic exposure. Thus, nuclear proteins from L*β*T2 cells specifically interact with the −167 site, but under these *in vitro* conditions Smad4 protein does not.

A Complex Containing Smad4 and the Homeodomain Proteins Pbx1 and Prep1 Binds to the −134 Region of the FSH*β* Gene

When an oligonucleotide encompassing the −134 site is used in EMSA with nuclear extract from L*β*T2 cells, a highly specific and distinctive doublet of bands is observed (Fig. 5A, *arrows*, lane 1). Unlabeled oligonucleotide successfully competes with the labeled probe for protein binding (lane 2), but oligonucleotide containing the 2-bp mutation (Table 1) that disrupts activin response of the reporter does not (lane 3). Labeled mutant oligonucleotide binds a different, faster migrating complex (lane 4), which is competed by inclusion of excess unlabeled mutant competitor (lane 5). Under these conditions, an antibody directed against

Smad4 protein has no effect on the observed bands (lane 6). Other studies in our laboratory focusing on the transcriptional regulation of the GnRH gene identified an identical doublet of bands in EMSA using a region of the GnRH promoter as labeled probe (Rave-Harel, N., and P. L. Mellon, manuscript in preparation). The proteins binding to this probe have been identified as complexes containing the TALE homeodomain proteins Pbx1a and 1b, which are alternatively spliced transcripts from a single gene, and their cofactor Prep1. These proteins (and their homologs in other species) have been shown to interact with, and modulate the function of, homeotic Hox proteins to regulate cell-specific gene transcription and diverse developmental pathways. However, involvement of these proteins in the regulation of TGFβ family target genes has not previously been described.

To determine whether Pbx1 and Prep1 proteins are also present in the distinctive complexes binding the −134 activin response element, EMSA was performed using nuclear extract from LβT2 cells and Pbx and Prep1 antibodies (Fig. 5A). Inclusion of an antibody that recognizes Pbx1a, −2, or −3, but not Pbx1b (lane 7), reduces the upper band of the doublet, whereas an antibody that specifically interacts with Pbx1b in EMSA results in a diminution of the lower band (lane 8). Inclusion of an antibody to Prep1 abolishes both bands of the doublet (lane 9). EMSA using the −973/−962 and −167 oligonucleotide probes and antibodies to Pbx1 and Prep1 did not detect the presence of these proteins in complexes binding to those activin response elements (data not shown). EMSA performed with each of the oligonucleotide probes and nuclear extracts from either vehicle or activin-treated LβT2 cells detected no difference in protein binding (data not shown). These data confirm that the −134 activin response element binds protein complexes containing Pbx1a and Prep1, and Pbx1b and Prep1. Furthermore, the same mutation that abolishes Pbx1/Prep1 binding also markedly disrupts activin responsiveness, implicating Pbx1 and Prep1 as novel mediators of activin regulation of FSHβ expression.

Pbx1 and Prep1 protein complexes have been shown to interact with equal affinity to both TGACAG and TGATTGAT consensus binding sites (23). Examination of the −134 region for homology to these consensus sequences reveals two overlapping regions of homology to the shorter sequence, a 5′-region between −136 and −131 that matches 5 of 6 bp of the consensus sequence, and a 3′-region between −132 and −127 that matches 4 of 6 bp (Fig. 5B, *bold and underscored*, respectively, in wild-type sequence). In addition, a 5 of 8 bp match to the longer consensus sequence can also be identified between −134 and −127 (*overscored* in wild-type sequence) that is encompassed by the two shorter potential sites. The 2-bp mutation (−134M in Table 1 and indicated in *lower case letters* in the M4 sequence in Fig. 5B), made with the original intention of disrupting the putative SBE, resides within all of these potential Pbx1/Prep1 binding sites and thus is not informative as to the bases required for Pbx1/Prep1 binding.

To further investigate the binding requirements of the Pbx1/Prep1 complex at this region, EMSA was performed using the wild-type −134 probe and competitor oligonucleotides containing 2-bp mutations that scan the sites (Fig. 5B, indicated in *bold lower case letters in sequence*). Inclusion of excess unlabeled wild-type oligonucleotide efficiently competes both bands of the doublet (lane 2). An oligonucleotide containing mutation M1 competes for protein binding only slightly less than the wild type (lane 3). Oligonucleotides with mutations M2–M5 are completely unable to compete with the labeled probe for protein binding (lanes 4–7), whereas the oligonucleotides containing mutations M6 and M7 are able to compete, but less well than the wild type (lanes 8 and 9). Thus, nucleotides that are critical for Pbx1 and Prep1 binding reside between −136 and −125 in the ovine gene.

Under our standard conditions using the −134 probe and 2 μg LβT2 nuclear proteins in the reaction mix, we observed no effect on the EMSA bands upon inclusion of an antibody to Smad4 protein. Because the Pbx1/Prep1 complex has been shown to be very stable and to bind

DNA with high affinity (24), we suspected that if a higher order Smad/Pbx1/Prep1 complex was formed, it would be comparatively unstable, and detectable in EMSA only with high concentrations of nuclear proteins present. To test this, we performed EMSA using the labeled −134 oligonucleotide probe and increasing amounts of LβT2 nuclear extract. No difference in the banding pattern is observed when we increase the amount of nuclear proteins to 8 or 14 μg (Fig. 6, lanes 1 and 2). However, when 20 μg of nuclear proteins are included in the reaction mix, a higher-order complex (*arrow*) is formed in addition to those containing Pbx1a/Prep1 and Pbx1b/Prep1 (lane 3). This higher-order complex is disrupted by inclusion of antibodies to Prep1 and Pbx1 (lanes 4 and 5). Significantly, this complex is abolished by inclusion of an antibody to Smad4 as well (lane 6), thus confirming that a complex containing Smad4, Pbx1, and Prep1 binds to this activin response element in the FSHβ gene.

Pbx1 and Prep1 Physically Interact with Smad2, Smad3, and Smad4

We have shown that Pbx1, Prep1, and Smad4 form a DNA-binding complex in EMSA. However, as described earlier, for technical reasons we were unable to identify either Smad2 or Smad3 as partners in the complex. To assess whether Pbx1 and Prep1 can physically interact with Smads 2 and 3, in addition to Smad4, we performed glutathione-*S*-transferase (GST) interaction assays. Bacterially expressed Smad2-GST, Smad3-GST, and Smad4-GST fusion proteins all are capable of interacting with *in vitro* translated Pbx1a (Fig. 7, lanes 3, 7, and 11) and Prep1 (lanes 4, 8, and 12), as well as with the positive control Smad4 (lanes 2, 6, and 10). None of these GST-fusion proteins interacts with the negative control green fluorescent protein (GFP) (lanes 1, 5, and 9), nor does the GST tag alone interact with any of the *in vitro* translated proteins (lanes 16, 17, and 18). These data suggest that the TALE/Smad complexes that bind to the −134 activin response element may contain Smad2 or Smad3 in addition to Smad4, Pbx1, and Prep1.

The Proximal Activin Response Elements Are Well Conserved across Species and Are Also Required for Activin Responsiveness of the Mouse Gene

Recently, an SBE was identified in the rat FSHβ gene that is required for full activin response (18). Comparison of this region and the corresponding regions of the ovine, porcine, bovine, mouse, and human FSHβ genes reveals that although this site is conserved in the mouse sequence, it is not present in any of the other species (Fig. 8A, *boxed sequence* at −268 in the rat sequence). Examination of the gene regions that correspond to the activin-responsive SBEs that we identified at −973/−962 in the ovine gene reveals that the −962 site is not conserved, and the −973 SBE is conserved only in the bovine and porcine FSHβ genes, but not in the mouse, rat, or human genes. Similarly, the SBEs we identified at −453/−442, which are not required for activin response, show limited conservation in the bovine and human genes, but not in the other species examined.

In contrast, comparison of the region of the oFSHβ gene that encompasses the two most proximal activin response elements with corresponding gene regions in the other species reveals that significant conservation exists over the entire promoter proximal region (Fig. 8A). The AGAC sequence at −163 is completely conserved, and the bases required for Pbx1/Prep1 binding (Fig. 8A, *boxed sequences*) are well conserved across species. This significant level of homology suggests that these activin responsive regions may be important for FSHβ regulation across species. Indeed, in EMSA using an oligonucleotide probe from the mouse gene that corresponds to the Pbx1/Prep1 binding site and nuclear extract from LβT2 cells, protein complexes form that are identical to those that bind to the ovine probe (Fig. 5). Competition experiments with oligonucleotides containing scanning block mutations identify the region containing homology to the Pbx1/Prep1 binding site as critical for the protein complex binding to the mouse oligonucleotide as well (data not shown).

To determine whether these conserved proximal sites are also required for activin regulation of the mouse gene, we performed transient transfections in LβT2 cells with a mouse (m) FSHβ-Luc plasmid, which contains 1000 bp of the mouse gene 5′-regulatory region driving luciferase expression. Expression of this reporter gene is stimulated approximately 3.5-fold by activin treatment compared with vehicle-treated samples (Fig. 8B). Mutations (2 bp) were made in the mouse reporter gene regions that correspond to the −167 and −134 activin response elements in the ovine gene, resulting in the M153 and M120 plasmids, respectively (Table 1). Both of these mutations profoundly disrupt activin responsiveness of the mFSHβ gene as well, resulting in levels of expression with activin treatment that are not significantly different from the vehicle-treated controls. These data confirm that the two proximal activin response elements identified in the ovine gene are functionally conserved in the mFSHβ gene as well.

Pbx1 and Prep1 Bind to the mFSHβ-Subunit Gene *in Vivo*

To verify that the interaction between Pbx1/Prep1 complexes and the −134 oligonucleotide observed in EMSA accurately represents *in vivo* interaction of these proteins with the endogenous mouse gene, chromatin immunoprecipitation assays were used to confirm Pbx1 and Prep1 binding to the regulatory region of the mFSHβ gene. Antibodies specific for Pbx1a/1b and Prep1 are capable of immunoprecipitating the cross-linked protein/DNA region that encompasses the Pbx1/Prep1 binding site in the mouse gene as revealed by subsequent PCR amplification of the precipitated DNA fragment (Fig. 9). These data indicate that Pbx1/Prep1 complexes interact with the FSHβ gene, both in EMSA and *in vivo*, and implicate these homeodomain proteins as important regulators of FSHβ expression across species.

DISCUSSION

Activin regulation of FSH expression is critical for normal reproductive function in both males and females across mammalian species. Previously, however, little was known about the molecular details of this regulation. Our characterization of the activin response elements in the oFSHβ gene and identification of Pbx1 and Prep1 as mediators of activin action contribute significantly to the understanding of this important physiological process at the molecular level.

A recent report identified an SBE at −268 in the rat FSHβ gene that is required for activin responsiveness. The sequence of this Smad-binding site is conserved in the mouse gene, but is absent from the other species we examined, including the oFSHβ gene. Instead, we find that activin regulation of oFSHβ-subunit gene expression involves three distinct regulatory regions. We identified two promoter proximal activin response elements at −167 and −134 in the ovine gene that are not only highly conserved across all species examined but are required for activin responsiveness of the mouse gene as well. Another, more distal, element containing two consensus Smad-binding sequences was identified at −973/−962. This site shows significant sequence conservation only in the bovine and porcine genes. In general, the regulation of the reproduction axis in mammalian species is similar and relies on complex feedback interactions of the same peptide and steroidal hormones. However, important species-specific differences exist in the timing of reproductive behavior and ovulation in mammals, ranging from the seasonally restricted reproductive pattern that occurs in many species, to the year-round recurring estrous cycles observed in rodents and menstrual cycles found in many primates, including humans. Our report provides insight into species-specific distinctions in the molecular mechanisms of activin regulation of FSH expression that may play a role in these differences.

The requirement of multiple promoter elements for full response has been reported for several other TGFβ superfamily target genes. TGFβ induction of the PAI1 gene depends on Smad3/4 binding to distal CAGA elements and Smad interaction with Sp1 protein at promoter proximal sites (25), and BMP2 activation of the basic helix-loop-helix gene *Id1* in muscle is dependent

on two overlapping Smad 1/5-specific GC boxes as well as three separate consensus Smad4 binding sites (26). It has been argued that the presence of multiple SBEs allows cooperative association between Smad proteins, thus overcoming their low DNA-binding affinity. In light of that, it is interesting to note that in the FSHβ gene, the distal activin response region has two Smad sites spaced 11 bp apart at −973 and −962. In addition, the SBE at −453/−442, which might play a role in the regulation of the FSHβ gene by other TGFβ family members, also consists of two Smad sites separated by 11 bp. It is possible that these adjacent sites allow Smads to physically interact and bind with higher affinity to the FSHβ gene as well.

Nuclear proteins in LβT2 nuclear extract bind strongly and specifically to the −167 site in EMSA; however, these proteins remain unidentified. Because mutation of this site abolishes activin response entirely in both the mouse and ovine genes, it is clearly a critical component in activin regulation of the FSHβ gene. However, although it contains significant homology to a consensus SBE, we were unable to demonstrate Smad-binding to this site in EMSA, using either LβT2 nuclear extracts (Fig. 4), or whole-cell extracts from Cos-1 cells in which Smad2 and −4 or Smad3 and −4 had been overexpressed (data not shown). It is possible that Smad protein interaction with this site is of very low affinity or occurs as part of a relatively unstable larger complex. Alternatively, activin regulation through this element may occur independently of Smad signaling. Although Smads are required for the regulation of nearly all TGFβ superfamily target genes identified to date, TGFβ is also capable of activating Rho family GTPases, MAPKs, and protein kinase B, leading to target gene regulation that is Smad independent (5,27). Further characterization of the −167 site, and identification of the proteins binding there, are required to determine whether it truly represents a Smad-independent activin response element.

Mutation of the −134 site similarly results in a profound disruption in activin responsiveness of both the ovine and mouse FSHβ genes. We identified the proteins binding to this site as complexes containing Smad4 and the TALE homeodomain proteins Pbx1 and Prep1. Mammalian Pbx1 was identified initially as an oncogenic Pbx/E2A fusion protein (28). Pbx1, and its homolog in *Drosophila*, Extradenticle, play important roles during development as partners of homeotic selector Hox proteins (29). More recently, Pbx1/Prep1 regulation of various genes, including the glucagon and somatostatin genes, has been described (30). Our characterization of Pbx1 and Prep1 as transcriptional regulators of the GnRH gene in the hypothalamus (Rave-Harel, N., and P. L. Mellon, manuscript in preparation), and the FSHβ gene in pituitary gonadotropes, identifies them as key modulators of the reproductive axis as well.

We showed that Pbx1 and Prep1 physically interact with the activin receptor-specific Smad2 and −3, in addition to Smad4. The cooperative association of Pbx1 and Prep1 with Hox proteins both increases the DNA binding affinity of the Hox proteins and modulates their target site selection (29). It is possible that Pbx1 and Prep1 function similarly in their association with Smad proteins at the −134 site of the FSHβ gene. We observed no changes in Pbx1/Prep1 binding in response to activin treatment in EMSA. Likewise, we detected no statistically significant difference in the densitometric measure of Pbx1 or Prep1 binding in the chromatin immunoprecipitation assays. However, because the LβT2 cells secrete activin, it is possible that this apparent constitutive binding is the result of the presence of an autocrine activin signaling loop within the LβT2 cell culture. Alternatively, Pbx1 and Prep1 may be capable of binding in the absence of stimulation and may act to recruit Smad proteins and/or stabilize their binding to the promoter after Smad nuclear translocation in response to activin signaling. Transcriptional regulation of the osteoprotegrin gene by a complex of Smads and the homeodomain protein Hoxc-8, a Pbx1 partner, in response to BMP signaling has been described (31). Our studies reporting the interaction of Pbx1 and Prep1 with Smad proteins in

the mediation of activin signaling suggest that a broader involvement of Smad proteins in target gene regulation by Pbx1/Prep1 and/or Hox complexes may exist.

A number of other homeodomain proteins have been implicated in transcriptional regulation by TGF*β* family members. Activin regulation of the *Xenopus goosecoid* gene requires Smad2 and −4*β* (a *Xenopus* Smad4 homolog) in association with the paired-like homeodomain proteins Mixer and Milk. Interaction of these two proteins (as well as the winged-helix proteins *Xenopus* Fast-1, human Fast-1, and mouse Fast-2) with Smad2 depends on a C-terminal, proline-rich Smad-interaction motif. Proteins with this motif are also found in Smad3/4 complexes (15). Examination of the Pbx1 protein sequence revealed no homology to the Smad-interaction motif. However, we have identified a region in the N terminus of Prep1 that contains homology to this motif, with significant conservation of critical amino acid residues. Interestingly, this region is directly adjacent to the HR1 domain of Prep1, which has been shown to be required for its interaction with Pbx1 (23).

In summary, we have demonstrated that activin regulation of the oFSH*β* gene is complex, requiring both distal Smad-binding sites and evolutionarily conserved promoter-proximal elements. In addition, we identify the homeodomain proteins Pbx1 and Prep1 as novel Smad partners and mediators of activin signaling and as important regulators of reproductive function at both the hypothalamic and pituitary levels of the reproductive axis. These studies provide important insight into the transcriptional regulation of FSH expression by activin and how this regulation converges and diverges across species.

MATERIALS AND METHODS

Plasmids

The ovine reporter plasmids oFSH*β*-Luc and −985 oFSH*β*-Luc have been previously described (32,33). All of the oFSH*β* reporter plasmids contain the indicated 5′-region in addition to the first intron and a portion of the first exon of the gene, with the endogenous AUG start codon mutated to allow the initiation of translation at the luciferase gene start codon. The −750 oFSH*β*-Luc, −562 oFSH*β*-Luc, and −108 oFSH*β*-Luc plasmids were made by cloning the *Sac*I/*Sal*I, *Nde*I (Kle-nowed)/*Bgl*II, and *Hin*dIII fragments from −985-Luc into the *Sac*I/*Xho*I, *Sma*I/*Bgl*II, and *Hin*dIII sites, respectively, of the pGL3 Basic plasmid (Promega Corp., Madison, WI). The −401-Luc plasmid was made by PCR amplification of the −401 to +759 fragment from −985 oFSH*β*-Luc, which was then cloned into the *Sma*I site of pGL3 Basic. The mouse reporter plasmid mFSH*β*-Luc (kindly provided by Dr. Varykina G. Thackray) was made by PCR amplification of the 1000-bp 5′-regulatory region from a mouse genomic clone, which was then ligated into the *Kpn*I and *Hin*dIII restriction sites of pGL3. The 2-bp point mutations (*lower case letters* in the primer sequences) in the ovine and mouse FSH*β* reporter genes were made using the primers listed in Table 1 and their reverse complements, and the QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. All deletions and mutations were verified by sequencing.

Transient Transfections and Luciferase and *β*-Galactosidase Assays

L*β*T2 cells were cultured as previously described (19). The day before transfection, cells were divided onto six-well plates at a dilution of one confluent 10-cm plate per 18 wells. FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) was used according to the manufacturer's protocol. Each well was transfected with 0.5 *μ*g of the luciferase reporter plasmid along with 0.1 *μ*g of a plasmid containing a *β*-galactosidase reporter gene regulated by the Rous sarcoma virus promoter (RSV-*β*Gal) as a control for transfection efficiency. Twelve hours after transfection, the cells were transferred to serum-free DMEM supplemented with 0.1% BSA, 5 mg/liter transferrin, and 50 nM sodium selenite. Sixteen hours later, either

activin A (Calbiochem, San Diego, CA) or vehicle alone was administered to the cells, with the final concentration of activin 10 ng/ml. After 24 h of treatment, cell extracts were prepared and assayed for luciferase and β-galactosidase activity as described previously (34). Transfections performed with the mFSHβ-Luc plasmids, wild type and with the mutations M153 and M120, followed the same protocol with the following exceptions: cells were divided onto 24-well plates and transfected with 0.2 μg of reporter and 0.1 μg of a control plasmid containing the β-galactosidase gene regulated by the thymidine kinase reporter (TK-βGal). All experiments were performed in triplicate, and results represent at least three independent experiments performed in at least two different thawed batches of LβT2 cells. The means of the ratios of luciferase to β-galactosidase for each plasmid and treatment were compared using ANOVA and the Tukey-Kramer honestly significant difference *post hoc* test. P values <0.05 were considered statistically significant.

EMSA

Nuclear extracts were prepared from LβT2 cells, and EMSA binding reactions were carried out with 2 μg of nuclear protein (except as indicated in Fig. 8) and 1 fmol of ^{32}P-labeled oligonucleotide (sequences of forward strands only are listed in Table 1) as previously described (34). In competition assays, 100 fmol of unlabeled oligonucleotide competitor and the labeled probe were added to the binding reaction at the same time. In supershift assays, 2 μg of Smad2, Smad3, Smad4, Prep1, Pbx1/2/3, Pbx1, or normal rabbit IgG antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were preincubated with the nuclear proteins in binding buffer at room temperature for 10 min before the addition of the probe. After probe addition, binding reactions were incubated an additional 5 min at room temperature before electrophoresis on a 5% nondenaturing polyacrylamide gel.

GST Interaction Assay

The Smad2-GST and Smad4-GST plasmids were provided by Dr. Masayuki Funaba, and the Smad3-GST plasmid was obtained from Dr. Rik Derynck. The Pbx1 and Prep1 expression plasmids were provided by Dr. Mark Kamps. The Smad4 and GFP expression plasmids were obtained from Dr. Rik Derynck and Dr. Douglass Forbes, respectively. ^{35}S-labeled proteins were produced *in vitro* using the TnT Coupled Reticulocyte Lysate System (Promega Corp.). Bacteria transformed with the GST plasmids were grown to an OD of 0.5, and then induced with 0.2 mM isopropyl-β-D-thiogalactoside overnight at room temperature. Bacterial pellets were sonicated in 0.1% Triton X-100, 5 mM EDTA in 1× PBS, and centrifuged, and the supernatant was bound to glutathione sepharose 4B resin (Amersham Pharmacia Biotech, Piscataway, NJ). The interaction assay was performed as previously described (35).

Chromatin Immunoprecipitation Assay

LβT2 proteins were cross-linked to genomic DNA by addition of formaldehyde directly to the cell medium to a final concentration of 1%. After the nuclear fraction was obtained, chromatin was sonicated to fractions of 1 kb average length in sonication buffer (50 mM HEPES, pH 7.9; 140 mM NaCl; 1 mM EDTA; 1% Triton X-100; 0.1% sodium deoxycholate; 0.1% sodium dodecyl sulfate). After preclearing with protein A beads, protein-DNA complexes were incubated overnight with Pbx1 or Prep1 antibodies and then precipitated with protein A beads (Amersham Pharmacia Biotech). After extensive washing [two times each with sonication buffer, high-salt sonication buffer (500 mM NaCl with other components as defined above), lithium chloride buffer (20 mM Tris, pH 8; 250 mM LiCl; 1 mM EDTA; 0.1% Nonidet P-40; 0.1% NaDOC), and Tris-EDTA buffer], cross-linking was reversed by addition of 300 mM NaCl and incubation at 65 C, and proteins were digested by incubation with Proteinase K. DNA was phenol-chloroform extracted, and ethanol precipitated, and the sequence of interest was then amplified by PCR. Primers sequences are listed in Table 1 and flank the 280-bp sequence in

the mFSHβ gene from −223 to +57. For quantification, [α^{32}P]dATP was included in the reaction mix. The PCR product was then electrophoresed on a 5% acrylamide gel in 0.5× Tris-borate-EDTA, the gels were dried and subjected to autoradiography, and band intensity was quantified using a PhosphorImager Optical Scanner Storm 860 (Molecular Dynamics Inc., Sunnyvale, CA) and the ImageQuant program (Molecular Dynamics, Inc.).

Acknowledgments

The authors thank Dr. Malcolm Low for the mFSHβ genomic clone and Suzanne B. R. Jacobs, Varykina G. Thackray, and Marjory L. Givens for helpful discussions and advice.

This work was supported by National Institute of Child Health and Human Development/National Institutes of Health (NIH) through cooperative agreement (Grant U54 HD12303) as part of the Specialized Cooperative Centers Program in Reproduction Research (to P.L.M.). This work was also supported by NIH Grant R37 HD20377 (to P.L.M.). J.S.B. was supported in part by NIH Grant T32 GM08666. N.R.-H. was supported by the Lalor Foundation. S.M.M. was supported by NIH Grant T32 DK07541. D.C. was supported by NIH National Research Service Award F32 HD41301and NIH Grant T32 DK07044 and the Lalor Foundation.

Abbreviations

BMP	Bone morphogenetic protein
GFP	green fluorescent protein
GST	glutathione-*S*-transferase
SBE	Smad-binding element

REFERENCES

1. Ling N, Ying S-Y, Ueno N, Shimasaki S, Esch F, Hotta M, Guillemin R. Pituitary FSH is released by a heterodimer of the β-subunits from the two forms of inhibin. Nature 1986;321:779–782. [PubMed: 3086749]
2. Carroll RS, Corrigan AZ, Gharib SD, Vale W, Chin WW. Inhibin, activin, and follistatin: regulation of follicle-stimulating hormone messenger ribonucleic acid levels. Mol Endocrinol 1989;3:1969–1976. [PubMed: 2516876]
3. Carroll RS, Kowash PM, Lofgren JA, Schwall RH, Chin WW. *In vivo* regulation of FSH synthesis by inhibin and activin. Endocrinology 1991;129:3299–3304. [PubMed: 1954905]
4. Matzuk MM, Kumar TR, Bradley A. Different phenotypes for mice deficient in either activins or activin receptor type II. Nature 1995;374:356–560. [PubMed: 7885474]
5. Attisano L, Wrana JL. Signal transduction by the TGF-β superfamily. Science 2002;296:1646–1647. [PubMed: 12040180]
6. Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S, Gauthier JM. Direct binding of Smad3 and Smad4 to critical TGFβ-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. EMBO J 1998;17:3091–3100. [PubMed: 9606191]
7. Johnson K, Kiskpatrick H, Comer A, Hoffman FM, Laughon A. Interaction of Smad complexes with tripartite DNA-binding sites. J Biol Chem 1999;274:20709–20716. [PubMed: 10400705]
8. Zawel L, Dai JL, Buckhaults P, Zhou S, Kinzler KW, Vogelstein B, Kern SE. Human Smad3 and Smad 4 are sequence-specific transcription activators. Mol Cell 1998;1:611–617. [PubMed: 9660945]
9. Zhang Y, Musci T, Derynck R. The tumor suppressor Smad4/DPC 4 as a central mediator of Smad function. Curr Biol 1997;7:270–276. [PubMed: 9094310]
10. Zhang Y, Feng XH, Derynck R. Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-β-induced transcription. Nature 1998;394:909–913. [PubMed: 9732876]
11. Wong C, Rougier-Chapman EM, Frederick JP, Datto MB, Liberati NT, Li JM, Wang XF. Smad3-Smad4 and AP-1 complexes synergize in transcriptional activation of the c-Jun promoter by transforming growth factor β. Mol Cell Biol 1999;19:1821–18230. [PubMed: 10022869]

12. Hua X, Liu X, Ansari DO, Lodish HF. Synergistic cooperation of TFE3 and smad proteins in TGF-β-induced transcription of the plasminogen activator inhibitor-1 gene. Genes Dev 1998;12:3084–3095. [PubMed: 9765209]

13. Yeo CY, Chen X, Whitman M. The role of FAST-1 and Smads in transcriptional regulation by activin during early *Xenopus* embryogenesis. J Biol Chem 1999;274:26584–26590. [PubMed: 10473623]

14. Howell M, Inman GJ, Hill CS. A novel *Xenopus* Smad-interacting forkhead transcription factor (XFast-3) cooperates with XFast-1 in regulating gastrulation movements. Development 2002;129:2823–2834. [PubMed: 12050132]

15. Germain S, Howell M, Esslemont GM, Hill CS. Homeodomain and winged-helix transcription factors recruit activated Smads to distinct promoter elements via a common Smad interaction motif. Genes Dev 2000;14:435–451. [PubMed: 10691736]

16. Webster JC, Pedersen NR, Edwards DP, Beck CA, Miller WL. The 5′-flanking region of the ovine follicle-stimulating hormone-β gene contains six progesterone response elements: three proximal elements are sufficient to increase transcription in the presence of progesterone. Endocrinology 1995;136:1049–1058. [PubMed: 7867558]

17. Strahl BD, Huang HJ, Sebastian J, Ghosh BR, Miller WL. Transcriptional activation of the ovine follicle-stimulating hormone β-subunit gene by gonadotropin-releasing hormone: involvement of two activating protein-1-binding sites and protein kinase C. Endocrinology 1998;139:4455–4465. [PubMed: 9794452]

18. Suszko MI, Lo DJ, Suh H, Camper SA, Woodruff TK. Regulation of the rat follicle-stimulating hormone β-subunit promoter by activin. Mol Endocrinol 2003;17:318–332. [PubMed: 12554780]

19. Pernasetti F, Vasilyev VV, Rosenberg SB, Bailey JS, Huang HJ, Miller WL, Mellon PL. Cell-specific transcriptional regulation of FSHβ by activin and GnRH in the LβT2 pituitary gonadotrope cell model. Endocrinology 2001;142:2284–2295. [PubMed: 11356674]

20. Dupont J, McNeilly J, Vaiman A, Canepa S, Combarnous Y, Taragnat C. Activin signaling pathways in ovine pituitary and LβT2 gonadotrope cells. Biol Reprod 2003;68:1877–1887. [PubMed: 12606325]

21. Otsuka F, Shimasaki S. A novel function of bone morphogenetic protein-15 in the pituitary: selective synthesis and secretion of FSH by gonadotropes. Endocrinology 2002;143:4938–4941. [PubMed: 12446622]

22. Huang HJ, Wu JC, Su P, Zhirnov O, Miller WL. A novel role for bone morphogenetic proteins in the synthesis of follicle-stimulating hormone. Endocrinology 2001;142:2275–2283. [PubMed: 11356673]

23. Berthelsen J, Zappavigna V, Ferretti E, Mavilio F, Blasi F. The novel homeoprotein Prep1 modulates Pbx-Hox protein cooperativity. EMBO J 1998;17:1434–1445. [PubMed: 9482740]

24. Berthelsen J, Zappavigna V, Mavilio F, Blasi F. Prep1, a novel functional partner of Pbx proteins. EMBO J 1998;17:1423–1433. [PubMed: 9482739]

25. Datta PK, Blake MC, Moses HL. Regulation of plasminogen activator inhibitor-1 expression by transforming growth factor-β-induced physical and functional interactions between smads and Sp1. J Biol Chem 2000;275:40014–40019. [PubMed: 11054406]

26. Lopez-Rovira T, Chalaux E, Massague J, Rosa JL, Ventura F. Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of Id1 gene. J Biol Chem 2002;277:3176–3185. [PubMed: 11700304]

27. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-β family signalling. Nature 2003;425:577–584. [PubMed: 14534577]

28. Kamps MP, Baltimore D. E2A-Pbx1, the t(1;19) translocation protein of human pre-B-cell acute lymphocytic leukemia, causes acute myeloid leukemia in mice. Mol Cell Biol 1993;13:351–357. [PubMed: 8093327]

29. Mann RS, Chan SK. Extra specificity from extradenticle: the partnership between HOX and PBX/EXD homeodomain proteins. Trends Genet 1996;12:258–262. [PubMed: 8763497]

30. Goudet G, Delhalle S, Biemar F, Martial JA, Peers B. Functional and cooperative interactions between the homeodomain PDX1, Pbx, and Prep1 factors on the somatostatin promoter. J Biol Chem 1999;274:4067–4073. [PubMed: 9933599]

31. Wan M, Shi X, Feng X, Cao X. Transcriptional mechanisms of bone morphogenetic protein-induced osteoprotegrin gene expression. J Biol Chem 2001;276:10119–10125. [PubMed: 11139569]

32. Strahl BD, Huang HJ, Pedersen NR, Wu JC, Ghosh BR, Miller WL. Two proximal activating protein-1-binding sites are sufficient to stimulate transcription of the ovine follicle-stimulating hormone-β gene. Endocrinology 1997;138:2621–2631. [PubMed: 9165057]

33. Vasilyev VV, Pernasetti F, Rosenberg SB, Barsoum MJ, Austin DA, Webster NJ, Mellon PL. Transcriptional activation of the ovine follicle-stimulating hormone-β gene by gonadotropin-releasing hormone involves multiple signal transduction pathways. Endocrinology 2002;143:1651–1659. [PubMed: 11956146]

34. Rosenberg SB, Mellon PL. An Otx-related homeodomain protein binds an LHβ promoter element important for activation during gonadotrope maturation. Mol Endocrinol 2002;16:1280–1298. [PubMed: 12040015]

35. Zappavigna V, Sartori D, Mavilio F. Specificity of HOX protein function depends on DNA-protein and protein-protein interactions, both mediated by the homeodomain. Genes Dev 1994;8:732–744. [PubMed: 7926763]

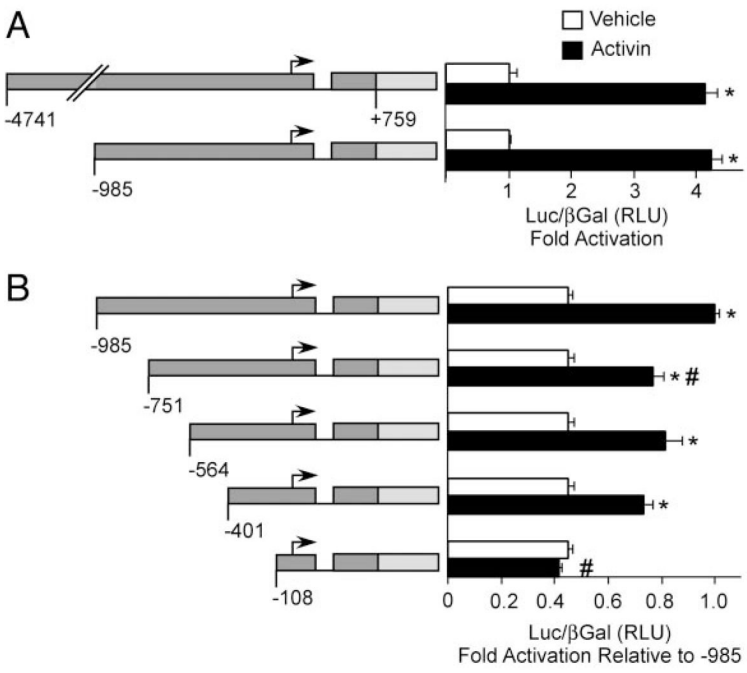

Fig. 1.

Full Activin Responsiveness of the FSH*β* Gene Requires Regions between −985 and −751, and between −401 and −108

Transient transfections were performed in L*β*T2 cells with reporter plasmids containing the indicated gene regions controlling luciferase expression. A Rous sarcoma virus-*β*Gal plasmid was cotransfected, and luciferase activity was normalized to *β*-galactosidase activity to control for transfection efficiency. For each reporter, relative light unit (RLU) values for activin-treated samples were divided by vehicle-treated controls to yield fold activation. Data are the means ± SEM of three independent experiments, each performed in triplicate. *, Activin-treated values that differ significantly from vehicle-treated controls; #, fold activation that differs significantly from that of the previous truncation. A, Activin responsiveness of reporter plasmids containing either the full-length −4741/+759 region of the oFSH*β* gene or a 5′-deletion to −985 was assayed. B, Activin responsiveness of reporter plasmids containing either the −985/+759 region or the indicated 5′-deletions was assayed. To control for differences in activin stimulation between different batches of L*β*T2 cells, fold activation for each reporter was normalized to that of the −985 oFSH*β*-Luc plasmid, which was set at 1.

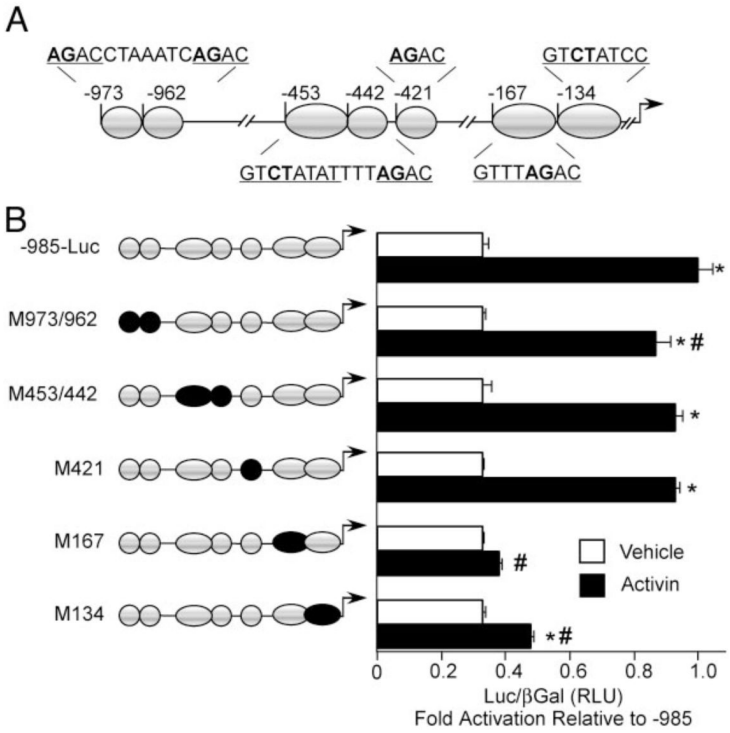

Fig. 2.

Mutation of Putative SBEs Disrupts Activin Responsiveness of the FSH*β* Gene

A, Sites in the −985 regulatory region with homology to the SBE consensus sequence. Nucleotides in *bold* indicate bases mutated for use in subsequent transfection and EMSA experiments (base changes are shown in *lowercase letters* in sequences listed in Table 1). B, Activin responsiveness of reporter plasmids containing either the wild-type −985 region of the oFSH*β* gene or the indicated point mutations was assayed in transiently transfected L*β*T2 cells. Data are the means ± SEM of three independent experiments, each performed in triplicate, and were normalized as described in Fig. 1. *, Values that differ significantly from vehicle-treated control; #, fold activation with activin treatment that differs significantly from fold activation of the wild-type −985 reporter. RLU, Relative light units.

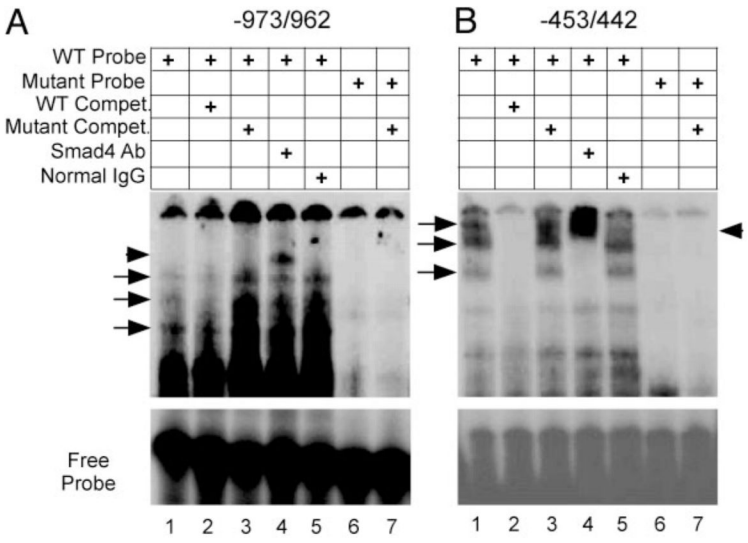

Fig. 3.

Smad4 Binds to the −973/−962 and −453/−442 Regions of the FSH*β* Gene

EMSA was performed using L*β*T2 nuclear extract and wild-type (WT) or mutant −973/−962 oligonucleotide probes (panel A) or wild-type or mutant −453/−442 oligonucleotide probes (panel B). Wild-type or mutant competitor oligonucleotides and antibodies (Ab) were included in the reactions as indicated. Specific protein complexes are indicated by *arrows*, and shifted complexes induced by inclusion of an antibody to Smad4 are indicated by *arrowheads*. Detection of the specific complexes required a week-long autoradiographic exposure, which resulted in overexposure of the free probe bands. To enable comparison of the specific activity of the individual probes, the free probe panels are from an overnight exposure of the same gels.

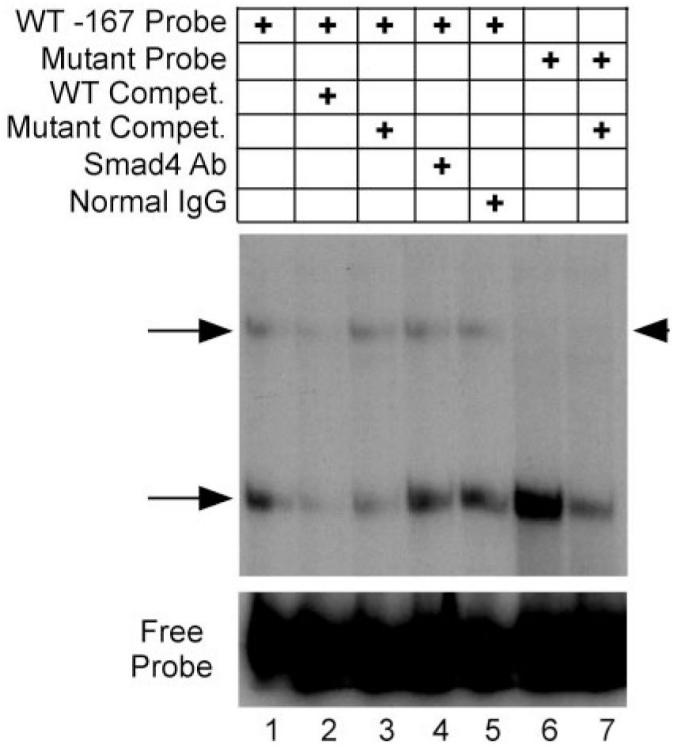

Fig. 4.

Specific LβT2 Nuclear Proteins Bind to the −167 Region of the FSHβ Gene

EMSA was performed using LβT2 nuclear extract and either the wild-type (WT) or mutant −167 oligonucleotide probe. Wild-type or mutant competitor oligonucleotides and antibodies (Ab) were included in the reactions as indicated. Specific protein complexes are indicated by *arrows*, and the complex disrupted by the mutation is indicated by an *arrowhead*.

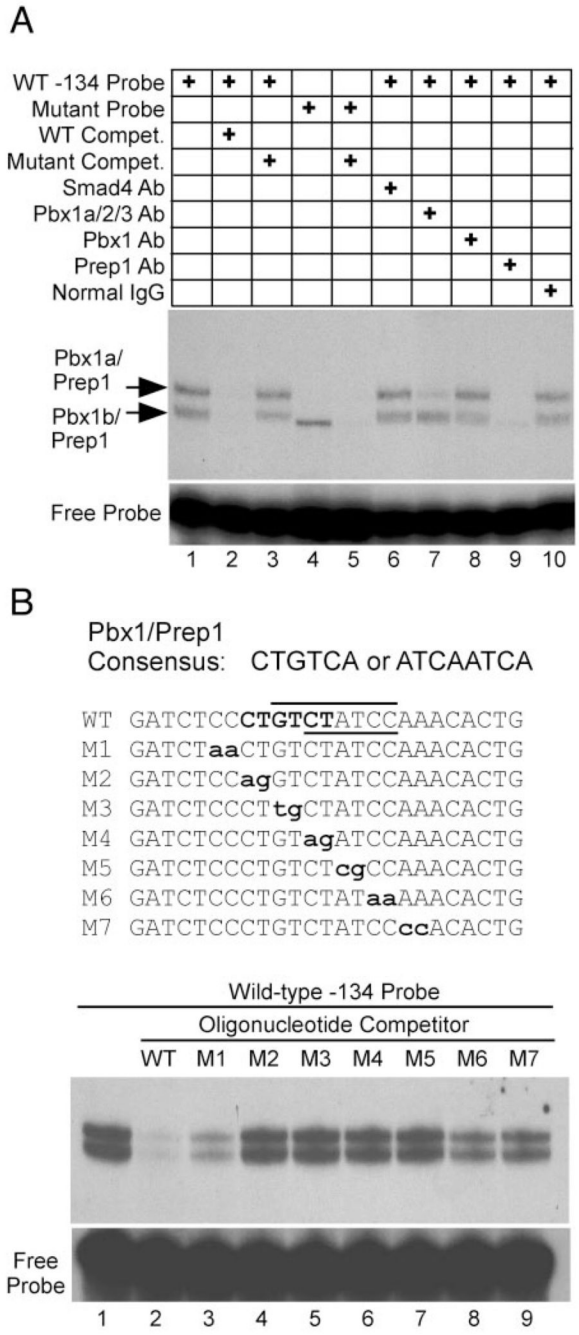

Fig. 5.

Pbx1 and Prep1 Bind to the −134 Region of the FSH*β* Gene

EMSA was performed using L*β*T2 nuclear extract and either the wild-type (WT) or mutant −134 oligonucleotide probe. A, Wild-type or mutant competitor oligonucleotides and antibodies (Ab) were included in the reactions as indicated. Pbx1a/Prep1 and Pbx1b/Prep1 complexes are indicated by *arrows*. B, Oligonucleotide sequences used to characterize the Pbx1/Prep1 binding site. Three distinct regions with homology to the Pbx1/Prep1 consensus are indicated in the wild-type sequence in *bold*, *underscored* and *overscored*. Mutations in the competitor oligonucleotides are indicated in *bold lowercase letters*. The 2-bp mutation that disrupts activin responsiveness in transfections is equivalent to the M4 sequence.

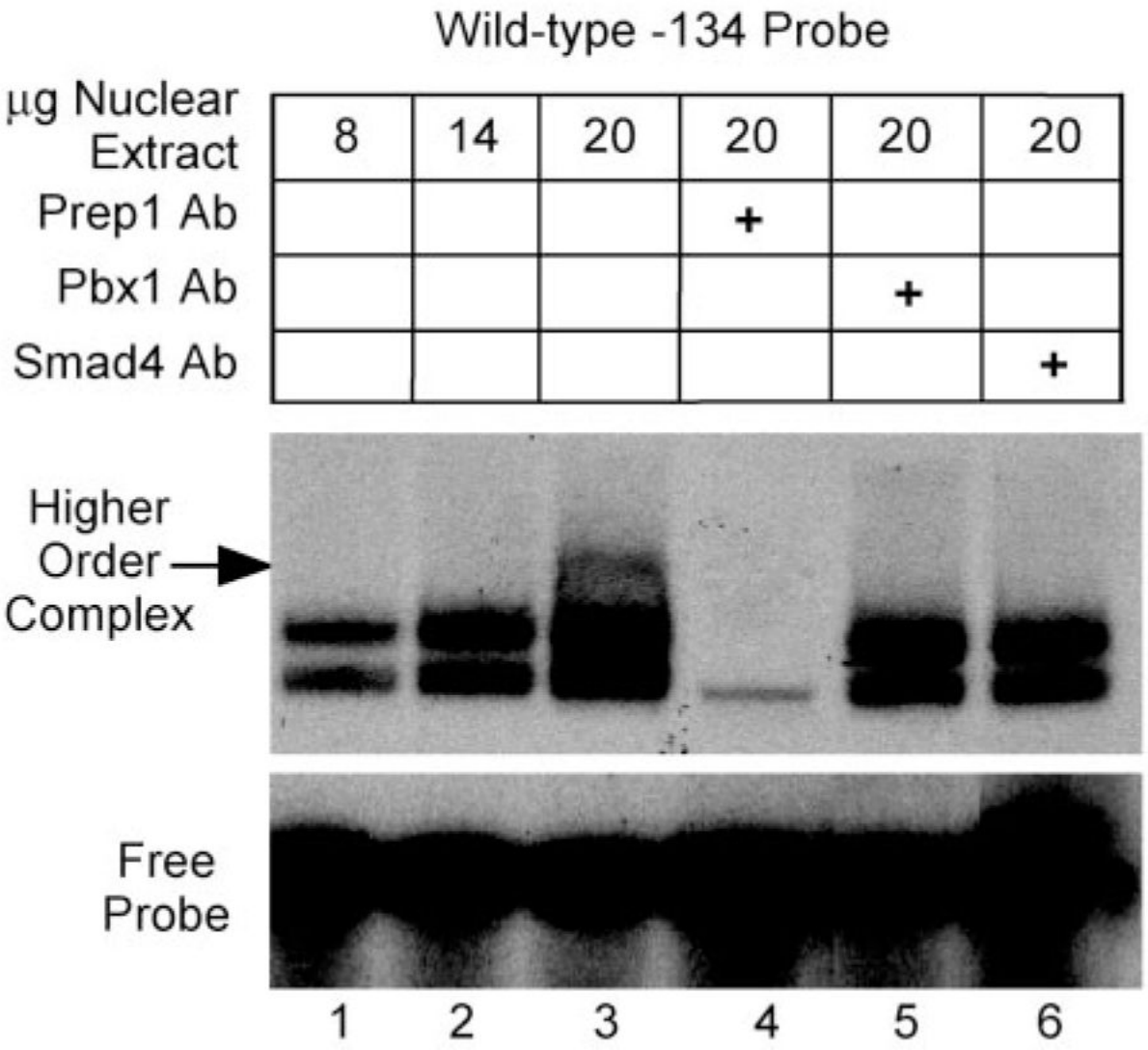

Fig. 6.

A Complex Containing Pbx1, Prep1, and Smad4 Binds to the −134 Region of the FSH*β* Gene

EMSA was performed using the wild-type −134 oligonucleotide probe and the indicated amounts of L*β*T2 nuclear extract. Antibodies (Ab) were included in the reactions as noted. A higher order complex containing Pbx1, Prep1, and Smad4 is indicated by an *arrow*. At this high concentration of extract, the Pbx1 antibody is only capable of reducing the intensity of the Pbx1/Prep1 doublet, not eliminating it.

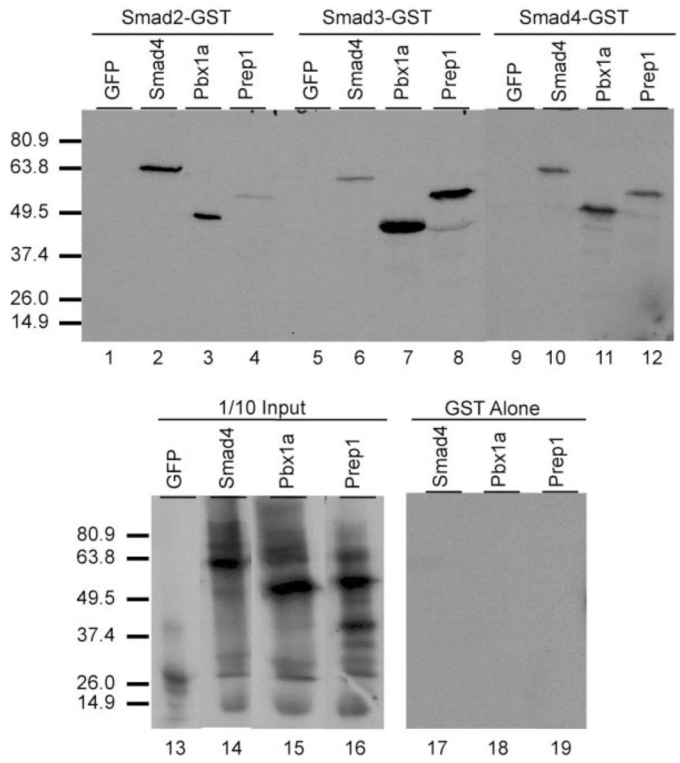

Fig. 7.

Pbx1 and Prep1 Interact with Smad2, Smad3, and Smad4

GST interaction assays were performed using the indicated bacterially expressed Smad-GST fusion proteins and ^{35}S-labeled *in vitro* translated GFP, Pbx1a, Prep1, and Smad4. One tenth of the protein input and the GST tag-alone negative control are shown in lanes 13–19.

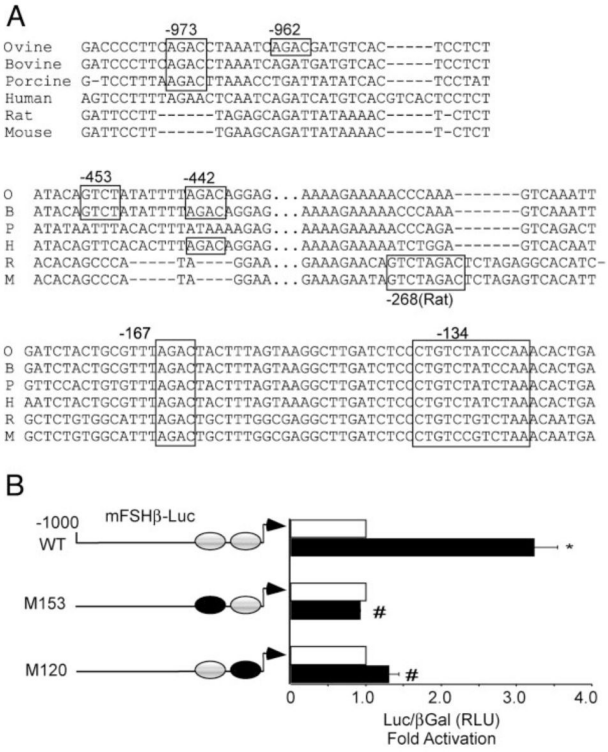

Fig. 8.

The Evolutionarily Conserved Proximal Activin Response Elements Are Also Required for Activin Regulation of the mFSH*β* Gene

A, Sequence comparison of activin response elements and SBEs in the ovine, bovine, porcine, human, rat, and mouse FSH*β* genes. Homology is indicated by the *boxed regions.* B, Reporter plasmids containing either the wild-type (WT) −1000 region of the mFSH*β* gene or the indicated 2-bp mutations corresponding to those made in the −167 and −134 sites of the ovine gene were transiently transfected in L*β*T2 cells and activin responsiveness was assayed. Data are the means ± SEM of three independent experiments, each performed in triplicate, and were normalized as described in Fig. 1. *, Values that differ significantly from vehicle-treated control; #, fold activation with activin treatment that differs significantly from fold activation of the wild-type reporter. RLU, Relative light units.

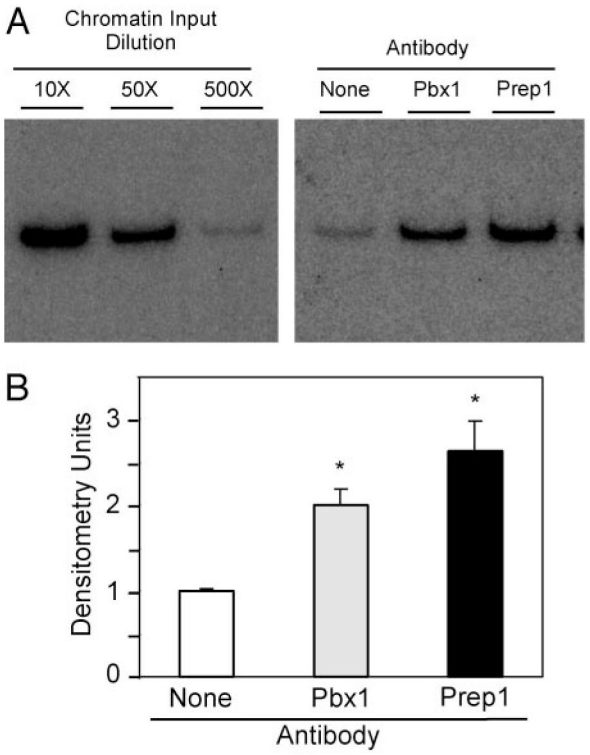

Fig. 9.

Pbx1 and Prep1 Bind to the mFSHβ Gene *in Vivo*

Chromatin immunoprecipitation was performed using cross-linked protein/chromatin from LβT2 cells and antibodies directed against Pbx1 and Prep1 proteins. PCR primers complementary to the mFSHβ 5′-regulatory region flanking the Pbx1/Prep1 binding site were used to detect precipitation of the genomic DNA. A, Representative gel of radioactively labeled PCR products amplified from control chromatin sample and chromatin precipitated with Pbx1 and Prep1 antibodies is shown. PCR products from reactions using input chromatin at 10-fold, 50-fold, and 500-fold dilutions are shown. B, Densitometry quantification of PCR products was performed. Data are the means ± SEM from three independently prepared chromatin samples, each performed in triplicate. *, Mean densitometry values that differ significantly from no-antibody control.

Table 1

Oligonucleotides Used in These Studies

Mutagenesis Oligonucleotides	
M973/962	5′ −989 AAGGGACCCCTTCctACCTAAATCctACGATGTCACTGCTCTAC −942 3′
M453/442	5′ −479 GAGGATCTGGATGTAAAGAGGAATACAGTagATATTTTActACAGGAGTCAGATCATG −423 3′
M421	5′ −440 GACAGGAGTCAGATCATGAActACTTTCTATTTGTTTTTTCTCTGC −395 3′
M167	5′ −181 CCTGATCTACTGCGTTTctACTACTTTAGTAAAGGCTTGATCTCCC −135 3′
M134	5′ −150 TAAGGCTTGATCTCCCTGTagATCCAAACACTGATTCACTTACAGCAAG −101 3′
M153	5′ −171 CATTTTCTGCTCTGTGGCATTTctACTGCTTTGGCGAGGCTTGAT −127 3′
M120	5′ −138 GCGAGGCTTGATCTCCCTGTCCGTagAAACAATGATTCCCTTTCA −94 3′
EMSA Oligonucleotides	
−973/962	5′ −979 CCCCTTCAGACCTAAATCAGACGATGTCACTC −948 3′
M973/962	5′ −979 CCCCTTCctACCTAAATCctACGATGTCACTC −948 3′
−453	5′ −460 GGAATACAGTCTATATTTTAGACAGGAGTC −431 3′
M453	5′ −460 GGAATACAGTagATATTTTctACAGGAGTC −431 3′
−167	5′ −173 CTACTGCGTTTAGACTACTTTAG −151 3′
M167	5′ −173 CTACTGCGTTTctACTACTTTAG −151 3′
−134	5′ −142 GATCTCCCTGTCTATCCAAACACTG −118 3′
M134	5′ −142 GATCTCCCTGTagATCCAAACACTG −118 3′
ChIP PCR Oligonucleotide Primers	
Forward	5′ −223 GGTGTGCTGCCATATCAGATTCGG −200 3′
Reverse	5′ +57 GCATCAAGTGCTGCTACTCACCTGTG +32 3′