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Homeodomain transcription factor Hesx1/Rpx occupies Prop-1 activation sites in porcine follicle stimulating hormone (FSH) β subunit promoter

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Abstract

Homeodomain repressor factor Hesx1/Rpx plays a crucial role in the formation of Rathke's pouch at the start of pituitary organogenesis and represses the Prop-1-dependent expression of Pit-1 gene, which promotes the differentiation of Pit-1-dependent hormone producing cells. Recently, we discovered a novel function of Prop-1 by which it activates the porcine follicle stimulating hormone β subunit (FSHβ) gene through Fd2 region (−852/−746). The present study aimed to determine whether Hesx1 exerts its role in the Prop-1-dependent activation of FSHβ gene.

Transient transfection assay for the porcine FSHβ promoter −985/+10, electrophoretic mobility shift assay (EMSA) and DNase I footprinting analysis for Fd2 region were carried out. Transfection assay in GH3 cells demonstrated that expression of Hesx1 alone does not change the promoter activity but the coexpression with Prop-1 represses the Prop-1-dependent activation of FSHβ promoter. Similar results were obtained for the mutant reporter vector deleting the region −745/−104 indicating that Fd2 region is a target site of Hesx1 as well as Prop-1. EMSA and DNase I footprinting analysis using recombinant Hesx1 and Prop-1 protein demonstrated that Hesx1 and Prop-1 certainly bind to the AT-rich regions in a different manner. These results suggest that Hesx1 blocks the advanced expression of FSHβ gene in the early stage of pituitary development, and Prop-1 thereafter appears and activates this gene.

Keywords: Prop-1; Hesx1; Gonadotropin; Pituitary; Gene regulation

Follicle stimulating hormone (FSH) and luteinizing hormone (LH), composed of common α and unique β subunits, together play important roles in mammalian reproductive function and development. During pituitary organogenesis, the regulatory mechanism of these genes is acquired by spatial and temporal expression of several transcription factors. Recently, we demonstrated a novel function of Prop-1 by which it directly activates the porcine follicle stimulating hormone β (FSHβ) subunit gene [1]. Prop-1 is the gene responsible for Ames dwarf mice and is an upstream transcription factor of Pit-1 that determines the development of Pit-1 lineage hormone-producing cells, somatotrope, lactotrope, and thyrotrope that produce growth hormone (GH), prolactin (PRL), and thyroid-stimulating hormone (TSH), respectively [2]. On the other hand, Hesx1/Rpx plays a critical role

Abbreviations: FSH, follicle stimulating hormone; LH, luteinizing hormone; GH, growth hormone; PRL, prolactin; TSH, thyroid-stimulating hormone; EMSA, electrophoretic mobility shift assay.

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in development of the pituitary primordium and antagonizes together with TLE1 corepressor the Prop-1-dependent activation of Pit-1 gene [3], presumably blocking the ongoing pituitary cell lineage differentiation by Prop-1 until its scheduled period. Our finding that Prop-1 activates the gene expression of FSHβ as well as that of Pit-1, suggests that reciprocal action between Hesx1 and Prop-1 on FSHβ gene is feasible. In this study, we attempted to clarify whether Hesx1 participates in the regulation of FSHβ gene expression. Transfection assay revealed that Hesx1 represses Prop-1-dependent activation of FSHβ gene through Fd2 region (−852/−746 of the porcine FSHβ promoter), although Hesx1 alone does not change the expression level. Electrophoretic mobility shift assay (EMSA) and DNase I footprinting analysis revealed that both Hesx1 and Prop-1 bind to similar sites in the Fd2 region with a different binding mode.

Materials and methods

Construction of vectors and preparation of recombinant protein. Reporter vectors were constructed by fusing FSHβ−985/+10 and FSHβΔ−985/+10 region lacking −745/−104 region to alkaline phosphatase expression vector, pSEAP2-Basic (BD Biosciences Clontech, Palo Alto, CA), as described previously [1]. cDNAs encoding for porcine Prop-1 (Accession No. NM_001001263) and mouse Hesx1 (Accession No. NM_010420) [4] (kindly provided by Dr. Rathjen) were inserted in the vectors, pcDNA3.1/Zeo+ (Invitrogen, San Diego, CA) for the transient transfection and pET32a (Novagen, Darmstadt, Germany) to obtain bacterial recombinant proteins.

Cell culture and transfection. GH3 cells which belong to the clonal somatomammotrope cell line of the rat pituitary [5], and endogenously express several pituitary specific transcription factors, were obtained from RIKEN Cell Bank (Tsukuba, Japan) and maintained in monolayer cultures as described previously [6] . Transfection of GH3 cells was performed after 24 h seeding at 1.0×10^4 cells/100 μl per well in a 96-well plate (Corning Inc., Corning, NY) using the 5 μl of DNA-FuGENE 6 complex containing 0.3 μl FuGENE6 (Roche Diagnostics GmbH, Mannheim, Germany), 10 ng reporter vectors and 10 ng expression vectors each per well. Total amount of DNA for transfection was adjusted to 50 ng/well with pcDNA3.1/zeo. After incubation for 24 h, an aliquot (5 μl) of cultured medium was assayed for SEAP activity using the Phospha-Light Reporter Gene Assay System (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions with a MiniLumat LB 9506 luminometer (Berthold, Wildbad, Germany). All values were expressed in means ± SEM of quadruplicate transfections in three independent experiments.

Electrophoretic mobility shift assay. Preparations of the Trx/His-tag fused recombinant proteins and FAM-labeled Fd2 fragment were carried out as previously described [1]. The binding reaction mixture was incubated at 30 °C for 30 min. It included 200 ng recombinant Prop-1, Hesx1 or Trx/His-tag, 100 fmol FAM-labeled Fd2 and 2 μg poly(dI–dC) with or without 20-fold amount of unlabeled Fd2 in 10 μl of 10 mM Hepes buffer (pH 7.9), containing 0.4 mM $MgCl_2$, 0.4 mM DTT, 50 mM NaCl, and 4% glycerol. Samples were then subjected to electrophoresis on a 4% polyacrylamide gel as described previously [1].

DNase I footprinting analysis. The 5′ FAM-labeled Fd2 fragment was incubated with 200 or 400 ng recombinant Prop-1 or Hesx1 in binding buffer under the same condition used for EMSA. After a 30 min incubation at 30 °C, 0.3 U RQ1 RNase-Free DNase (Promega Madison, WI) 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 formamide 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

Hesx1 represses Prop-1-dependent activation of porcine FSHβ promoter

Transfection assay using GH3 cells showed that Hesx1 alone did not affect the FSHβ promoter (−985/+10), whereas Prop-1 promoted its activity by 5.3-fold at 20 ng vector (Fig. 1A). The empty reporter vector used as a negative control did not exhibit any obvious effect of Prop-1 or Hesx1. Prop-1 enhanced the FSHβ promoter activity dose-dependently, whereas Hesx1 repressed the Prop-1-dependent activation. Maximal decrease was observed with cotransfection of each 20 ng Hesx1 and Prop-1 from 5.3- to 2.7-fold (Fig. 1B).

To determine whether Hesx1 and Prop-1 share the Fd2 region (−852/−746) as a target site, a truncated FSHβΔ−985/+10 lacking −745/−104 region was assayed (Fig. 2). The Prop-1-dependent activation of the FSHβ promoter was similarly repressed by increasing the amount of Hesx1, indicating the responsiveness of Hesx1 to Fd2.

Both Prop-1 and Hesx1 bind to same Fd2 region

EMSA was carried out for Hesx1 and Prop-1 using FAM-labeled Fd2 (Fig. 3). A specific shift band with different mobility for Hesx1 and Prop-1 appeared in the presence of excessive poly(dI–dC) (2 μg), while Trx/His-tag protein alone, in which recombinant proteins were constructed, did not have any binding ability to Fd2 (data not shown). Then, the addition of an excess amount of unlabeled Fd2 (20-fold molar excess) decomposed the shift bands and free-DNA bands and multiple fast migrating bands with patterns different from those of Hesx1 and Prop-1 appeared (Fig. 3).

To determine the binding sites of Hesx1 and Prop-1, DNase I footprinting analysis was carried out for Fd2 and demonstrated a marked reduction of signals by Prop-1 and Hesx1 in comparison with Fd2 alone (Fig. 4). With each 400 ng protein, the region −840/−798 for Hesx1 and −840/−785 for Prop-1 were protected from nuclease digestion, indicating that both proteins have several binding sites in those regions. The decreasing amount of recombinant proteins (200 ng) exhibited limited profiles of −840/−810 for Hesx1 and −840/−798 for Prop-1, reflecting each preferential binding region. The differences in the signal intensities and profiles in the wide region −840/−798 were the differences in binding between Hesx1 and Prop-1. DNase I footprinting analysis with the mixture of 200 ng Prop-1 and Hesx1, respectively, showed an almost similar profile to that with 400 ng Prop-1 (Fig. 4).