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Prop-1 Activation of Porcine FSHβ Subunit Gene Requires AT-rich Recognition Sites

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Abstract. We have recently cloned the pituitary specific transcription factor Prop-1 as a regulator of the porcine follicle-stimulating hormoneβ (pFSHβ) gene [1]. This study aimed to establish the characteristics of porcine Prop-1. The deduced amino acid sequence showed considerable diversity in the N-terminal and moderate change in the C-terminal regions among mammalian Prop-1s except for the homeodomain in the middle region. Prop-1 binds to two thirds of the 5' region of Fd2 located between the −852 and −746 base upstream of the pFSHβ gene, which shows a high AT-content (80%). The transfection assay of the pituitary tumor-derived cell line, LβT2, using the chimera genes fused the upstream of the pFSHβ gene as a reporter vector, showed that Prop-1 activates FSHβ promoter but not SV40 promoter. The gene expression of Prop-1 during porcine pituitary ontogeny was observed from fetal days 40 through 110, and its postnatal expression increased even further. Thus, this study demonstrated that Prop-1 definitely stimulates the porcine FSHβ gene by acting on the AT-rich Fd2 region and plays a role in the fetal and postnatal pituitary.

Key words: FSH, Transcription factor, Prop-1, Porcine anterior pituitary, Gonadotropin, Gene regulation

Introduction

The synthesis and secretion of gonadotropins (follicle stimulating hormone, FSH, and luteinizing hormone, LH), are restricted to the same cells called gonadotropes in response to several extracellular signals and physiological requirements. Various investigations have revealed that the gene expression of three subunits of gonadotropin expressing common regulatory factors are involved under different controls. Several approaches have so far succeeded in confirming the molecular mechanisms and transcription factors governing basal and cell-specific expressions of the glycoprotein hormone α subunit (α-GSU) and LHβ genes [2-6], but relatively little knowledge has been accumulated about the molecular mechanisms regulating FSHβ gene expression [7-12]. More recently, we have found for the first time that one of the Fd2 binding proteins [13] is the pituitary specific transcription factor, Prophet of Pit-1 (Prop-1) [1]. Since this factor is the gene responsible for an inherited defect in the dwarf mouse, Ames [14] and a combined pituitary hormone deficiency (CPHD) with hypogonadism [15, 16], our findings provide a novel approach and breakthrough to a better understanding of the control mechanisms not only for FSHβ gene expression but also for the etiology of dwarf/CPHD.

In this study, we demonstrated that Prop-1 binds to the AT-rich sequence of Fd2, resulting in transactivation of the FSHβ gene. RT-PCR of pituitary ontogeny demonstrated that the transcripts of Prop-1 gene persist throughout the fetal and postnatal periods

Materials and methods

1) Sequence analysis

Previously cloned porcine Prop-1 cDNA [1] was prepared from E. coli by the alkaline mini-preparation method. The fluorescence labeled dye-terminator reaction was employed using the Big Dye terminator system (Applied Biosystems, Foster City, CA), according to the instruction manual.

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2) Electrophoretic mobility shift assay (EMSA)

Construction of porcine Prop-1 cDNA in the pET32a vector (Novagen, Darmstadt, Germany) and the production and isolation of recombinant Prop-1 are described in our previous paper [1]. FAM-labeled DNA fragments were produced by PCR using FAM-labeled oligonucleotide 5'-primer. The binding reaction mixture included 100 fmol of FAM-labeled probe DNA (10 ng) and 100 ng of porcine recombinant Prop-1 with 250 or 2000 ng poly (dI-dC) in 10 μl of 10 mM HEPES buffer, pH 7.9, containing 0.4 mM $MgCl_2$, 0.4 mM DTT, and 50 mM NaCl, and 4% glycerol was incubated at 30℃ for 30 min. Samples were then subjected to electrophoresis on a 4% polyacrylamide gel as described in our previous paper [13].

The consensus sequence of the paired homeodomain with Q9 in the third α-helix (PRDQ9), ACTAATTGAATTAGC [14], was used as a control for the EMSA of Prop-1.

3) Expression vector and reporter vector constructs

Porcine Prop-1 cDNA was ligated into the *Eco*RI and *Xho*I sites of a mammalian expression vector, pcDNA3.1Zeo$^+$ (Invitrogen, Carlsbad, CA). Reporter gene constructs were generated by ligation of the upstream regions of the porcine FSHβ gene (Fβ3-Basic: −985/+10, ΔFβ3-Basic: deletion mutant of Fβ3-Basic from −745 to −104 bp, Fβ6-Basic: −103/+10) into pSEAP2-Basic (BD Bioscience Clontech) and Fd2, −852/−746, into pSEAP2-Promoter (BD Bioscience Clontech) (Fd2-promoter).

4) Cell culture, DNA transfections, and reporter gene assays

LβT2 cells, which were established from the mouse pituitary tumor [17] and kindly provided by Dr. P. Mellon, were cultured and used for transfection assay as described previously [1]. Each 5 μl of cultured medium was assayed for secreted alkaline phosphatase activity using the Phospha-Light Reporter Gene Assay System (Applied Biosystems).

5) RT-PCR analysis of Prop-1 expression during porcine ontogeny

Total RNAs were extracted from the porcine anterior pituitaries of German Landrace pigs with intact gonads of both sexes collected at fetal days (f40, f50, f65, f82, f95 and f110), postnatal days (p8, p60, p160 (prepubertal), and p230 (sexually mature)) [18].

Specific primer sets for porcine Prop-1 and cyclophilin A were synthesized as follows; Prop-1 forward 5'-CTACAGAAACCTCTCTGGCGTAGG-3', and reverse 5'-TTGCTTCCTCTGCTTAGCTCTGCG-3', and cyclophilin A forward 5'-TGGTGACTTYACACGCCATAATG-3', and reverse 5'-ATTCCTGGACCCRAAACGCTCC-3'. Amplifications and analysis on agarose gel (2%) were described previously [18].

Results

1) Nucleotide sequence of Prop-1

The nucleotide sequence of the clone revealed the entire sequence of 966 bp, including the open reading frames of 681 bases of 226 amino acids (DDBJ accession no. AB187272). Fig. 1 shows the predicted amino acid sequence of porcine Prop-1, consisting of 226 amino acids by alignment with the known amino acid sequences of other mammalian Prop-1s. The central DNA-binding HD and

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Pig   : MEAEGRREQGKPRKGRVCSSLWPEGYPAAGTLTARVDISTRPYRNLSGVGAGRPRLSPQGGQRGRPHSRRRHRTTFSPAQLEQLESAFGRNQYPDIWARE 100
Man   : ----R--QA---K-E--G---L--RH--T--P-TT--S-AP-C-R-P-A-G--S-F----------------------V---------------------
Rat   : ---QR-SQ---QT-EP--GRSL--SQA-S---IST--R-PETSKR---T-L---K-C--   --------------N-----G---------------V--
Mouse: ---QR-SH---QT-EHA-GRSL--PRV-S---IST--R-SEA--R---TEL---K-C--   --------------N-----------------------

Pig   : GLARDTGLSEARIQVWFQNRRAKQRKQERSLLQPLAHLSPATFSGFLPEPPACPYSYPTPPPPMTCFPHPYNHALPSQPSTGGSFARHPQSEDWYPTLHP 200
Man   : S-------------------------------------A--S----ST------AA----V-------S-----------A--LSH-----------
Rat   : ---Q--------------------------------T-----------S-SSPY--T-T-----VP--------------C--A-LTLPA-P---------
Mouse: ---Q--------------------------------I----T---------SS-Y--T-G-----AP------S-S-------AA-L-LPP-P---------

Pig   : TPTGHLPCPPAPPVLPLSLEPPKSWN 226
Man   : A-A-------P--M-------S----
Rat   : -H----A---P--MFS----T-----
Mouse: A---------P--MF-----T-----
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Fig. 1. Comparison of amino acid sequences of Prop-1. Amino acid sequence of porcine Prop-1 was compared with those of human, rat and mouse. Each amino acid identical to that of porcine Prop-1 is indicated by a hyphen (-), and open spaces represent gaps to optimize homology. Homeodomain is underlined.

carboxyl terminus of the activation domain [14] were respectively well and moderately conserved, while the amino terminus of the inhibitory domain showed a considerable divergence.

2) In vitro binding assay by EMSA

To confirm the binding of Prop-1 to Fd2, EMSA was carried out using 5 subfragments of Fd2 (Fd2-1~Fd2-5) designed to overlap each other. As seen in Fig. 2, PRDQ9 showed a single shift band even in the presence of 250 ng of poly (dI-dC). Fd2-1, -2, and -3 exhibited shift bands and binding was not prevented by 2000 ng of poly (dI-dC). In contrast, Fd2-4 showed a shift band that was lost following 2000 ng of poly (dI-dC) (data not shown). In addition, Fd2-1, -2, and -3 mainly showed two shift bands, and Fd2-4 a single one, while no binding at all was observed only in Fd2-5, which is not AT-rich.

3) Transcriptional activation of porcine FSHβ gene by Prop-1 in LβT2 cells

Transfection of pSEAP2-Basic and pSEAP2-Promoter with or without expression vectors of porcine Prop-1 showed similar expression level of SEAP gene as that in LβT2 cells (Fig. 3), indicating the absence of endogenous activation by unexpected regulatory elements present in the vector construct. The expression level did not increase following the transfection of Fd2-Promoter, indicating that the SV40 promoter did not work properly in combination with Fd2. Reporter vectors of Fβ3-Basic, ΔFβ3-Basic, and Fβ6-Basic as well as the transcriptional activity of Prop-1 were examined. Unlike Fβ6-Basic, Fβ3-Basic and ΔFβ3-Basic showed a significant activation ($P<0.01$) of 2.2-fold and 2.5-fold, respectively, by cotransfection with the expression vector of porcine Prop-1.

4) RT-PCR analysis of Prop-1 gene expression during porcine pituitary ontogeny

RNAs prepared from fetal and postnatal porcine anterior pituitaries of both sexes were observed during all periods examined for both genders, though some changes and sex differences were detected (Fig. 4). The level of Prop-1 expression in male fetuses decreased temporally at f82 and f110, and showed a slight postnatal increase. In females, the amounts of Prop-1 transcript kept almost similar levels with no marked change during the fetal period. Interestingly, the postnatal expression level in females increased markedly.

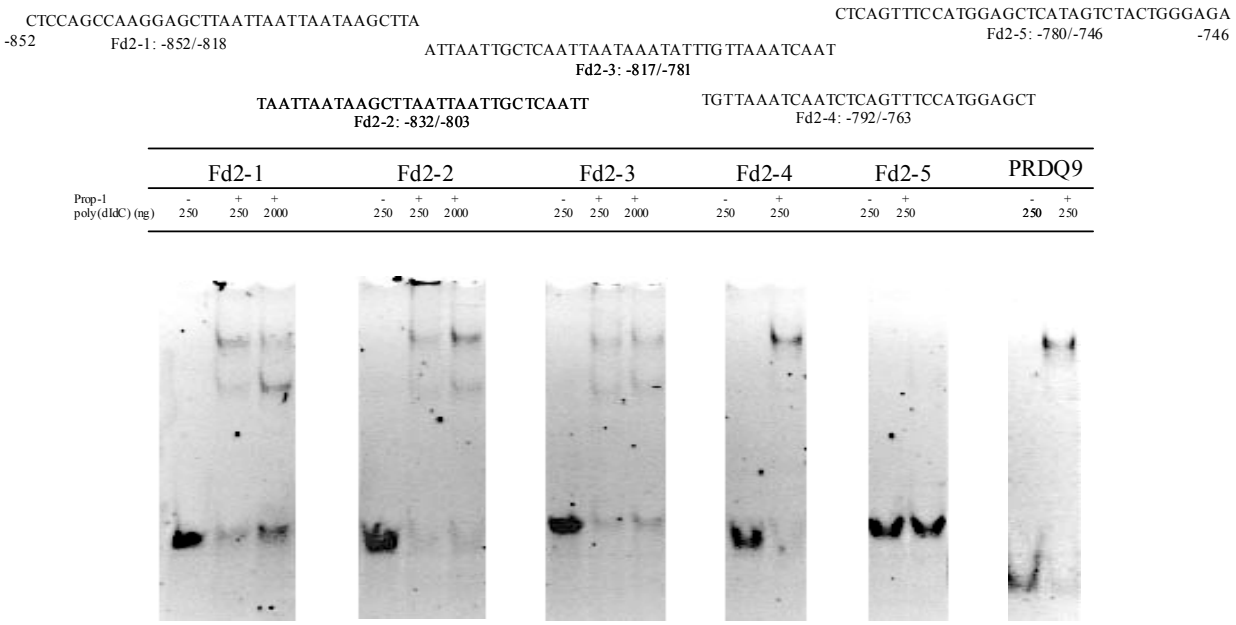

Fig. 2. Electrophoretic mobility shift assay. Binding between recombinant Prop-1 and FAM-labeled Fd2 subfragments was separated on a 4% polyacrylamide gel followed by visualization with a fluorescence viewer. Composition of each binding mixture is listed above the electrophoretic pattern. Paired homeodomain response element (PRDQ9), ACTAATTGAATTAGC [14], was used as a control for EMSA.

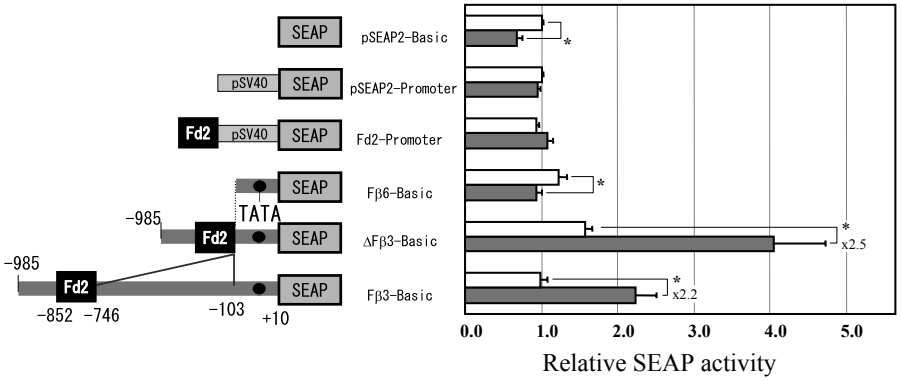

Fig.3. Transcriptional activation of FSHβ gene by Prop-1 in LβT2 cells. Porcine Prop-1 cDNA in pcDNA3.1$^+$ vector (closed bar) or pcDNA3.1$^+$ vector alone (open bar) was cotransfected with pSEAP2-Basic, pSEAP-Promoter, Fd2-Promoter, Fβ6-Basic, ΔFβ3-Basic, and Fβ3-Basic vectors into LβT2 cells. After 48-h incubation, an aliquot of cultured medium was assayed for secreted alkaline phosphatase activity. Each activity was normalized using that obtained from cotransfection of pcDNA3.1+ vector alone with pSEAP2-Basic. Asterisks show a significant difference ($P<0.01$) compared with the values with Prop-1-pcDNA3.1 and pcDNA3.1$^+$ vector alone.

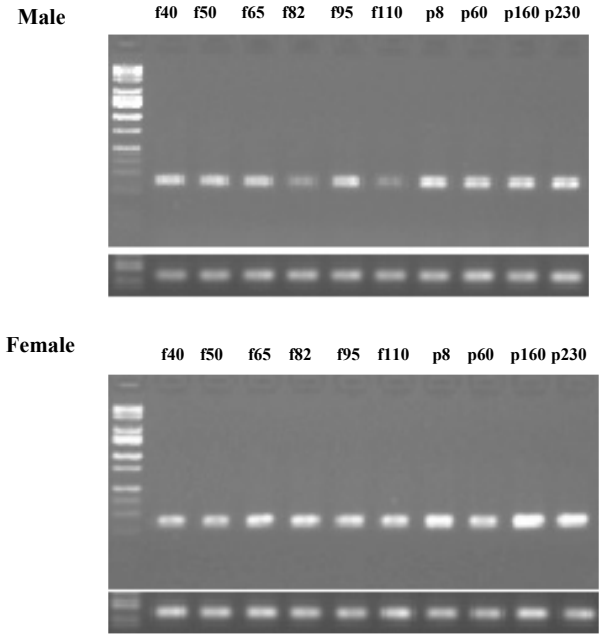

Fig. 4. Ontogeny of porcine Prop-1. Total RNAs prepared from fetal and postnatal porcine pituitaries of both sexes were analyzed by RT-PCR. Amounts of cDNA sample, which produce the same amounts of PCR product for cyclophilin A (shown in lower panel), were subjected to PCR reaction for Prop-1.

Discussion

In this study we demonstrated that Prop-1 acts as a transcription factor for the FSHβ gene by binding to the multiple binding sites of an AT-rich region about −800 bp upstream of the porcine FSHβ gene. In addition, RT-PCR revealed a continuous Prop-1 gene expression during pituitary organogenesis, and an elevated expression postnatally, indicating that Prop-1 participates in pituitary development by acquiring a capacity for hormone production and maintaining the mature pituitary function.

Our examination of sub-fragments of Fd2 showed that Prop-1 binds to four of five of them (Fig. 2). It is noteworthy that the binding of Prop-1 showed two shift bands, one of which corresponded to the shift band of PRDQ9 known to be bound with two molecules [14], indicating a binding of one or two Prop-1 molecules. In addition, these sub-fragments are AT-rich and contain a core motif TAAT/ATTA of the binding site for homeodomain transcription factor [19], except for Fd2-4, which has no core motif and shows only a weak binding affinity.

Transfection assay of LβT2 cells showed a significant transcriptional activation of the SEAP gene (Fig. 3). It is noteworthy, that the fusion of Fd2 to the SV40 promoter did not work, whereas the region −100 bp upstream of porcine FSHβ gene clearly acted as a promoter for transcriptional activation by Fd2, indicating the importance of the combination of the endogenous promoter with Fd2. In addition, Fd2 functioned as a *cis*-acting element in both Fβ3-Basic and ΔFβ3-Basic, indicating its enhancer activity.

Prop-1 has been an indispensable transcription factor for the development of Pit-1 lineage hormone-producing cells [14]. During the ontogeny of murine Prop-1 gene expression, the Prop-1 transcript could first be detected as early as embryonic day 10 (e10) when Rathke's pouch is devel-

oping. Prop-1 gene expression reached its maximum level by e12, followed by a decline to an extremely low level by e14.5 with the distribution of the dorsal/ventral gradient [14]. During anterior pituitary development, the differentiation of hormone-producing cell types takes place in a temporal sequence in distinct regions. In the murine anterior pituitary, both LH- and FSH-producing cells finally appear last of all at e16.5 and e17.5 in the ventral region where the expression level of Prop-1 is low [20]. Thus, a spatially and temporally unique expression of Prop-1 at an early stage is essential for the determination and differentiation of pituitary hormone-producing cells. However, we demonstrated by RT-PCR of porcine pituitary ontogeny that the Prop-1 gene is expressed throughout the fetal (from f40 through f110) and postnatal periods. We have also observed that Prop-1 mRNAs are present at high levels in the pituitary tumor-derived cell lines LβT2 and LβT4 (unpublished observation). Our data suggested that Prop-1 might participate in the development of hormone-producing cells up to the birth and in the control of gene regulation of pituitary hormones during the postnatal period.

In this study, we have demonstrated that Prop-1 is a transcription factor for FSHβ gene. It is known that combined pituitary hormone deficiency (CPHD) caused by a defective Prop-1 gene is accompanied by hypogonadism as well as by a defect in Pit-1-dependent lineage cells. Therefore, the present finding provides a novel insight into our understanding of CPHD defects.

In summary, we have confirmed the function of Prop-1 in regulation of the FSHβ gene, its marked presence in postnatal pituitaries, and its role in developing porcine fetal pituitaries, suggesting that Prop-1 is involved in the pituitary function in addition to the determination of cell lineages that produce pituitary hormones. Finally, the present results also provide new information for an improved understanding of CPHD.

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