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Multiple binding sites for nuclear proteins of the anterior pituitary are located in the 5′-flanking region of the porcine follicle-stimulating hormone (FSH) β-subunit gene

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Abstract

Gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), are synthesized specifically in the gonadotropes of the anterior pituitary. The aim of this study was to investigate nuclear factors that bind specifically to the porcine FSH β-subunit gene. We examined nuclear protein binding to 2.75 kilobase pairs (kbp) of DNA adjacent to the porcine FSH β-subunit gene: about 2.32 kbp of upstream DNA and 0.43 kbp of downstream DNA. The upstream region contains only TATA box, CACCC element, and some imperfect sequences of cAMP-responsive element, activator protein-1 binding site, and activator protein-2 binding site. Gel mobility shift assay using nuclear proteins extracted from the porcine anterior pituitary revealed that the proteins bound to a limited region of DNA, 107 bp long (designated as Fd2), located about −800 bp upstream from the transcription initiation site. Competitive binding assays demonstrated that the protein binding was sequence specific; the addition of excess amounts of several putative regulatory sequences and plasmid (non-homologous) DNA fragments did not reduce the binding. Furthermore, all five subfragments of Fd2 were also bound by the pituitary nuclear proteins, showing that the entire region of Fd2 is involved in this interaction. Southwestern blotting demonstrated that at least seven protein species of 110, 98, 78, 63, 52, 42, and 35 kDa recognize Fd2. Nuclear proteins from several other porcine tissues were also able to bind to the Fd2 fragment but the gel shift patterns were different and the bindings were weak, although only the cerebellum showed a pattern of binding that was similar to that of the anterior pituitary. These data suggest that multiple proteins of the anterior pituitary recognize a specific region of the porcine FSH β-subunit gene.

Keywords: Pituitary; Follicle-stimulating hormone; FSH; DNA-binding protein; Glycoprotein hormone; Pig

1. Introduction

Follicle-stimulating hormone (FSH) regulates the development of reproductive organs and gametogenesis and is a member of the glycoprotein hormone family, which includes luteinizing hormone (LH), thyroid-stimulating hormone, and placental chorionic gonadotropin of primates and horses. FSH is composed of two subunits, a common α subunit and a unique β subunit as other glycoprotein hormones (Pierce and Parsons, 1981). Three genes, the α-subunit, FSH β-subunit, and LH β-subunit genes, are expressed in the same type of pituitary cell (Liu et al., 1988) and regulated by gonadotropin-releasing hormone (GnRH) (Papavasiliou et al., 1986; Kato et al., 1989), steroid hormones, activin, and inhibin (Gharib et al., 1990) in different ways. The regulatory mechanism of gene expression is a major issue of the gonadotropin function. Although the structures of the gonadotropin subunit genes have been determined for various mammalian species including our previous studies of the pig genes (Hirai et al., 1990; Ezashi et al., 1990; Kato et al., 1991), the gene regulation, especially for FSHβ, is still unclear.

The anterior pituitary is composed of a number of cell types including five types of endocrine cell, which differentiate in a cell lineage from the primordial cells of Rathke's pouch (Jacobson et al., 1979). The corticotropes, which produce pro-opiomelanocortin, and thyrotropes, which produce thyroid-stimulating hor-

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mone, differentiate first followed by the gonadotropin-producing cells, gonadotropes, which specifically express the LH β-subunit and FSH β-subunit genes, as demonstrated in rat (Simmons et al., 1990), mouse (Japòn et al., 1994), and pig (Ma et al., 1994, 1996; Granz et al., 1997). Differentiation then proceeds to somatotropes, which produce growth hormone, and mammotropes, which produce prolactin. During this differentiation process, the pituitary hormone genes acquire cell-type specific expression patterns via the hierarchical action of specific transcription factors. Recent studies of pituitary ontogeny have shown that many transcription factors are involved in the development of pituitary function (Treier and Rosenfeld, 1996; Tremblay et al., 1998). Some transcription factors are essential for pituitary development and for the differentiation of hormone-producing cells. Simultaneously, the same transcription factors and others regulate gene expression of pituitary hormones.

The expression of three related gonadotropin subunit genes by gonadotropes is controlled by common and/or gene-specific signals. To identify the molecular mechanisms that regulate the expression of each gonadotropin subunit gene we need to know which transcription factors determine subunit gene-specific expression and the responsiveness of each gene to extracellular signals. Several factors that recognize and specifically regulate the α-subunit gene have been identified (Barnhart and Mellon, 1994; Roberson et al., 1994; Steger et al., 1994; Jackson et al., 1995). In the past few years several transcription factors for the LH β-subunit gene have been reported (Lee et al., 1996; Garrel et al., 1997; Tremblay et al., 1998). By contrast, regulatory factors that act on FSH β-subunit gene have not yet been identified, although several extracellular signals are known to modulate the expression of this gene (Tsujii and Winters, 1995; Kaiser et al., 1995; Besecke et al., 1996; Armstrong and Childs, 1997; Strahl et al., 1997, 1998). The lack of an appropriate cell line that produces the FSH β-subunit has hampered research in this direction. Nevertheless, several lines of evidence, obtained by studying transgenic mice, have demonstrated that the 5′-flanking region of the FSH β-subunit gene contains a nucleotide sequence that confers cell- and/or gene-specific transcription (Kumar et al., 1992; Markkula et al., 1995a,b). Other approaches are required to identify the molecular mechanism by which the FSH β-subunit gene is regulated.

The aim of the present study was to identify nuclear proteins from the porcine anterior pituitary that bind specifically to the 5′-flanking region of the FSH β-subunit gene. We found that at least seven proteins bound to an upstream sequence that was about 100 base pairs (bp) long.

2. Materials and methods

2.1. Fragmentation and labeling of the porcine FSH β-subunit gene

The entire nucleotide sequence of the porcine FSH β-subunit gene (10 kbp) had been determined in a previous study (Hirai et al., 1990). We began this study with a fragment consisting of about 2.75 kbp including the region upstream (from −2323 to +428, F4-3 clone), from which we produced nine smaller fragments (Fa–Fi) by restriction enzyme digestion and by polymerase chain reaction (PCR) using specific primers. The location of several elements present in the upsteam region and position of upstream fragments used in this study are shown in Fig. 1. Fa and Fb were prepared by digestion of F4-3 with *Eco*RI and *Bam*HI, and with *Xmn* I and *Eco*RI, respectively. To obtain the other fragments, oligonucleotide primers (Table 1) were designed based on the nucleotide sequence of F4-3 and were synthesized using an Applied Biosystems DNA synthesizer (model 380A; Foster City, CA, USA). Each fragment (Fc–Fi) was amplified in a reaction mixture (100 μl) containing a deletion subclone of F4-3 as a template, two primers (a set of T7 primers and the fragment-specific primers; 50 pmol each) and 1 U of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT, USA). PCR was for 30 cycles of denaturation (94°C, 1 min), annealing (50°C, 2 min) and extension (74°C, 3 min) using a programmable thermal cycler (MJ Research Inc., Watertown, MA, USA). The amplified PCR products were separated using a Suprec-02 centrifuge filter (Takara Shuzo, Kyoto, Japan). Then the DNA fragments were labeled with [α-^{32}P]-dCTP (3000 or 6000 Ci/mmol) by a replacement reaction with T4 DNA polymerase.

2.2. Preparation of subfragments and labeling

The Fd fragment (−982 to −618 relative to the transcription start site) was divided into three parts (Fd-l, Fd-2, and Fd-3), which were prepared by PCR as described above using synthetic oligonucleotides (Table 1) as the primers. The Fd2 fragment was cloned into the *Sma* I site of pBluescript SK$^+$. After digesting the clone with *Eco*RI and *Bam*HI, the Fd2 fragment was purified by 1% agarose gel electrophoresis. The recessive 3′ ends of the Fd2 fragment were labeled by the fill-in reaction using Klenow fragments in the presence of dATP, dGTP, and dTTP, each at 0.2 mM, and [α-^{32}P] dCTP. Other fragments prepared by PCR were labeled by a replacement reaction using T4 DNA polymerase, followed by gel filtration through a Nick column (Pharmacia LKB, Uppsala, Sweden) to remove free nucleotides. Typical specific activities ranged from 5×10^4 to 7×10^4 cpm/ng of DNA.

2.3. Preparation of nuclear extracts

Nuclear extracts of porcine anterior pituitaries were prepared at 4°C according to the method of Miskimins et al., (1985). Fifty porcine anterior pituitaries were obtained from a local slaughterhouse and homogenized in 15 ml of HEPES buffer (10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, pH 7.9) containing 0.5 M sucrose, 0.1 mM EDTA, 5 mM $MgCl_2$, 0.5% (v/v) Triton X-100, 1 mM dithiothreitol (DTT), 50 mM NaCl, 0.2 mM p-amidinophenyl methanesulfonyl fluoride hydrochloride, 5 μg/ml antipain, 2 TIU/ml aprotinin, 2 μg/ml chymostatin and 5 μg/ml leupeptin. The homogenate was centrifuged at 25 000 × *g* for 10 min at 4°C. The pellet of intact nuclei was washed once in HEPES buffer and suspended in an equal volume of HEPES buffer containing 0.6 M NaCl and 5 mM spermidine. The suspension was stirred for 1 h and then centrifuged at 25 000 × *g* for 30 min at 4°C. Solid ammonium sulfate was added to the supernatant to 50% saturation. The precipitate was collected by centrifugation at 20 000 × *g* for 15 min and was dissolved in a minimum volume of buffer containing 10 mM HEPES (pH 7.9), 1 mM $MgCl_2$, 1 mM DTT, 40% (v/v) glycerol, 50 mM NaCl, and 0.5 mM EDTA followed by dialysis against the same buffer. After any precipitate had been removed by centrifugation, aliquots of the extract were stored at −80°C until use. Nuclear extracts were also prepared, as described above, using similar weights (about 25 g) of other porcine tissues.

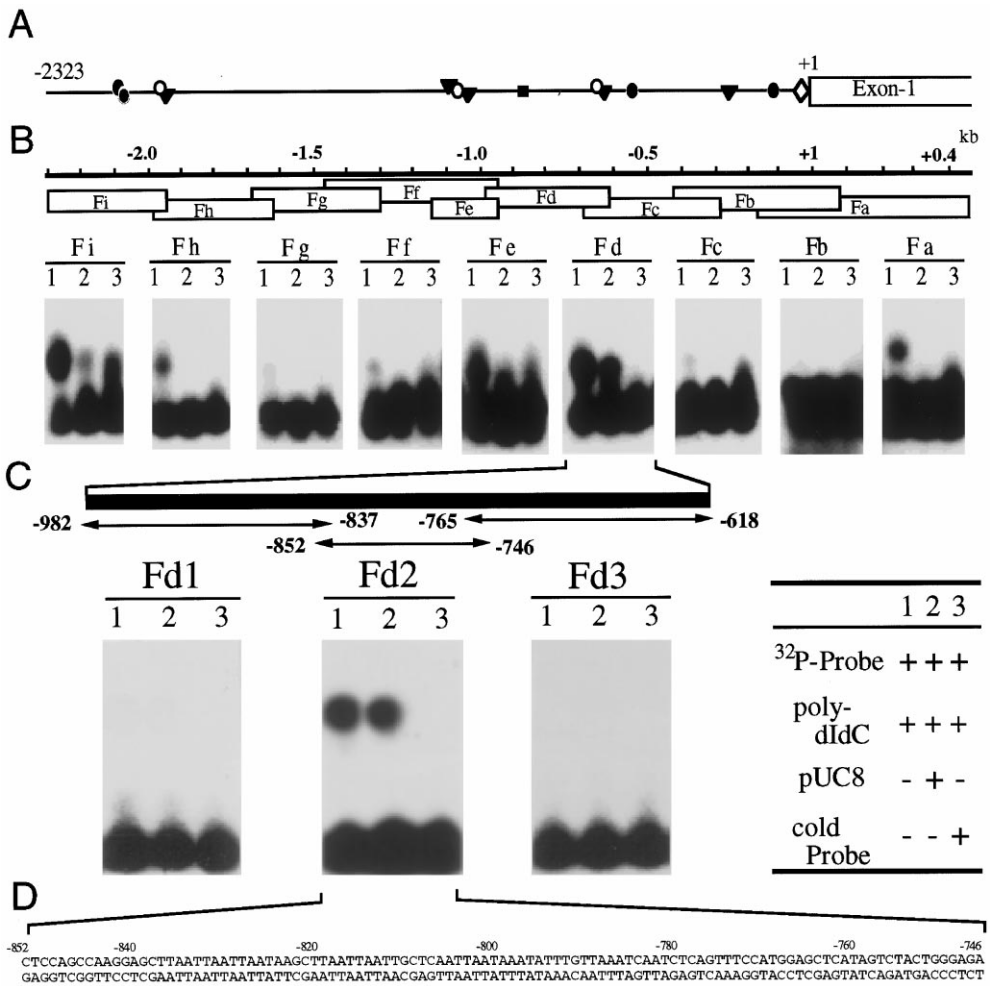

Fig. 1. Gel mobility shift assay of FSH β-subunit gene upstream sequences binding to proteins in nuclear extracts of porcine anterior pituitary. (A) Putative regulatory elements in the region upstream from of the FSH β-subunit gene (Kato et al., 1991) are illustrated. The sequences of a cAMP-responsive element (■), activator protein-1 binding site (●), activator protein-2 binding site (○), CACCC element (▼), and TATA box (◇) are indicated. The transcription initiation site is indicated by +1. (B) Gel mobility shift assay of FSH β-subunit gene upstream fragments. Nine fragments, Fa to Fi, covering the 2.75 kbp were prepared by enzymatic digestion or PCR. Labeled fragments were incubated with porcine anterior nuclear extract and the protein complexes were separated on a 1% agarose gel (lane l). Non-homologous DNA (*Hae*III-digested pUC8, lane 2) and unlabeled homologous DNA fragments (lane 3) were added at a 100-fold excess (w/w). The composition of the reaction mixture is shown on the right in the lower panel. (C) Gel mobility shift assay of fragments of Fd. Three fragments (Fdl, Fd2, and Fd3) were examined by gel mobility shift under the conditions described above. (D) Nucleotide sequence of the Fd2 fragment of the porcine FSH β-subunit gene.

Table 1
Oligonucleotide PCR primers used to construct fragments of the 5′-flanking region of the FSH β-subunit gene and consensus regulatory elements

PCR primers for fragments of the 5′-flanking region of the FSH β-subunit gene		
Fc	5′-GGTTTTTCTTTTGTATATTTAATTTGG-3′	
Fd	5′-TGTCCCCTATACTACTCCCAC-3′	
Fe/Ff	5′-TTTACTGTCGGTGTTTGGGGA-3′	
Fg	5′-TTTGGGAAAATGATAAGGAGT-3′	
Fh	5′-AAAGCCAGTTTGTCAGGAGGA-3′	
Fi	5′-TTGTAGTTGCCAAGGGCAGGA-3′	
T7 primer	5′-GTAATACGACTCACTATAGGGCG-3′	
PCR primers for Fd fragments		
	Upper strand	Lower strand
Fdl	5′-AAACCCTG-CAACTGGTCCCC-3′	5′-AGCTCCTTGGCTG-GAGAA-3′
Fd2	5′-CTCCAGCCAAG-GAGCTTAAT-3′	5′-TCTCCCAGTAGAC-TATGAGC-3′
Fd3	5′-GCTCATAGTC-TACTGGGAGA-3′	The primer for Fd was used
PCR primers for Fd2 subfragments		
	Upper strand	Lower strand
Fd2-1	5′-CTCCAGCCAAG-GAGCTTAATTAATT-3′	5′-TAAGCTTATTAAT-TAATTAAGCTCC-3′
Fd2-2	5′-TAATTAATAAGCT-TAATTAA-3′	5′-AATTGAGCAAT-TAATTGC-3′
Fd2-3	5′-ATTAATTGCT-CAAT-TAATAAATATT-3′	5′-ATTGATTTAA-CAAATATTTAT-TAAT-3′
Fd2-4	5′-TGTTAAAT-CAATCTCAGTTT-3′	5′-AGCTCCATG-GAAACTGAGAT-3′
Fd2-5	5′-CTCAGTTTCCATG-GAGCTCATAGTC-3′	5′-TCTCCCAGTAGAC-TATGAGCTCCAT-3′
PCR primers for the consensus regulatory elements		
	Upper strand	Lower strand
CRE	5′-CTAGCTTG-GCTGACGTCA-GAGAC-3′	5′-TC-GAGTCTCTGACGTCA GCCAAG-3′
AP1-site	5′-TAGTGAT-GAGTCAGCCGGAT-CAAGC-3′	5′-AGCTTGATCCG-GCTGACTCATCA-3′
AP2-site	5′-CACGGGC-CGCGGGGCGGTCA-GATCAA-3′	5′-TTGATCTGACCGC-CCCGCGGCCCGTG-3′
Pit1-site	5′-GATCCTGTGTA-CATTTATGC-3′	5′-GATCCCATG-CATAAATGTAC-3′
GSE	5′-TGTTTCATAG-GCTGACCTTGAGGT-CAACCTGCA-3′	5′-GTTGACCTCAAG-GTCAGCCTATGAAA-CACTGCA-3′

2.4. Gel mobility shift assay

The reaction mixture contained nuclear protein (2.5 μg), labeled DNA probe (1 to 2 fmol), and poly(dI-dC) (2 μg) in 10 mM HEPES buffer (pH 7.9) containing 50 mM NaCl, 400 μM DTT, 400 μM $MgCl_2$, 4% glycerol (total 10 μl). For competitive binding assays the same reaction mixture was used except for the addition of unlabeled homologous or non-homologous DNA as a competitor. The pre-binding mixture, without the DNA probe, was incubated for 15 min at room temperature. Then the labeled DNA probe (approximately 50 000 cpm, 1 to 2 fmol) was added and the mixture was incubated for 30 min at 30°C. The mixture was fractionated by electrophoresis on a 1% agarose gel or by native 4% polyacrylamide gel electrophoresis (PAGE; acrylamide to bisacrylamide ratio, 30:1) in 50 mM Tris, 380 mM glycine (pH 8.3), containing 2 mM EDTA. PAGE was for 2 h at 100 V. The gel was dried and exposed to an X-ray film with an intensifying screen at − 80°C.

2.5. Southwestern blotting

Southwestern blotting was performed by the method of Silva et al. (Silva et al., 1987). Nuclear protein (20–100 μg) was separated by SDS-PAGE (8%) and the gel was incubated twice at 1-h intervals in refolding buffer: 50 mM Tris–HCl (pH 7.0), 50 mM NaCl, 20 mM EDTA, 0.1 mM DTT, and 4 M urea. The proteins were transferred electrophoretically to a nitrocellulose filter. The filter was incubated overnight with gentle agitation at 4°C in binding buffer (10 mM HEPES, pH 7.9, 50 mM NaCl, 400 μM DTT, 400 μM $MgCl_2$, and 4% glycerol) containing 5% nonfat dry milk. Hybridization of the labeled DNA probe (4×10^6 cpm/ml) was carried out in binding buffer containing 0.25% nonfat dry milk and poly(dI-dC) (10 μg/ml). Then the filter was washed four times, at 20-min intervals, with binding buffer containing 0.25% nonfat dry milk. The filter was exposed to the Imaging plate (Fuji Film, Tokyo, Japan) and the radioactivity was quantified by analysis of the radiograph (Image Analyzer BAS2000, Fuji Film).

3. Results

3.1. Binding of nuclear proteins to DNA fragments of the 5′-flanking region of the porcine FSH β-subunit gene

We analyzed a 2.75 kbp region that covered about 2.32 kbp of the region proximal to the transcription start site of the FSH β-gene, which included a region of 5.56 kbp that contains several putative regulatory elements (Hirai et al., 1990) (Fig. 1A). Nine fragments covering 2.75 kbp (from − 2323 to + 428, Fa to Fi) of the upstream region were generated by PCR or enzymatic digestion and labeled with [α-^{32}P]dCTP. Gel mobility shift assays of nuclear proteins from the porcine

anterior pituitary were performed in the presence or absence of unlabeled homologous DNA fragments or *Hae*III-digested pUC8 DNA as a non-homologous competitor (Fig. 1B). Mobility shift bands were observed for the fragments Fi, Fh, Ff, Fe, Fd, and Fa, however, most of these shifts were prevented by the addition of *Hae*III-digested pUC8 DNA. Only the shift caused by the Fd fragment was still observed in the presence of a 100-fold excess of non-homologous DNA. Furthermore, the shifted band disappeared when unlabeled Fd was added to the reaction mixture, indicating that the shift was a result of sequence-specific binding.

Further gel mobility shift assays were performed for three subfragments of the Fd sequence (Fd1, Fd2, and Fd3). Fd1 and Fd3 did not cause a mobility shift, whereas Fd2 caused a mobility shift that was not prevented by the addition of *Hae*III-digested pUC8 DNA DNA, although a decrease in band intensity (by about 75%) was observed. When unlabeled Fd2 was added to the reaction mixture the labeled Fd2 was displaced from the nuclear protein binding (Fig. 1C). The nucleotide sequence of Fd2 is from -852 to -746 relative to the transcription start site (Fig. 1D). Fd2 has a high content of adenine and thymine and has several interesting sequences of inverted and direct repeats.

To estimate the binding affinity of pituitary nuclear proteins for Fd2, the gel mobility shift assay was repeated with increasing amounts of labeled Fd2 on a 1% agarose gel. Equilibrium binding was obtained at 40 pg Fd2/μg nuclear protein. The Scatchard analysis indicated the presence of two dissociation constants (Kd) of 1.76×10^{-10} and 2.56×10^{-8} (data not shown).

3.2. Binding specificity of Fd2

Binding specificity was examined by gel mobility shift assay on a 4% polyacrylamide gel (Fig. 2) and eight bands (I–VIII) of nuclear protein that bound Fd2 were observed. When increasing amounts of unlabeled Fd2 (10–200-fold by mass) were introduced, bands I–VI were reduced in intensity (Fig. 2A). Remarkably, the ^{32}P-signals of high molecular mass protein complexes that did not enter the gel were absent in samples with excess cold Fd2. By contrast, adding a 200-fold (by mass) excess of unlabeled consensus regulatory elements did not alter the gel mobility shift pattern, including the signal at the top of the gel. In addition, a 100-fold (by mass) excess of unlabeled *Hae*III-digested pUC8 DNA did not alter the gel mobility pattern although it caused a slight decrease in band intensity (data not shown). These results indicate that multiple proteins bind to Fd2, but it is possible that degradation of the binding proteins caused the multiple bands. When labeled consensus regulatory elements, cAMP regulatory element (CRE), activator protein-1 binding site (AP1-site), and activator protein-2 binding site (AP2-site), were used in the gel mobility shift assay (Fig. 2B), only 1 or 2 bands appeared, indicating that the nuclear protein fraction used in this study did not undergo significant proteolytic degradation.

3.3. Binding of Fd2 subfragments

Five subfragments of Fd2 (Fd2-1, $-852/-818$; Fd2-2, $-832/804$; Fd2-3, $-817/781$; Fd2-4, $-792/-763$; Fd2-5, $-780/-746$) were constructed (Fig. 3).

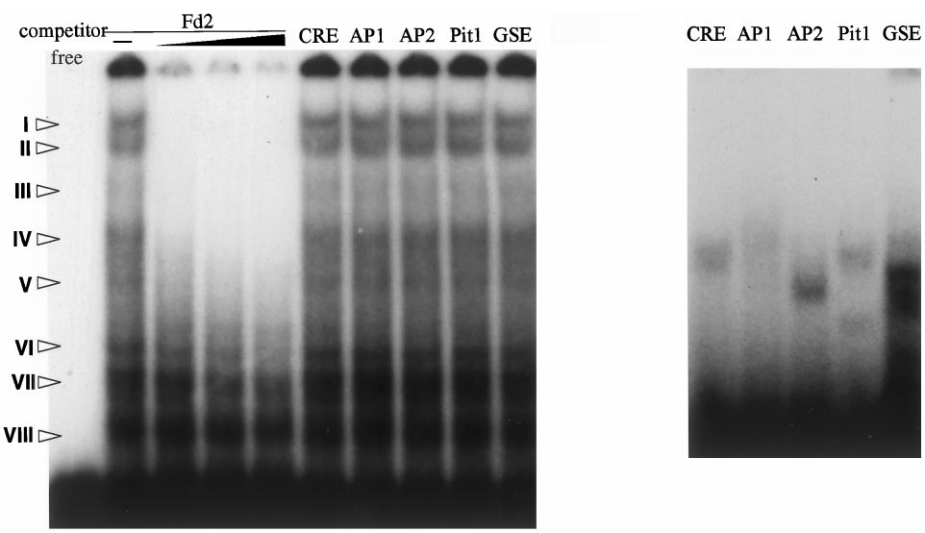

Fig. 2. Specificity of nuclear protein binding to Fd2. The mobility of porcine pituitary nuclear proteins incubated with Fd2 was analyzed on a 4% polyacrylamide gel. (A) Labeled Fd2 was incubated with nuclear proteins in the absence or presence of unlabeled competitors. Increasing amounts of unlabeled Fd2 (10-, 50-, and 200-fold excess) and unlabeled competitors of consensus regulatory elements (200-fold excess) were added to the reaction mixture. Only the probe (free) was applied to the left-hand lane. (B) The gel mobility shift assay was carried out with consensus regulatory elements under the same conditions as described above.

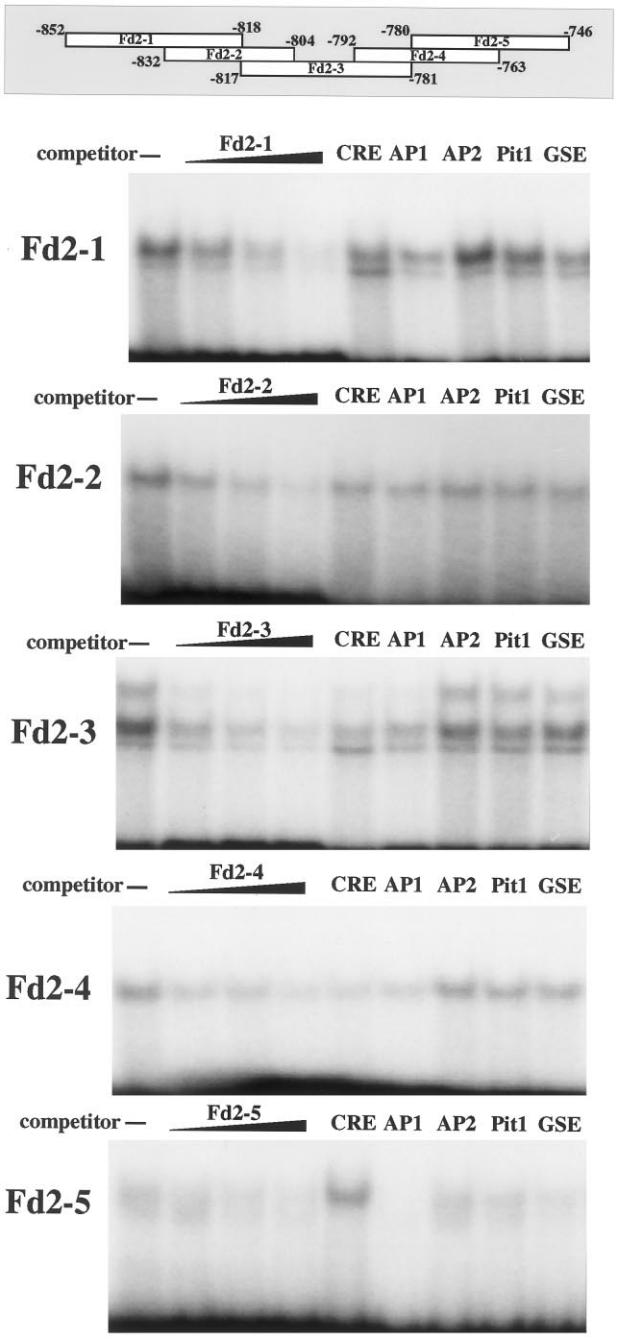

Fig. 3. Gel mobility shift assay of Fd2 subfragments. The positions of five subfragments of Fd2 were determined (upper panel). The labeled Fd2 subfragments were incubated with nuclear extracts in the absence or presence of unlabeled competitors: unlabeled Fd2 (10-, 50-, and 200-fold molar excess) and unlabeled competitors of each consensus regulatory element (200-fold excess).

When the subfragments were examined by gel mobility shift assay all five subfragments showed mobility shifts (Fig. 3). Fd2-1 caused shifts in at least two bands, which were not affected by the addition of unlabeled consensus regulatory elements except for slight decrease in band intensity caused by AP1-site and an increase in band intensity caused by AP2-site. Fd2-2 showed a single shifted band, which was not affected by the presence of regulatory elements. Fd2-3 caused three band shifts, one of which was reduced in intensity by the addition of CRE and AP1-site, although the AP2-site had no effect. Fd2-4 showed a single shifted band, which was considerably reduced in intensity by the addition of CRE and AP1-site. Fd2-5 showed a diffuse shifted band, which was absent in the presence of AP1-site and Pit-1 binding site (Pit1-site) and was reduced in intensity by GSE (gonadotrope-specific element). AP2-site did not affect the band shifted by Fd2-5 however CRE increased its intensity, the same as AP2-site increased the binding of Fd2-1. In all cases for five subfragments, the consensus regulatory elements (CRE, AP1-site, AP2-site, GSE, and Pit site) did not prevent the shift bands completely. These results indicate that the entire sequence of the Fd2 fragment contributes to the binding of nuclear factors.

3.4. Competition of Fd2 subfragment binding to Fd2-nuclear protein binding

The experiments described above showed that each Fd2 subfragment can bind a nuclear protein(s), so we added unlabeled Fd2 subfragments to the reaction mixtures containing labeled Fd2 to test the specificity of the interactions (Fig. 4). Fd2-1 showed reduced intensity of band IV together with slight decreases in the other shifted bands. Fd2-2 showed reduced intensity of band IV but the other shifted bands were not affected. Fd2-3 showed the strongest competition; bands II–V were completely lost and band I was reduced considerably. Note that the high molecular mass protein complexes that did not enter the gel were reduced by the addition of unlabeled Fd2-3. Fd2-4 and Fd2-5 did not show obvious reductions in band intensity.

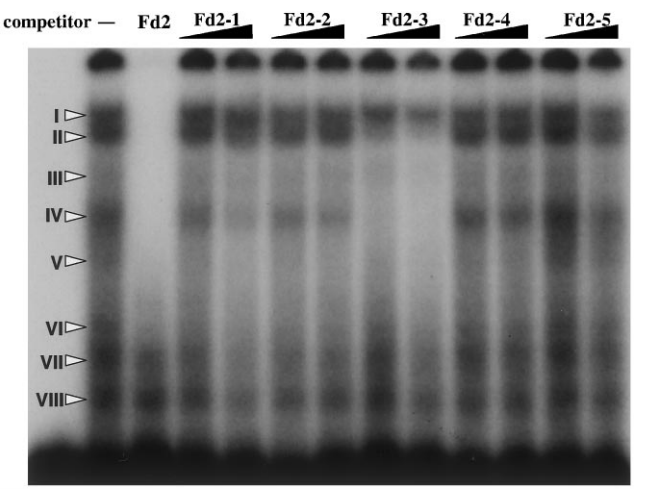

Fig. 4. Competition of Fd2 subfragment binding to Fd2-nuclear protein binding. Gel mobility shift assay of Fd2 subfragments in the absence (left lane) or presence of unlabeled Fd2 (200-fold molar excess) or unlabeled subfragments (50- and 200-fold molar excess).

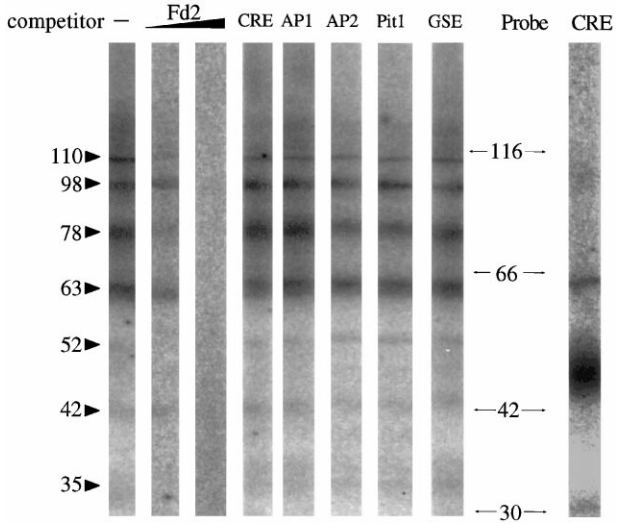

Fig. 5. Southwestern blotting of Fd2-binding proteins. Proteins of porcine anterior pituitary nuclear extracts (100 μg) were resolved by 8% SDS-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose filter. The filters were incubated with labeled Fd2 in the absence or presence of unlabeled Fd2 (50- and 100-fold molar excess) and consensus regulatory elements (100-fold molar excess). Masses (kDa) of the binding proteins were estimated from the molecular mass markers and are indicated on the left.

3.5. Southwestern blotting of Fd2-binding proteins

Southwestern blotting was performed to estimate the molecular masses of the nuclear proteins that bind to Fd2. The proteins had molecular masses of 110, 98, 78, 63, 52, 42, and 35 kDa (Fig. 5). These bands disappeared in the presence of a 100-fold excess of unlabeled Fd2. On the other hand, the addition of unlabeled consensus regulatory elements did not reduce the band intensities. Labeled CRE was used to examine the intactness of pituitary proteins under the conditions of the gel mobility shift assay. CRE labeled a major band at 46 kDa, which is similar to the molecular mass of known CRE-binding proteins (Montminy and Bilezikjian, 1987), and minor bands at higher molecular masses.

3.6. Gel mobility shift assay of other porcine tissues

To verify whether other porcine tissues contain the Fd2-binding proteins, nuclear extracts prepared from other tissues were examined by gel mobility shift assay (Fig. 6). The results demonstrate that nuclear proteins that bind to labeled Fd2 were present in some tissues. Extensive binding was observed for extracts from the cerebrum, cerebellum, kidney, and spleen, as well as from the anterior pituitary, although the binding was reduced to some extent by the addition of an excess amount of unlabeled *Hae*III-digested pUC8 DNA. Extracts from the hypothalamus, intermediate, and posterior lobes of the pituitary, heart, and testis also bound to Fd2, but most of the labeled Fd2 bound to the intermediate and posterior lobes and heart proteins was removed by the addition of *Hae*III-digested pUC8. Interestingly, the cerebellar proteins showed a gel mobility shift pattern that was similar, but not identical, to the pattern for the anterior pituitary. Extracts from some tissues, including the brain, showed a small amount of binding complex that did not enter the gel, similar to the anterior pituitary. Extracts from pancreas and liver did not cause a shift of the Fd2 band. Although binding proteins were present in some tissues, the gel mobility shift patterns differed from that of the anterior pituitary. These results indicate that the anterior pituitary contains distinct protein species that bind to Fd2.

4. Discussion

We have demonstrated in this study that a specific region, called Fd2, of the 5′-flanking region of the porcine FSH β-subunit gene (107 bp long) is bound by several anterior pituitary nuclear proteins. Competitive binding experiments demonstrated that the entire length of Fd2 is involved in nuclear protein binding, indicating that the FSH β-subunit gene is regulated by multiple proteins interacting with each other.

Several fragments of the upstream region of the FSH β-subunit gene caused mobility shifts by binding with nuclear proteins but most of them showed reduced band intensity when unlabeled *Hae*III-digested pUC8 DNA was added to the reaction mixture (Fig. 1), indicating that the binding was not sequence specific. The competition with *Hae*III-digested pUC8 DNA expected to eliminate the binding to similar sequence of

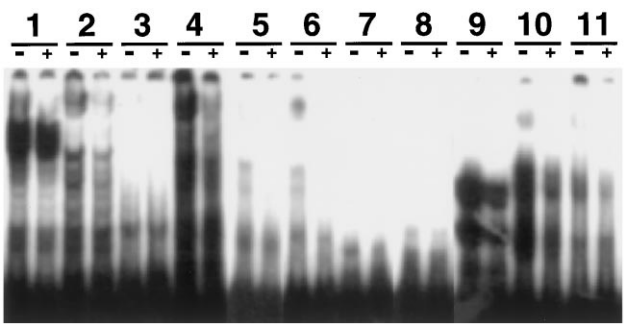

Fig. 6. Gel mobility shift assay of Fd2 incubated with nuclear extracts from various porcine tissues. The mobility shift in a 4% polyacrylamide gel was assayed using nuclear extracts (2.5 μg) prepared from several porcine tissues in the absence (-) or presence (+) of a 100-fold excess (w/w) of unlabeled *Hae*III-digested pUC8. Lane 1, cerebrum; lane 2, cerebellum; lane 3, hypothalamus; lane 4, anterior pituitary; lane 5, intermediate and posterior lobes of the pituitary; lane 6, heart; lane 7, pancreas; lane 8, liver; lane 9, kidney; lane 10, spleen; and lane 11, testis.

CRE, AP-1 site, and other sequences present in pUC8 DNA. The labeled Fd fragment however, caused band shifts that were retained in the presence of excess amount of *Hae*III-digested pUC8 DNA and were lost in the presence of excess amount of unlabeled Fd fragment, indicating that Fd fragment bound to several nuclear proteins in a sequence-specific manner. This fragment, which covers 365 bp, was further divided into three parts (Fig. 1C) to identify the sequence essential for binding. We demonstrated that the binding site is wholly located on the Fd2 fragment.

The specificity of the binding was confirmed by competitive binding with several putative regulatory elements, which did not displace Fd2 (Fig. 2A). By contrast, the labeled Fd2 was displaced by the addition of excess unlabeled Fd2, indicating that the binding was sequence specific. Further analyses of five subfragments that covered the entire Fd2 sequence demonstrated that each subfragment had a characteristic binding pattern in the gel mobility shift assay (Fig. 3). It is apparent from these results that the entire Fd2 sequence interacts with pituitary nuclear proteins. When the Fd2 subfragments were added individually to reaction mixtures containing labeled Fd2 only Fd2-5 failed to displace Fd2 (Fig. 4). Adding Fd2-3, in particular, resulted in a considerable reduction in Fd2 binding to the pituitary proteins. This result suggests that the strength of Fd2 binding may be determined by the Fd2-3 sequence, although no individual subfragment completely prevented Fd2 binding.

The Fd2 region does not contain known consensus regulatory elements such as CRE, AP1-site or steroid hormone-responsive elements (Hirai et al., 1990). A sequence (TAAATCA) similar to AP1-site (TGA(CG)TCA) is present in Fd2-2 and Fd2-3, but competition by several putative regulatory elements did not change the protein mobility pattern of Fd2 (Fig. 2). However, some of the Fd2 subfragments were affected by the addition of putative regulatory elements (Fig. 3). Fd2-5 was completely displaced by the addition of AP1-site and AP1-site also considerably reduced Fd2-1, Fd2-3, and Fd2-4 binding. These observations indicate that proteins that bind AP1-site may be involved in Fd2-protein complex formation. On the other hand, AP2-site and CRE increased the binding of Fd2-1 and Fd2-5, respectively, to the pituitary proteins. However, the interaction of the binding proteins and consensus regulatory elements may not be substantial, because the Fd2 was not displaced by the addition of excess amounts of regulatory elements (Fig. 2).

We observed a large protein complex that bound Fd2 but did not enter the acrylamide gel in the gel mobility shift assay (Fig. 2A and 4). However, this complex was not formed by putative regulatory elements (Fig. 2B). Addition of excess amount of unlabeled Fd2 as a competitor displaced labeled Fd2, whereas addition of the putative regulatory elements did not. These facts suggest that this large complex may be formed by sequence-specific binding of Fd2 and pituitary proteins. Excess amount of each unlabeled Fd2 subfragment did not displace the labeled Fd2 fragments from the large complex, except for a 100-fold molar excess of Fd2-3, suggesting that the proteins bound to the Fd2 subfragments, if any, may be held in the large complex by interactions with other proteins that bind to another part of Fd2. Alternatively, some nuclear proteins may have more than one binding domain for the Fd2 sequence.

Gel mobility shift assay of nuclear extracts from 11 porcine tissues revealed interesting characteristics of the Fd2-binding proteins. The binding pattern of the anterior pituitary was different from those of the other tissues except for the cerebellum, indicating that the pituitary proteins that bind to Fd2 are specifically expressed in this tissue and perhaps in the cerebellum. The possibility that proteins similar to the Fd2-binding proteins of the pituitary are present in neural tissues is interesting because several known proteins are essential for the development of both the brain and pituitary (Seidah et al., 1994; Zhadanov et al., 1995; Valerius et al., 1995; Treier and Rosenfeld, 1996; Sornson et al., 1996; Gage and Camper, 1997; Sheng et al., 1997).

We have demonstrated that a definite region (Fd2, $-852/-746$) upstream from the porcine FSH β-subunit gene is bound by multiple nuclear proteins that are expressed specifically in the anterior pituitary and perhaps also in the cerebellum. The entire sequence of Fd2 (about 100 bp) participated in the binding, although the region of strongest binding was located in the middle of the sequence. Southwestern blotting revealed that at least seven protein species bind specifically to Fd2. These are the first data to show that multiple proteins bind to the 5′-flanking region of the FSH β-subunit gene.

Similarly, the α subunit gene contains a definite regulatory region for multiple transcription factors. The sequence between -130 and -180 contains multiple regulatory sequences, including the trophoblast-specific element, GATA and CRE (Steger et al., 1994). It is also known that the glucocorticoid receptor inhibits CRE binding by CRE-binding protein (Steger et al., 1994). SF-1, which was recently identified as a regulatory factor of steroidogenic organs (Lala et al., 1992), plays a role in the expression of the α-subunit gene (Barnhart and Mellon, 1994). SF-1 recognizes a GSE that is located in the cluster of regulatory elements upstream from the α-subunit gene. There are other important regulatory elements for Lim-homeodomain LH-2 homologue (Roberson et al., 1994) and Ptx1 (Smidt et al., 1997) in the region downstream from the multiple regulatory sequences of the α-subunit gene. On the other hand, the upstream region close to the transcrip-

tion initiation site of the growth hormone gene contains regulatory elements for several transcription factors that interact with each other to precisely modulate simultaneous extracellular signals (Theill and Karin, 1993). This accumulated knowledge indicates that multiple regulatory elements are important for the induction of specific and maximal expression by many transcription factors that bind to their respective elements. Our observation that multiple proteins specifically recognize a limited region of about 100 bp of the FSH β-subunit gene suggests a molecular mechanism that specific binding and interaction between multiple binding proteins modulate this gene, although a better understanding of the regulatory proteins is required.

It is well known that genes encoding pituitary hormones are expressed in specific cell types. Transcription of those genes is regulated by specific transcription factors that recognize respective regulatory elements (Maniatis et al., 1987; Mitchell and Tjian, 1989). Recently, many transcription factors have been identified as essential regulators of pituitary development and maintenance as well as being regulators of gene expression of pituitary hormones (Treier and Rosenfeld, 1996; Tremblay et al., 1998). Ptx1, LH-2, and SF-1 (binds to GSE), in particular, may play critical roles in the expression of the α-subunit and LH β-subunit genes by binding to the upstream sequence (Bach et al., 1995; Tremblay et al., 1998). By contrast, knowledge of the regulatory factors for the FSH β-subunit gene is limited so far, although it has been reported that Ptx1 activates expression of this gene (Tremblay et al., 1998) and that the multiple progesterone response elements (Webster et al., 1995; O'Conner et al., 1998) and an AP1-site (Strahl et al., 1998, 1997) in the 5′-flanking region of the FSH β-subunit gene are functional. Despite the establishment of many pituitary lineage cell lines (Akerblom et al., 1990; Windle et al., 1990; Alarid et al., 1996; Thomas et al., 1996) a cell line that produces FSH is still not available, which is hampering studies of the molecular mechanism that regulates this gene. Therefore, an approach that searches for gene-specific binding proteins may provide new information. The finding of the present study, that many proteins recognize a limited region upstream from the FSH β-subunit gene, provides a clue for understanding the mechanisms that regulate FSH β-gene expression.

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