Pituitary transcription factor Prop-1 stimulates porcine pituitary glycoprotein hormone α subunit gene expression

Takanobu Sato, Kousuke Kitahara, Takao Susa, Takako Kato and Yukio Kato

Laboratory of Molecular Biology and Gene Regulation, Department of Life Science, School of Agriculture, Meiji University, 1-1-1 Higashi-mita, Tama-ku, Kawasaki, Kanagawa 214-8571, Japan

(Requests for offprints should be addressed to T Sato; Email: yukato@isc.meiji.ac.jp)

Abstract

Recently, we have reported that a Prophet of Pit-1 homeodomain factor, Prop-1, is a novel transcription factor for the porcine follicle-stimulating hormone β subunit (FSHβ) gene. This study subsequently aimed to examine the role of Prop-1 in the gene expression of two other porcine gonadotropin subunits, pituitary glycoprotein hormone α subunit (αGSU), and luteinizing hormone β subunit (LHβ). A series of deletion mutants of the porcine αGSU (up to −1059 bp) and LHβ (up to −1277 bp) promoters were constructed in the reporter vector, fused with the secreted alkaline phosphatase gene (pSEAP2-Basic). Transient transfection studies using GH3 cells were carried out to estimate the activation of the porcine αGSU and LHβ promoters by Prop-1, which was found to activate the αGSU promoter of −1059/+12 bp up to 11.7-fold but not the LHβ promoter. Electrophoretic mobility shift assay and DNase I footprinting analysis revealed that Prop-1 binds to six positions, −1038/−1026, −942/−928, −495/−479, −338/−326, −153/−146, and −131/−124 bp, that comprise the A/T cluster. Oligonucleotides of six Prop-1 binding sites were directly connected to the minimum promoter of αGSU, fused in the pSEAP2-Basic vector, followed by transfecting GH3 cells to determine the cis-acting activity. Finally, we concluded that at least five Prop-1 binding sites are the cis-acting elements for αGSU gene expression. The present results revealed a notable feature of the proximal region, where three Prop-1-binding sites are close to and/or overlap the pituitary glycoprotein hormone basal element, GATA-binding element, and junctional regulatory element. To our knowledge, this is the first demonstration of the role of Prop-1 in the regulation of αGSU gene expression. These results, taken together with our previous finding that Prop-1 is a transcription factor for FSHβ gene, confirm that Prop-1 modulates the synthesis of FSH at the transcriptional level. On the other hand, the defects of Prop-1 are known to cause dwarfism and combined pituitary hormone deficiency accompanying hypogonadism. Accordingly, the present observations provide a novel view to understand the hypogonadism caused by Prop-1 defects at the molecular level through the regulatory mechanism of αGSU and FSHβ gene expressions.

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Introduction

The pituitary glycoprotein hormones, LH, FSH, and thyroid-stimulating hormone (TSH), are a family of heterodimeric proteins that consist of a common α subunit (αGSU) noncovalently associated with a hormone-specific subunit (Pierce & Parsons 1981). In the pituitary gland, LH and FSH are synthesized within gonadotropes, while TSH is synthesized in thyrotropes. In addition, this αGSU is also expressed in the primate and equine placenta to produce chorionic gonadotropins (Fiddes & Goodman 1981). Thus, αGSU gene is expressed within two different cell types in the pituitary and in different tissue in a cell-type specific as well as tissue-specific manner. Meanwhile, the expression of specific β-subunit genes, LHβ and FSHβ, are differently regulated (Papavasiliou et al. 1986, Kato et al. 1989). To clarify the specific regulatory mechanism of αGSU and specific β-subunit genes, several investigations have been conducted and several regulatory elements and transcription factors were reported (for review, see Brown & McNeilly 1999, Savage et al. 2003, Jorgensen et al. 2004).

We recently demonstrated that one of the DNA-binding proteins for the Fd2 region (−852/−746 bp) of the porcine FSHβ gene (Kato et al. 1999) is a pituitary-specific transcription factor, Prophet of Pit-1 (Prop-1; Aikawa et al. 2004). Our finding may throw light on a novel aspect that may be helpful in clarifying the regulatory mechanism of the FSHβ gene and also serve to pose the question of whether Prop-1 regulates other subunit genes of gonadotropins, αGSU and LHβ, which are expressed in gonadotropes as well as FSHβ gene.

The Prop-1 gene was originally identified as the gene responsible for a heritable form of murine Ames dwarfism (df; Sornson et al. 1996). Similarly, recessive mutations in the human Prop-1 gene result in combined pituitary hormone deficiency (CPHD) with hypogonadism (Wu et al. 1998). Sornson et al. demonstrated that Prop-1 is essential as an upstream transcription factor
of Pit-1 that determines the development of Pit-1 lineage hormone-producing cells, somatotrope, lactotrope, and thyrotrrope that produce growth hormone (GH), prolactin (PRL), and TSH. It is notable that the defect of murine Prop-1 causes Ames dwarfism, which reduces levels of FSH and LH (Tang et al. 1993) in addition to inducing a deficiency in GH, PRL, and TSH as shown in human CPHD. Accordingly, such evidence encouraged us to undertake this investigation into whether Prop-1 participates in the regulation of the genes encoding gonadotropin subunits.

In this study, we have examined the stimulation of the promoter activity of both αGSU and LHβ genes by Prop-1 using transient transfection assay, followed by analyzing the Prop-1-binding site by electrophoretic mobility shift assay (EMSA) and DNase I footprinting. Our results show that the promoter of αGSU gene was activated by Prop-1, whereas that of LHβ gene was not. EMSA and DNase I footprinting demonstrated that the αGSU promoter has at least six Prop-1-binding sites.

**Materials and methods**

**Construction of reporter vectors and expression vector**

To obtain serial truncated upstream regions of the porcine αGSU gene (accession number: D00768; Kato et al. 1991) and porcine LHβ gene (accession number: D00579; Ezashi et al. 1990; Fig. 1B and E), specific primer sets for PCR were designed and synthesized (Table 1). PCR was carried out in a reaction mixture (5 μl) containing two required primers (5 pmol each) and 0.125 U AmpliTaqGold DNA polymerase (PE Applied Biosystems, Foster City, CA, USA) with 32–36 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 30 s), and extension reaction (72 °C, 2 min) steps. The resulting amplified products were ligated to the secreted alkaline phosphatase (SEAP) plasmid vector, pSEAP2-Basic (Clontech Laboratories, Inc., Mountain View, CA, USA), which has no eukaryotic promoter, resulting in reporter vectors αGSU(−1059/+12), αGSU(−798/+12), αGSU(−540/+12), αGSU(−239/+12), αGSU(−100/+12), and αGSU(−53/+12).

![Diagram of porcine αGSU, LHβ promoters, and DNA fragments.](image-url)
for αGSU promoter, and LHβ(−1277/+7), LHβ(−950/+7), LHβ(−676/+7), LHβ(−331/+7), and LHβ(−57/+7) for LHβ promoter. Using oligonucleotides listed in Table 2, we have constructed expression vectors containing putative Prop-1-binding sites and their mutants as follows. The −1059/−1015, −946/−909, −503/−474, −359/−319, −163/−142, and −147/−114 bp regions of αGSU, and their mutants were directly connected to the minimum αGSU promoter vector, αGSU(−53/+12), in forward orientation resulting in αGSU(−1059/−1015), αGSU(−1059/−1015)-m, αGSU(−946/−909), αGSU(−946/−909)-m, αGSU(−503/−474), αGSU(−503/−474)-m, αGSU(−359/−319), αGSU(−359/−319)-m, αGSU(−163/−142), αGSU(−163/−142)-m, αGSU(−147/−114), and αGSU(−147/−114)-m. The Prop-1 consensus binding element (PRDQ9; Sormson et al. 1996) and its mutant were also connected to αGSU(−53/+12) resulting in αGSU(PRQ9) and αGSU(PRQ9)-m. The integrity of all DNA fragments inserted to vectors was confirmed by DNA sequencing on the ABI PRISM 310 (PE Applied Biosystems).

Porcine Prop-1 cDNA (Aikawa et al. 2004) was ligated to the EcoRI/XhoI site of the mammalian expression vector, pcDNA3.1/Zeo+ (Invitrogen) to construct the Prop-1 expression vector, Prop-1/pcDNA3.1.

### Table 1

<table>
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<th>Fragment</th>
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<td>Reverse</td>
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<td></td>
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<td>−239/+12</td>
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*Table 1 Primers used to generate porcine αGSU and LHβ vectors and FAM-labeled αGSU fragment*

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**Cell culture, transfection, and reporter gene assay**

LβT2 and GH3 cells were used for transient transfection assay. LβT2 cells, which endogenously express gonadotropin genes of αGSU, LHβ, and FSHβ (Pernasetti et al. 2001), are the pituitary gonadotrope cell line established by targeted oncogenesis in transgenic mice (Alarid et al. 1996) and were kindly provided by Dr P Mellon. GH3 cells are the clonal somatomammotrope cell line of the rat pituitary (Bancroft et al. 1969), which endogenously express several pituitary specific transcription factors, including Pit-1 and pituitary hormones of GH and PRL, but not αGSU, FSHβ, or LHβ. GH3 cells were obtained from RIKEN Cell Bank (Tsukuba, Japan). The maintenance of cells was performed in monolayer cultures in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO-BRL, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; HyClone, Logan, UT, USA) and antibiotics (Sigma-Aldrich Co.) for LβT2 cells or in DMEM/F-12 medium (GIBCO-BRL) supplemented with 10% (v/v) horse serum (JRH Biosciences, Lenexa, KS, USA), 2.5% (v/v) FBS (JRH Biosciences), and antibiotics (Sigma-Aldrich Co.) for GH3 cells in humidified 5% CO2–95% air at 37 °C. LβT2 cells were plated at 1·0×10^5 cells/100 μl per well in a 96-well plate (Corning Inc., Corning,
NY, USA). Then, 24 h after seeding, cells were transfected with the complex of 0.2 μl Lipofectamine2000 (Invitrogen) and 20 ng reporter vector or pSEAP2-Basic DNA with or without Prop-1/pcDNA3.1 per well in quadruplicate (n = 4) in two independent experiments according to the manufacturer’s instructions. In GH3 cells, the complex of 0.3 μl FuGENE6 (Roche Diagnostics GmbH) and 10 ng reporter vectors was assayed for SEAP activity using the Phospha-Light (Roche Diagnostics GmbH) and 10 ng reporter vectors or pSEAP2-Basic DNA with or without Prop-1/pcDNA3.1 per well in quadruplicate. Samples were then subjected to electrophoresis on a 4% polyacrylamide gel as described in our previous paper (Kato et al. 1999).

**Table 2 Synthetic oligonucleotides used to generate porcine αGSU vectors containing putative Prop-1-binding elements and mutations in these elements**

<table>
<thead>
<tr>
<th>PRDQ9</th>
<th>5’-CGCGTGTGCTGAATTAGTAAGCGCA-3’</th>
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<tr>
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<td>5’-CGCGTGTGCTGAATTAGTAAGCGCA-3’</td>
<td>3’-ACAGCAATTAACTATACCACCGTCGTCG-5’</td>
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<tr>
<td>5’-CGCGTGTGCTGAATTAGTAAGCGCA-3’</td>
<td>3’-ACAGCAATTAACTATACCACCGTCGTCG-5’</td>
</tr>
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</table>

The sequence in wild type where mutation was introduced in bold letters. Mutated sequence generated is underlined. m, Mutated oligonucleotide.

**Electrophoretic mobility shift assay**

Production and purification of the Trx/His-tag fused recombinant porcine Prop-1 and Trx/His-tag protein (Tag protein) were carried out as previously described (Aikawa et al. 2004). Purified recombinant porcine Prop-1 and Tag protein were analyzed on 10% SDS-PAGE followed by staining with Bio-Safe Coomassie Blue G-250 (Bio-Rad Laboratories). To accomplish EMSA, FAM-labeled DNA fragments (Fig. 1C) were produced by PCR using 5’ FAM-labeled oligonucleotide primer (Table 1). The binding reaction mixture included 100 ng recombinant porcine Prop-1 or Tag protein, 100 fmol FAM-labeled DNA and 2 μg poly(dI-dC) in 10 μl of 10 mM HEPES buffer (pH 7.9), containing 0.4 mM MgCl2, 0.4 mM DTT, 50 mM NaCl, and 4% glycerol, and was incubated at 30 °C for 30 min. Samples were then subjected to electrophoresis on a 4% polyacrylamide gel as described in our previous paper (Kato et al. 1999).

**DNase I footprinting assay**

The 5’ FAM-labeled DNA fragments were incubated with 200 or 400 ng recombinant porcine Prop-1 in binding buffer under the same conditions used for EMSA. After a 30-min incubation at 30 °C, 0-2 or 0-4 U RQ1 RNase-Free DNase (Promega) was added, and the mixture was incubated for 5 min at 25 °C. The reaction was stopped by the addition of EDTA to a final concentration of 100 mM, and proteins were then removed by phenol–chloroform extraction. DNA fragments were precipitated, dissolved in 10 μl formamid...
containing 0.5 μl ROX-labeled GS-500 (PE Applied Biosystems) as a molecular size marker, and analyzed on a GeneScan analyzer equipped with an ABI PRISM 310 (PE Applied Biosystems).

Results

Prop-1 activates porcine αGSU promoter, but not porcine LHβ promoter

Several regulatory elements of the porcine αGSU promoter (Kato et al. 1991) and the porcine LHβ promoter (Ezashi et al. 1990) have been identified in the proximal region (Fig. 1A and D). To investigate whether Prop-1 regulates the transcription of the αGSU and LHβ genes, we constructed each series of reporter vectors by fusing sequential deletion mutants of the promoter regions, K1059/C12 bp for αGSU (Fig. 1B) and K1277/C7 bp for LHβ (Fig. 1E), to pSEAP2-Basic and assayed the promoter activities by transfection in two pituitary cell lines, LβT2 cells and GH3 cells, with or without Prop-1/pcDNA3.1. The real-time PCR analysis using total RNA isolated from cultured cells revealed that Prop-1 gene expresses in LβT2 cells, but not in GH3 cells (Aikawa et al. 2006).

In LβT2 cells, as shown in Fig. 2, the basal promoter activity of porcine αGSU was 47-, 89-, 58-, and 7-fold higher than that of pSEAP2-Basic, for αGSU (−1059/+12), αGSU (−798/+12), αGSU (−540/+12), and αGSU (−239/+12) respectively. αGSU (−100/+12) and αGSU (−53/+12) showed the same basal level as that of pSEAP2-Basic. In the presence of Prop-1/pcDNA3.1, the promoter activity in reporter vectors of αGSU promoter up to −239 bp was not enhanced. The promoter activity of αGSU (−540/+12), in spite of the high basal level, was enhanced significantly to 1.4-fold (P<0.01) by Prop-1 and the further distal promoter was also likely to be enhanced. However, the basal promoter activity of porcine LHβ gene in LβT2 cells was lower than or equal to that of pSEAP2-Basic without obvious enhancement by Prop-1.

As shown in Fig. 3, in GH3 cells, the basal promoter activity of all reporter vectors of αGSU promoter was equal to or lower than that of pSEAP2-Basic. In the presence of Prop-1/pcDNA3.1, the reporter vectors of αGSU promoter up to −100 bp showed the same expression level of SEAP gene as that of pSEAP2-Basic.

Figure 2 Transient transfection assay of porcine αGSU and LHβ promoters in LβT2 cells. Truncated promoters of (A) αGSU and (B) LHβ fused with SEAP gene in pSEAP2-Basic vector (shown in left panel) were transfected in LβT2 cells with/without Prop-1 expression vector. An aliquot of cultured medium was used for SEAP assay. Reporter gene activities are indicated relative to pSEAP2-Basic vector. Shaded and solid bars indicate values with pcDNA3.1 and Prop-1/pcDNA3.1 respectively. Data (mean±s.d.) are means of quadruplicate transfections in two independent experiments. Asterisk (*) indicates statistical significance by Student’s t-test (P<0.01).
Activation of the expression level of SEAP gene by Prop-1 was 4.3-fold for aGSU(K239/C12), 9.4-fold for aGSU(K540/C12), 9.7-fold for aGSU(K798/C12), and 11.7-fold for aGSU(K1059/C12). aGSU(K540/C12) and aGSU(K239/C12) showed a similar expression level in the presence of Prop-1/pcDNA3.1, but different activation of 9.4 and 4.3 respectively. This fold-change is caused by a decrease in the basal expression level. These results indicate that Prop-1-responsive elements are located in regions K239/K101, K540/K240, and K1059/K799 bp. It is noteworthy that, although porcine pituitary glycoprotein hormone basal element (PGBE) is located at K345/K301 bp (Schoderbek et al. 1993), no typical elements have so far been identified in the K1059/K541 bp region.

In contrast, Prop-1 failed to activate the LHb promoter, showing almost the same expression level as pSEAP2-Basic.

**Prop-1 binds to aGSU promoter**

Recombinant porcine Prop-1 and Tag protein were isolated from *E. coli*, and the purities were confirmed by 10% SDS-PAGE (Fig. 4A). The binding activity of recombinant porcine Prop-1 was assayed in comparison with Tag protein as a negative control using FAM-labeled aGSU fragments (Fig. 1C) by EMSA in the presence of about a 200-fold excess amount of poly(dI-dC) as a competitor to prevent nonspecific binding.

**Figure 3** Transient transfection assay of porcine aGSU and LHb promoters in GH3 cells. Truncated promoters of (A) aGSU and (B) LHb (shown in left panel) fused with SEAP gene in pSEAP2-Basic vector were transfected in GH3 cells with/without Prop-1 expression vector, followed by assaying reporter gene activities and processing data as described in Fig. 2.

**Figure 4** EMSA of Prop-1 binding of aGSU gene promoter. (A) Protein used for EMSA was analyzed on 10% SDS-PAGE. Lane 1, protein molecular marker (in kilo Daltons, kDa); lane 2, Trx/His-tag protein (20 kDa); lane 3, Trx/His-tag fused Prop-1 (43 kDa). (B) FAM-labeled fragments of porcine aGSU gene were analyzed on 4% PAGE after binding with recombinant Prop-1 or Tag protein under the condition described in Materials and methods. Arrow (→) indicates the shift band.
binding (Fig. 4B). Prop-1 certainly binds to \(-1059/\-740\), \(-540/\-190\), and \(-239/\+12\) bp fragments, giving multiple shift bands. Only small amounts of shift bands were observed in \(-798/\-501\) bp fragment. Since Tag protein did not give any shift band, these shift bands were created by the binding with Prop-1 itself. These binding features are consistent with the results of the transfection experiment described earlier.

**Prop-1 binds to A/T rich sequences in αGSU promoter**

Transfection assay and EMSA demonstrated the presence of responsive elements for Prop-1 in the αGSU promoter between \(-1059\) and \(-101\) bp. However, there was no consensus Prop-1-binding site, so-called PRDQ9 TAATgATTA (Sornson et al. 1996), in this region. DNase I footprinting analysis was performed to determine the nucleotide sequence of Prop-1-binding sites using 5′ FAM-labeled fragments of the αGSU promoter. FAM-labeled DNA fragments with or without the binding of Prop-1 were digested by DNase I, and the digested fragments were recovered, followed by separation on capillary electrophoresis and analysis on the GeneScan Analyzer using ABI PRISM 310 (Fig. 5A–E). In the distal region over \(-800\) bp, a loss of signals caused by the protection against DNase I digestion by Prop-1 binding was observed in the region \(-1045/\-1026\) bp (5′-ACTAATTCATATC-3′) (Fig. 5A), and an increase in signals caused by conformational change against DNase I digestion by Prop-1 binding was found in the region \(-1020/\-1015\) bp. The region \(-942/\-928\) bp (5′-AGAAATGAACCTGATTA-3′; Fig. 5B) also lost signals, and an increase in signals was found in the adjacent region at \(-927/\-921\) bp. In the region \(-540/\-190\) bp, which showed maximal binding to Prop-1, decreased signals were observed in the regions \(-495/\-479\) bp (5′-CATCCTTATGACATATC-3′; Fig. 5C), and \(-338/\-326\) bp (5′-AGCTAATTAAATG-3′; Fig. 5D). It is noteworthy that the latter sequence overlaps with that of Lhx2-binding site (5′-TACTTAGCTAATTA-3′, K343/330; Roberson et al. 1994). In addition, the increased signals by Prop-1 binding appeared in the regions \(-476/\-474\) and \(-325/\-314\) bp. Finally, in the proximal region, decreased signals were observed in the regions \(-153/\-146\) bp (5′-AGATAAGA-3′) and \(-131/\-124\) bp (5′-TGCTAATTAAATG-3′; Fig. 5E). Adjacent to these regions, the increased signals were also observed in \(-119/\-113\) bp. The DNase I footprinting analysis for \(-798/\-501\) bp did not show any marked change of signals (data not shown).

Prop-1-binding sequences identified by DNase I footprinting are summarized in Table 3. They consist

![Figure 5 DNase I footprinting analyses. DNase I digests were prepared with or without recombinant porcine Prop-1 (lower panel and upper panel respectively), followed by analysis using a capillary sequencer with GeneScan system. Solid bar indicates Prop-1-binding region. Nucleotide sequence and number are shown below and summarized in Table 3. Gray bar indicates signal increased by Prop-1 binding. Nucleotide sequence and number are shown in box.](https://www.endocrinology-journals.org)
of an A/T cluster. Especially four binding sequences contain TAAT motif of conserved homeodomain-binding sites. Taken together, the binding sequences identified were compatible with the results of the transfection assay showing that possible responsive regions are included in −1059/−799, −540/−240, and −239/−101 bp (Fig. 3A).

Regulatory role of Prop-1-binding sites

DNase I footprinting demonstrated that Prop-1 strongly binds to the region −338/−326 bp located in PGBE (−345/−301 bp; Schoderbek et al. 1993) with marked sensitivity to DNase I (Fig. 5D). In addition, five other Prop-1-binding sites were identified as described earlier. Hence, each cis-acting activity regardless of its position was examined using the consensus PRDQ9 as a positive control. We generated the reporter vectors constructed by directly ligating the wild type and mutated sequences of Prop-1-binding site and PRDQ9 to the minimum αGSU promoter vector, αGSU(−53/+12), followed by a transfection assay of GH3 cells with/without Prop-1 (Fig. 6). Synthetic oligonucleotides used to generate wild type and mutated-binding sequences were listed in Table 2. With PRDQ9, Prop-1 activated the SEAP gene expression up to 2.3-fold, and the mutation decreased its activation to the level of αGSU(−53/+12; Fig. 6A). The expression levels of SEAP gene in the reporter vector constructed with wild-type sequence of other six Prop-1-binding sites were increased to 1.8- to 3.4-fold by Prop-1 (Fig. 6B). The mutation generated in five binding sites significantly decreased the Prop-1 activation level to almost the same level of αGSU(−53/+12), though αGSU(−359/−319)m still preserved substantial activity. However, αGSU(−503/−474) showed a decrease from 1.8- to 1.6-fold, which was not significant. The mutation introduced to −1059/−1015, −946/−909, −163/−142, and TAATT in −147/−114 bp are the core sequences of Prop-1 activation. On the other hand, the mutation introduced to −359/−319 bp, in which the maximum induction by Prop-1 was observed remained the Prop-1 activation to show only a 20% decrease in expression level, indicating that not only TAAT but also other A/T-rich sequences contribute to Prop-1 activation in the −359/−319 bp region. These data revealed that at least five of six Prop-1-binding sites play the role of a cis-acting element, regardless of their position.

Discussion

Prop-1 is known as an indispensable transcription factor for the development of Pit-1 lineage hormone-producing cells by regulating the expression of Pit-1 gene (Sornson et al. 1996). To our knowledge, this study provides the first evidence that the transcription of αGSU gene is involved in the role of Prop-1. The present results further indicate that Prop-1 plays extensive roles in the production of FSH, since we reported that Prop-1 is also a transcription factor for FSHβ gene (Aikawa et al. 2004). Yet, Prop-1 failed to activate the LHβ promoter and our finding is that Prop-1 regulates the gene expression of αGSU and FSHβ but not LHβ, may explain the data showing that the content of FSHβ mRNA in the pituitary of Ames dwarf mice caused by Prop-1 defects decreased to 5.4%, whereas that of LHβ mRNA was 58.5% of the levels in normal controls (Tang et al. 1993). Our data might explain that the Prop-1 gene defect introduces hypogonadism, revealing low gonadotropin content, especially for FSH. However, since we examined only at around 1 kb upstream of the porcine LHβ gene, the evidence for a Prop-1 response in this gene is not yet conclusive.

The αGSU gene expresses in a cell type or tissue-specific manner by several trans-acting factors interacting with their respective cis-acting elements (Maurer et al. 1999). The major regulatory elements identified are shown in Fig. 1A. This study revealed that Prop-1 binds to several positions and promotes αGSU promoter activity. Our comprehensive investigations using transfection assay, EMSA, DNase I footprinting, and estimations of cis-acting activities demonstrated that Prop-1 binds to six cis-acting elements, i.e., −1038/−1026, −942/−928, −495/−479, −338/−326, −153/−146, and −131/−124 bp.

In the distal region, no information on typical regulatory elements has yet been provided, while we recently demonstrated the importance of the distal region of porcine αGSU promoter for the high level and cell-type-specific expression of αGSU gene (Aikawa et al. 2005a). Indeed, the study of cis-acting activity revealed two cis-acting elements of Prop-1 located at

<table>
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<th>Sequence</th>
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<td>AGATAAGA</td>
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<tr>
<td>−131/−124</td>
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</table>

TAAT/ATTA motifs are indicated by bold letters, and one-base mismatches are underlined. The consensus sequence of Prop-1 binding, PRDQ9, has two TAAT/ATTA motifs in the palindromic sequence.
The mutation of the region at −1038/−1026 and −942/−928 bp. The mutation of the region at −503/−474 showed a nonsignificant decrease of Prop-1-dependent activation (1.8-fold) to 1.6-fold, indicating that its activity in this region, if any, is weak. This finding may provide new knowledge that demonstrates the regulatory factor and its target sequence in the distal region of αGSU promoter. In comparison with the nucleotide sequences of the corresponding positions among other mammalian αGSU promoters, this porcine sequence is not well conserved, indicating a possibility of species specificity.

The proximal region is well conserved among mammalian species, containing a number of regulatory elements important for the basal transcription of αGSU gene (Delegeane et al. 1987, Silver et al. 1987, Jameson et al. 1988, Heckert et al. 1995). The present study revealed that three cis-acting elements of Prop-1 located in the proximal region at −338/−326, −153/−146, and −131/−124 bp. These three elements were compared with the elements already identified in the proximal region of human αGSU (Fig. 7). The element −338/−326 bp mostly overlapped the LIM homeodomain transcription factor-binding element (Roberson et al. 1994), which is characterized as an important regulatory element for the basal transcription of αGSU gene in the pituitary. The element −153/−146 bp completely overlapped with GATA (element for GATA-binding proteins; Steger et al. 1991). Another Prop-1-binding site, −131/−124 bp, overlaid half of the junctional response element (JRE), which is originally characterized as an important element for placenta-specific expression (Andersen et al. 1990) and recognized by the homeobox factor, Distal-less 3 (Dlx 3; Roberson et al. 2001). Our study further reveals an interesting feature of the regulatory mechanism of αGSU gene expression, i.e., that three of
the Prop-1-binding sites are shared with other transcription factors, LIM homeodomain transcription factors, GATA-binding proteins, and JRE-binding proteins. The interaction or synergy with other regulatory factors is of interest for future investigation.

In addition, we observed a cell-type-dependent expression that the response of the \( \alpha \)GSU promoter in the gonadotrope cell line, L\( T2 \), and the somatomammotrope cell line, GH3, were different (Figs 2 and 3). The basal expression level of \( \alpha \)GSU gene in L\( T2 \) cells was extremely high, while that in GH3 cells was low. As recently reported (Aikawa et al. 2006), Prop-1 gene expression was observed only in L\( T2 \) and L\( T4 \) cells, but not in GH3 cells. The already high levels of Prop-1 in the L\( T2 \) cells may explain the limited response of the \( \alpha \)GSU promoter when Prop-1 was overexpressed in this cell line, compared with the \( \alpha \)GSU promoter response to Prop-1 overexpression in the GH3 cells. This fact suggests that one cell type with Prop-1 should be sufficient to express the \( \alpha \)GSU gene in the gonadotropes.

Generally, most homeodomain factors recognize nucleotide sequences containing a TAAT core motif (Catron et al. 1993, Damante et al. 1994, Jagla et al. 1994, Pomerantz & Sharp 1994). Prop-1 is a homeodomain transcription factor and known to bind to the consensus sequence, PRDQ9 (5'-TAATgAATTA-3', a palindromic sequence of a TAAT core motif; Sornson et al. 1996). However, the binding regions of Prop-1 found in the porcine \( \alpha \)GSU promoter show a variety of sequences and lengths (Table 3). Four of the six sequences contain a TAAT motif, and the others contain A/T clusters of 5'-AAAT-3' or 5'-TTAT-3'/5'-ATAA-3'. Prop-1 certainly recognizes these sequences, and stimulates the promoter activity of \( \alpha \)GSU except for -495/-479 (Fig. 6). Taken together, Prop-1 may have broader binding properties. In fact, the systematic evolution of ligands by exponential enrichment (SELEX) analysis for porcine Prop-1 demonstrated that Prop-1 can bind firmly to a variety of AT-rich sequences, though the strongest binding sequence is 5'-TAATnnnATTA-3' (personal communication). Mutation analysis of sites -946/-909, -163/-142, and -147/-114 also confirmed that Prop-1 binds to AAAT/ATTG, ATAA/TTAT, and TAAT/ATTA.

In the development of the pituitary gland, Prop-1 expression is first detected on e10-10.5 at the dorsal portion of the gland and reaches a maximum by e12, after which its expression expands to the full caudomedial area, where Pit-1 is first expressed on e13-5 (Sornson et al. 1996). On the other hand, \( \alpha \)GSU expression is first detected on e11-5 in the anteroventral aspect of Rathke’s pouch and expands to the pars tuberalis by e12-5. After e13-5, dorsal and lateral gradients of its expression appear in cells of the anterior lobe with increasing age, although \( \alpha \)GSU-expressing cells remain in the pars tuberalis (Japon et al. 1994, Lanctot et al. 1999). This expression pattern of \( \alpha \)GSU coincides with that of TSH\( B \) expression that first appears transiently in the pars tuberalis on e12-5 prior to preceding Pit-1 gene activation, and then appears in another region of the anterior lobe on e15-5. This transient TSH\( B \) expression in the pars tuberalis is Pit-1-independent and disappears by the day of birth (Japon et al. 1994). Thyrotrope embryonic factor is a candidate for potential activator of TSH\( B \) expression in the pars tuberalis, and Pit-1 is required to activate TSH\( B \) gene in the caudomedial area (Lin et al. 1994). Although many transcription factors for activating \( \alpha \)GSU expression have been reported as reviewed in Savage et al. (2003), factors which serve as an activator of \( \alpha \)GSU expression limited to the pars tuberalis have not been clarified. \( \alpha \)GSU expression in the anterior lobe appears on e13-5 when and where Pit-1 expression is introduced by Prop-1. This expression pattern of \( \alpha \)GSU in the developmental pituitary together with our data in this report indicates that Prop-1 might be one of the transcription factors that activates the limited expression of \( \alpha \)GSU in the anterior lobe of the pituitary.
Several findings support the Prop-1 function in an adult pituitary. We histochemically demonstrated the presence of Prop-1 in the adult porcine gonadotrope and other cells (Aikawa et al. 2004). Furthermore, Prop-1 mRNAs are detected in the fetal as well as the postnatal pituitaries (Aikawa et al. 2005b), and in the pituitary-tumor-derived cell lines LβT2 and LβT4 (Aikawa et al. 2006). In the adult human pituitary, Prop-1 mRNAs have also been detected (Nakamura et al. 1999). Taken together, Prop-1 might play multiple roles in the early stage of pituitary development and in the control of αGSU and FSHβ gene expressions in postnatal pituitaries.

In summary, this study demonstrated for the first time that the paired-like homeodomain transcription factor Prop-1 participates in the regulation of αGSU gene but not that of LHβ gene. Together, with our preceding study, showing that Prop-1 directly regulates FSHβ gene (Aikawa et al. 2004), we have now demonstrated that Prop-1 modulates the synthesis of FSH at the transcriptional level.

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