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—Original Article—

Search for regulatory factors of the pituitary-specific transcription factor PROP1 gene

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Abstract. Pituitary-specific transcription factor PROP1, a factor important for pituitary organogenesis, appears on rat embryonic day 11.5 (E11.5) in SOX2-expressing stem/progenitor cells and always coexists with SOX2 throughout life. PROP1-positive cells at one point occupy all cells in Rathke's pouch, followed by a rapid decrease in their number. Their regulatory factors, except for RBP-J, have not yet been clarified. This study aimed to use the 3 kb upstream region and 1st intron of mouse *prop1* to pinpoint a group of factors selected on the basis of expression in the early pituitary gland for expression of *Prop1*. Reporter assays for SOX2 and RBP-J showed that the stem/progenitor marker SOX2 has cell type-dependent inhibitory and activating functions through the proximal and distal upstream regions of *Prop1*, respectively, while RBP-J had small regulatory activity in some cell lines. Reporter assays for another 39 factors using the 3 kb upstream regions in CHO cells ultimately revealed that 8 factors, MSX2, PAX6, PIT1, PITX1, PITX2, RPF1, SOX8 and SOX11, but not RBP-J, regulate *Prop1* expression. Furthermore, a synergy effect with SOX2 was observed for an additional 10 factors, FOXJ1, HES1, HEY1, HEY2, KLF6, MSX1, RUNX1, TEAD2, YBX2 and ZFP36Ll, which did not show substantial independent action. Thus, we demonstrated 19 candidates, including SOX2, to be regulatory factors of *Prop1* expression.
Key words: Pituitary, PROP1**,** SOX2**,** Stem/progenitor cell, Transcription factor

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The pituitary gland is a major endocrine organ that plays important roles in the growth, metabolism, reproduction, stress response and homeostasis of all vertebrates. The adenohypophysis (anterior and intermediate lobes of the pituitary gland) develops by invagination of the oral ectoderm and acquires the ability to synthesize and secrete many hormones by differentiation into the respective hormone-producing cells under spatiotemporal regulation of various transcription factors. Among them, *Prop1*, Prophet of PIT1, is specifically expressed in the adenohypophysis and plays a crucial role in the differentiation of hormone-producing cells [1]. A single nucleotide replacement in *Prop1* of the *Ames* dwarf mouse results in abnormal pituitary expansion caused by a defect in migration of the progenitor cells from Rathke's pouch into the developing anterior lobe and in failure of the hormone-producing cells to differentiate [1, 2]. Persistent expression of *Prop1* interferes with anterior pituitary cell differentiation and increases the susceptibility to pituitary tumors [3]. In addition, PROP1 is likely important for dorsal-ventral patterning but not for cell proliferation and cell survival [4].

Recently, several investigators successively reported the relation between PROP1 and pituitary stem/progenitor cells by analyses of stem cell fractions separated by fluorescence activated cell sorting and pointed out the presence of a pituitary stem/progenitor niche [5–7]. On the other hand, we demonstrated that PROP1 starts its expression in SOX2-positive pituitary stem/progenitor cells and that SOX2 is consistently present in PROP1-positive cells [8]. In addition, PROP1-positive cells form a stem/progenitor cell niche in the parenchyma of the rat adult anterior lobe [9], as was elaborated on by further characterizations in subsequent reports [10–14]. PROP1 emerges in SOX2-positive cells early in the rat at embryonic day 11.5 (E11.5) and, after 2 days, occupies all cells in the pituitary primordium of Rathke's pouch [8]. Thereafter, PROP1 quickly fades away in the process of differentiating into committed cells before SOX2 disappearance and hormone appearance in PIT1-positive cells [8], indicating the presence of potent and prompt regulation mechanisms for *Prop1* expression. Much less is known about the regulatory mechanism, despite a study by Ward *et al*. [15] to determine the tissue-specific mechanism of *Prop1* expression using comparative genomics. They intensively analyzed three highly conserved regions and found orientation-specific enhancer activity but not a

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pituitary-specific element. Knockout of *Rbp-J*, a primary mediator of Notch signaling, revealed a decrease of *Prop1* expression [16], but information regarding transcription factors for *Prop1* expression is still limited.

In the present study, we attempted to discover potential regulatory factors and to examine whether SOX2 participates in *Prop1* expression by reporter assay. Ultimately, the present study demonstrated that the 5'-upstream region and 1st intron of *Prop1* show cell type-dependent transcriptional activity and that SOX2 can modulate *Prop1* expression. In addition, it was revealed that 18 other transcription factors, many of which are involved in early pituitary organogenesis, participate in modulation through the 5'-upstream region of *Prop1*.

Materials and Methods

Construction of reporter vectors and expression vectors

To obtain serial truncated fragments of the 5'-upstream region and the 1st intron of the mouse *Prop1* gene (Accession number: NM_008936.1), specific primer sets for PCR were designed and synthesized (Table 1). The resulting products were ligated to the upstream site of the secreted alkaline phosphatase (SEAP) gene in the pSEAP2-Basic vector or pSEAP2-Promoter vector (BD Biosciences Clontech, Palo Alto, CA, USA), respectively. This resulted in the following reporter vectors: *Prop1* (–2993/+21), *Prop1* (–1840/+21), *Prop1* (–1270/+21), *Prop1* (–771/+21), *Prop1* (–443/+21), *Prop1* (–154/+21), *Prop1* (+338/+519), *Prop1* (+338/+790), *Prop1* (+338/+1112) and *Prop1* (+338/+1383).

For construction of expression vectors, a full-length open reading frame encoding a number of transcription factors, listed in Supplementary Table 1 (online only), was obtained by PCR amplification using a rat pituitary cDNA library or cDNA clones from the FANTOM DNABook of mouse transcription factors (DNAFORM, Yokohama, Japan) and cDNA clones obtained by distribution and was cloned in frame into the mammalian expression vector pcDNA3.1Zeo$^+$ (pcDNA3.1, Invitrogen, Carlsbad, CA, USA). In the case of non-mouse clones, the amino acid sequence similarity between species was confirmed to be more than 92%.

Cell culture

CHO, GH3, AtT20, LβT2 and Tpit/F1 cells were used for transient transfection assay. CHO (established from Chinese hamster ovaries) [17], GH3 (a pituitary tumor-derived cell line expressing *Gh* and *Prl*) [18] and AtT20 (a pituitary tumor-derived cell line expressing proopiomelanocortin) [19] were obtained from the RIKEN Cell Bank (Tsukuba, Japan). LβT2 cells, which express gonadotropin genes of αGSU, LHβ and FSHβ [20, 21], were provided to us by Dr. P. L. Mellon (University of California, San Diego, CA, USA). Tpit/F1 cells, which were established from a mouse pituitary tumor and do not express any pituitary hormone [22], were provided to us by Dr. K. Inoue (Saitama University, Japan).

The conditions for cell culture, transfection procedures and reporter assays performed by measurement of the secreted alkaline phosphatase activity of the reporter gene products in the culture media were described in a previous paper [23]. Cell maintenance was performed in monolayer cultures in F-12 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and Antibiotic Antimycotic Solution (Sigma-Aldrich, St. Louis, MO, USA) for CHO cells, Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% (v/v) FBS (Gibco) and antibiotics for LβT2 cells or DMEM/F-12 (1:1) medium supplemented with 10% (v/v) horse serum (SAFC Biosciences, St. Louis, MO, USA), 2.5% (v/v) FBS (SAFC Biosciences) and Antibiotic Antimycotic Solution (Sigma-Aldrich) for AtT20, GH3 and Tpit/F1 cells. All cell lines were cultured in humidified 5% CO_2–95% air at 37 C, except for Tpit/F1 cells, which were cultured at 33 C, since this cell line was established from transgenic mouse cells immortalized with a temperature-sensitive mutant T-antigen active at 33 C [24].

Transfection and reporter assay

For transient transfection, cells were plated onto a 96-well plate (Corning, Corning, NY, USA) at a density of 1–2 × 10^4 cells/100 μl/well. Transfection was performed 24 h after seeding using a mixture of 2.5–4 μl of DNA (10–30 ng reporter vectors and 10 ng expression vectors by adjusting the total DNA to 50–80 ng with empty pcDNA3.1) and FuGENE 6 (0.3 μl; Roche Diagnostics GmbH, Mannheim, Germany) or Lipofectamine 2000 (0.2 μl; Invitrogen, Carlsbad, CA, USA) per well. The cell number per well, transfectant, amount of DNA and medium are listed in Table 2. After incubation for 24–72 h, an aliquot (5 μl) of cultured medium was assayed for SEAP activity using the Phospha-Light Reporter Gene Assay System (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions with a MiniLumat LB 9506 luminometer (Berthold, Bad Wildbad, Germany).

All values are expressed as means ± SD from quadruplicate transfections of two to three independent experiments. The statistical significance between the activity of each reporter vector and that of the control was determined by Student's *t*-test with the F-test. A value of $P < 0.01$ was considered significant.

In situ hybridization and immunohistochemistry

In situ hybridization was performed according to a previous report [25]. The full-length DNA of rat *Rpf1* (*Pou6f2*) was amplified by PCR with a primer set (5'-ATGATAGCTGGACAAGTCAGTAAGCCC-3' and 5'-TGCTTCCTTCTGATCTATGAACGGTGTG-3'), and cRNA probes for it were prepared by labeling it with digoxigenin (DIG) using a Roche DIG RNA Labeling kit (Roche Diagnostics, Penzberg, Germany). Cryosections (7 μm thickness) from the sagittal plane were hybridized with DIG-labeled cRNA probes at 55 C for 16 h and visualized with alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics) using 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Roche Diagnostics). Immunohistochemistry was performed according to our previous report [8] with a primary antibody for guinea pig antiserum against rat PROP1 (1:1,000 dilution) produced in our laboratory.

Results

Basal transcriptional activity of the 5′-upstream region and 1st intron of Prop1

Mouse *Prop1* is composed of three exons and two introns and has three regions with high conservation between several mammals

Table 1. List of primers used for construction of fragments of the 5'-upstream region and 1st intron of *Prop1*

5'-upstream region		
Forward primer	–2993	5'-aataacgcgtCTAAGATTCAGAGCCAAGCTAG-3'
	–1840	5'-aatacgcgtTCTGAGGAACAAGGAGAGTAAAG-3'
	–1270	5'-aatacgcgtGGAGATCAGGTTGTCCTATGGT-3'
	–771	5'-aatacgcgtAATCAGAGTGTACTCGGAACTC-3'
	–443	5'-aatacgcgtATGTCCTCCTCTCCACTCGC-3'
	–154	5'-aatacgcgtTAAAGGAGAAAGAAAGGCAGC-3'
Reverse primer	+21	5'-aatactcgagGCTAGATACCTGTTTTCTCACAG-3'
1st intron		
Forward primer	+338	5'-aatacgcgtGTGAGTGAATCCCCAGGATG-3'
Reverse primer	+519	5'-aatactcgagTTCTCAACCTGTAAAGCGAA-3'
	+790	5'-aatactcgagAGACACCTGGGAAGGTGGGT-3'
	+1112	5'-aatactcgagGTCTATCAATGACGTCTCTGGC-3'
	+1383	5'-aatactcgagCTATGGAGGGAGAAAAACGGA-3'

Uppercase letters indicate sequences of the gene to be amplified. Lowercase letters indicate adaptors containing recognition sequences for restriction enzymes *Mlu* I (acgcgt) in forward primers and *Xho* I (ctcgag) in reverse primers.

Table 2. List of conditions for cell cultures using a 96-well plate/100 µl medium

Cell line	Cells/well	Transfectant	Reporter vector (ng/well)	Expression vector (ng/well)	Total DNA amount * (ng/well)	Medium
CHO	1.0×10^4	FuGENE 6	10	10	50	F12
AtT20	1.0×10^4	LF2000**	30	10	70	DMEM/F12
LβT2	2.0×10^4	FuGENE 6	30	10	60	DMEM
GH3	1.0×10^4	FuGENE 6	10	10	50	DMEM/F12
Tpit/F1	1.5×10^4	FuGENE 6	30	10	70	DMEM/F12

* The total DNA amount was adjusted by addition of empty pcDNA3.1 to the reporter and expression vectors. ** Lipofectamine 2000.

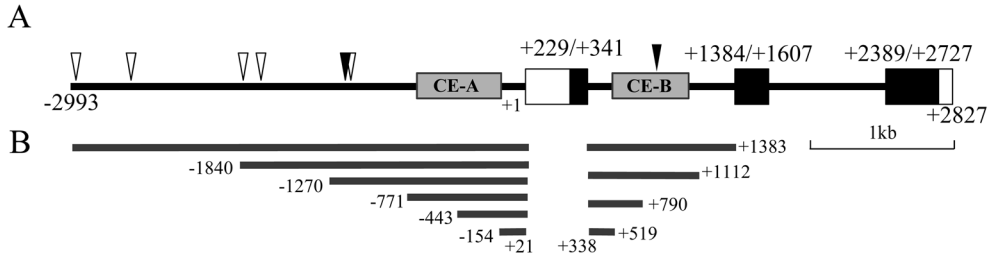

Fig. 1. Diagram of the structure of mouse *Prop1*. A. Coding and untranslated regions are indicated with closed and open boxes, respectively. Solid lines indicate the 5'-upstream region and introns. Nucleotide numbers from the transcription start site (+1) are indicated below the diagram, and those of coding regions are indicated above the closed boxes. Putative binding sequence of SOX2 (WCAAWG; W = A or T) and RBP-J (GTGGGAA/CACCCTT) are indicated by open and closed inverted triangles, respectively. Shaded boxes (CE-A and CE-B) represent regions evolutionally conserved among the human, chimpanzee, pig, dog, cattle, mouse and rat [15]. B. Truncated constructs of the 5'-upstream region and 1st intron are shown with the nucleotide number. A scale bar (1 kb) is shown below the diagram.

[15]: CE-A in the 5'-upstream –733/–155 base (b), CE-B in the 1st intron +593/+1073 b and CE-C in the 3'-downstream +2927/+5123 b. In Fig. 1A, except for the 3'-downstream region, the diagram indicates the structure of mouse *Prop1* with putative binding sites for SOX2 (open arrowheads, WCAAWG; W = A or T) [26, 27] and RBP-J (closed arrowheads, GTGGGAA/CACCCTT) [28], which regulates *Prop1* expression [16].

To examine transcriptional activity of the 5'-upstream region and 1st intron of *Prop1*, we constructed deletion mutants of both regions as indicted in Fig. 1B, followed by transfection in CHO, GH3, AtT20, LβT2 and Tpit/F1 cells. Basal transcriptional activity of the truncated upstream region showed low SEAP activity in comparison

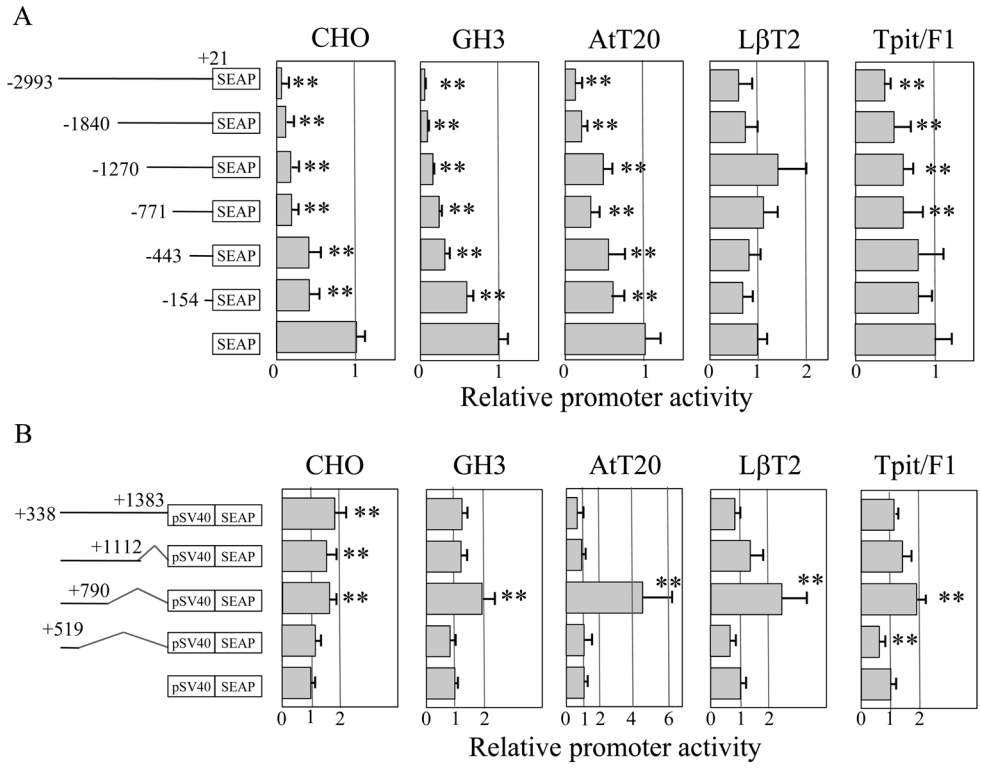

Fig. 2. Basal transcriptional activity of the 5'-upstream region and 1st intron of the mouse *Prop1*. Diagrams of truncated regions of the 5'-upstream region fused with the pSEAP2-Basic vector (A) and the 1st intron fused with the pSEAP2-Promoter vector containing an SV40 promoter (pSV40) (B) are indicated in the left panels. Kinked lines in the 1st intron (B) indicate deleted regions. Transfection assays were performed, as described in Materials and methods, in CHO, GH3, AtT20, LβT2 and Tpit/F1 cells with quadruplicated transfections in two to three independent experiments, and a representative result (means ± SD) is shown as the relative activity against that of an empty vector. The statistical significance between the activity of each reporter vector was determined by Student's *t*-test. ** $P < 0.01$.

with that of pSEAP2-Basic vector in CHO, GH3, AtT20 and Tpit/F1 cells, except for LβT2 cells (Fig. 2A). Decreased activity along with an increased length of the upstream region indicated that the upstream –2993/+21 b of *Prop1* itself has the ability to suppress its leaky expression, while LβT2 cells did not show a remarkable change. On the other hand, deletion of +791/+1112 b in the 1st intron increased the transcriptional activity in GH3, AtT20 and LβT2 cells (Fig. 2B). Notably, the increase was reduced by deletion of +520/+790 b, indicating the presence of a positive regulatory element in the +520/+790 b and a negative one in some cell types in the +791/+1112 b.

Regulation of transcriptional activity of the 5′-upstream region and 1st intron of Prop1 by SOX2

Based on the basal transcriptional activity, we examined whether SOX2 modulates *Prop1* expression by co-transfection of a *Sox2* expression vector. While Tpit/F1 cells did not have an apparent effect on SEAP activity, SOX2 modulated the transcriptional activities in four cell types (Fig. 3A). SOX2 decreased the activity in AtT20 and LβT2 cells continuously along with increasing the length of the upstream region by 0.5-fold and 0.2-fold, respectively. It acted repressively within –154/+21 in both CHO and GH3 cells but also stimulated the expression of *Prop1* (–2993/+21), *Prop1* (–1840/+21) and *Prop1* (–1270/+21) in both CHO and GH3 cells. Of note, the –2993/–1841 b region showed a remarkable increase of expression in CHO cells.

A reporter assay for the 1st intron of *Prop1* with a SOX2 expression vector was also examined. As shown in Fig. 3B, although there were some effects of SOX2 on the transcriptional activity of each construct in CHO, GH3, AtT20 and Tpit/F1cells, no remarkable influence of SOX2 was present.

Regulation of transcriptional activity of the 5′-upstream region and 1st intron of Prop1 by SOX2 and RBP-J

RBP-J is the only factor known to regulate *Prop1* expression [16]. Hence, we sought to find the effect RBP-J has on the transcriptional activity of the 5'-upstream region constructed in the pSEAP2-Basic vector and the 1st intron of *Prop1* constructed in the pSEAP2-Promoter vector in the absence or presence of SOX2. RBP-J alone had 0.6- and 0.7-fold repressive effects about in AtT20 and Tpit/F1 cells, respectively, and stimulated the 5'-upstream region by about 1.7-fold in LβT2 cells (Fig. 4A). For the 1st intron, RBP-J had a repressive effect only in CHO cells (about 0.6-fold; Fig. 4B). As a whole, the involvement of RBP-J is likely to be small. Double

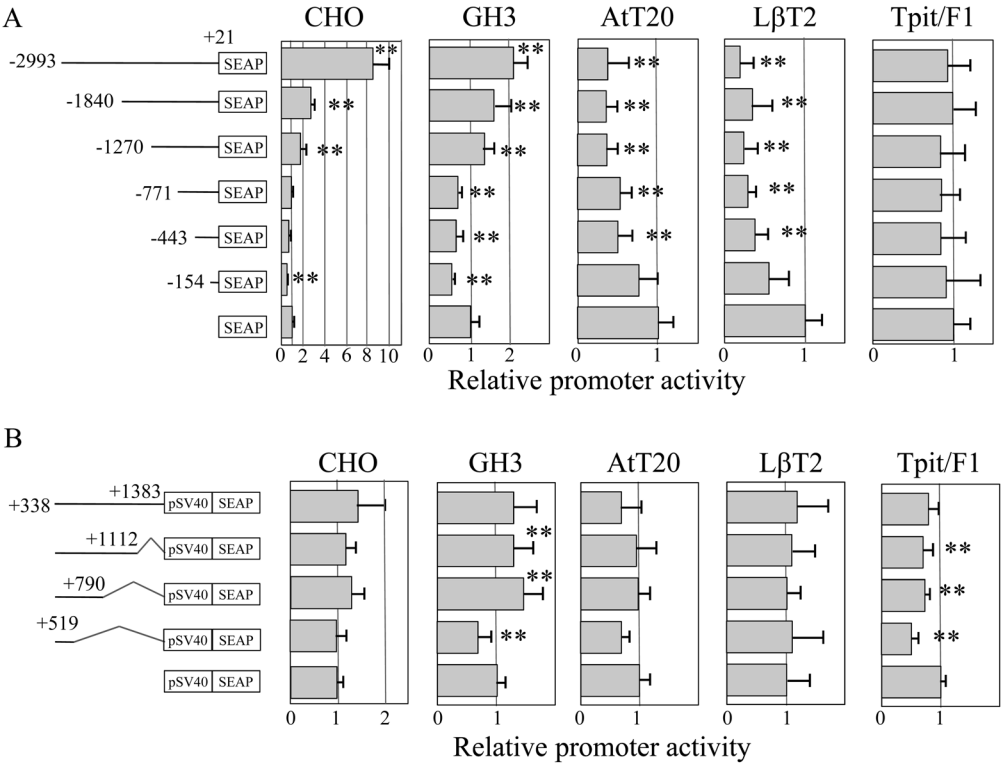

Fig. 3. Effect of SOX2 on the 5'-upstream region and 1st intron of mouse *Prop1*. The diagrams shown in the left panel are the same as described in Fig. 2. Transfection assays with expression vector of SOX2 were performed with the same conditions as described in Fig. 2, with quadruplicated transfections in two to three independent experiments. A representative result (means ± SD) is shown, and statistical significance was determined as described in Fig. 2. ** $P < 0.01$.

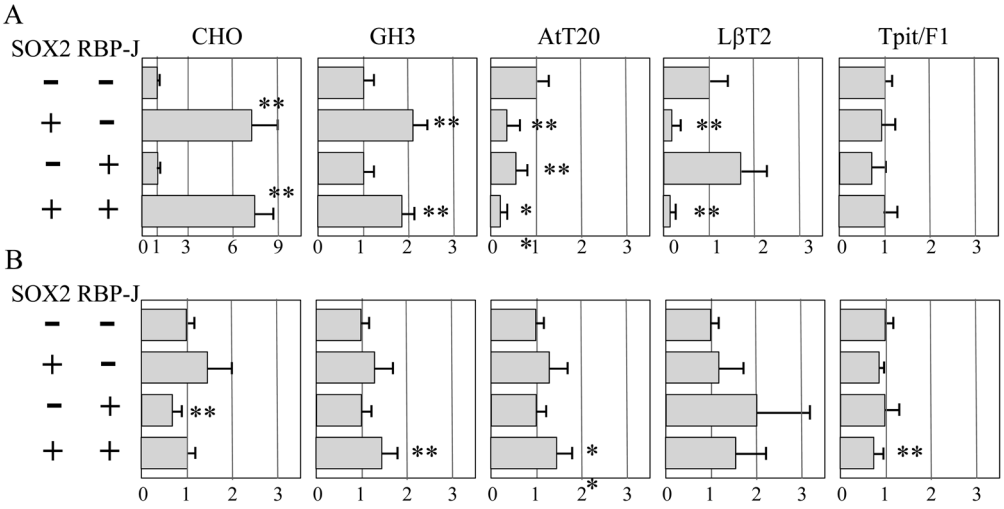

Fig. 4. Effect of SOX2 and RBP-J on the 5'-upstream region and 1st intron of mouse *Prop1*. Transfection assays for 5'-upstream region –2993/+21 (A) and 1st intron (B) in CHO cells were performed with quadruplicated transfections in two to three independent experiments in the absence (–) and/or presence (+) of SOX2- and RBP-J-expression vectors, as indicated in the left panel. A representative result (means ± SD) is shown, and statistical significance was determined as described in Fig. 2. ** $P < 0.01$.

Table 3. Reporter assay of transcription factors for *Prop1* (–2993/+21)

	A	B	C	D	Function	Ref.
	SOX2 only	Factor only	SOX2+Factor	C/A		
SOX2-dependent stimulation						
FOXJ1	9.9 ± 1.6**	0.7 ± 0.0**	15.6 ± 1.3**	1.6	Ependymal cell/astrocyte differentiation	[40]
HES1	9.0 ± 1.2**	0.8 ± 0.1	21.3 ± 1.9**	2.4	Maintain stemness of the stem cell	[41, 42]
HEY1	7.3 ± 1.0**	1.3 ± 0.2	28.0 ± 2.9**	3.8	Maintain stemness of the stem cell	[41, 42]
HEY2	7.3 ± 1.0**	0.8 ± 0.1	15.7 ± 1.1**	2.2	Maintain stemness of the stem cell	[41, 42]
SOX2-dependent suppression						
KLF6	9.9 ± 1.6**	1.2 ± 0.2	7.3 ± 1.3	0.7	Regulator of *Prrx2* in the pituitary	[43]
MSX1	8.2 ± 0.9**	0.9 ± 0.2	3.7 ± 0.6**	0.5	Pituitary organogenesis	[44]
RUNX1	7.3 ± 1.0**	0.8 ± 0.1	2.8 ± 0.4**	0.4	Hematopoietic/hair follicle stem cells	[45, 46]
TEAD2	9.9 ± 1.6**	1.3 ± 0.4	3.1 ± 0.2**	0.3	Vessel/neural tube/heart organogenesis	[47]
YBX2	7.6 ± 0.5**	1.4 ± 0.2**	2.8 ± 0.5**	0.4	Stability of germ cell mRNAs	[48]
ZFP36L1	7.3 ± 1.0**	1.1 ± 0.1	1.8 ± 0.1**	0.2	Vessel/neural tube/heart organogenesis	[49]
SOX2-independent regulation						
MSX2	6.6 ± 1.1**	7.7 ± 1.0**	7.5 ± 2.0	1.1	Cell survival/apoptosis	[50, 51]
PAX6	7.3 ± 1.0**	5.8 ± 0.1**	7.9 ± 1.2	1.1	Early embryonic pituitary factor	[52]
PIT1	9.5 ± 1.9**	4.8 ± 0.5**	13.8 ± 1.7	1.5	Generate GH-, PRL- and TSH- cells	[53]
PITX1	8.3 ± 0.8**	9.6 ± 2.7**	13.0 ± 0.6**	1.6	Pan-pituitary activator	[54]
PITX2	8.3 ± 0.8**	16.6 ± 4.3**	14.4 ± 1.5**	1.7	Pituitary formation/cell specification	[55]
RPF1	9.9 ± 0.6**	3.6 ± 0.3**	13.5 ± 1.8**	1.4	Retina/pituitary transcription factor	[13, 56]
SOX8	7.6 ± 0.5**	0.4 ± 0.1**	6.3 ± 1.1	0.8	Organogenesis	[57]
SOX11	9.3 ± 0.4**	9.2 ± 0.9**	7.2 ± 1.2	0.8	Neurogenesis and targets *TEAD2*	[58, 59]

All values are expressed as means ± SD of quadruplicate transfections from two to three independent experiments with reproducible data. Representative data are shown. Statistical analyses for Table 3 were performed as follows. Column A: significance between the values with and without SOX2. Column B: significance between the values with and without factors. Column C: significance between the values with SOX2 + factor and with SOX2 only. ** $P < 0.01$.

transfection of SOX2 and RBP-J expression vectors revealed that RBP-J does not have a notable effect on the modulation of SOX2 in both the 5'-upstream region and 1st intron of *Prop1* in CHO cells.

Regulation of transcriptional activity of the 5′-upstream region of Prop1 by SOX2 and other pituitary transcription factors

The results described above indicate that the 5'-upstream region of *Prop1* contains a responsive region(s) for SOX2. As pituitary organogenesis progressed by temporospatial expression of various transcription factors, we focused on 39 additional factors, most of which were assumed to be expressed in the early developmental period based on investigating of microarray data for rat embryonic pituitary cDNA libraries at E14.5 and E15.5 (data not shown). We then examined the factors for their effects on the transcriptional activity of *Prop1*.

Reporter assays using CHO cells were performed for *Prop1* (–2993/+21) by co-transfection of expression vectors without or with a SOX2 expression vector, and their results are summarized in Table 3. Since the assays were performed in different experiments because of a large number of samples, the value of a single effect of SOX2 differed in each experiment (6.6- to 9.9-fold; Table 3, column A). Single transfection of expression vectors showed that 31 factors had little effect (only 0.7- to 1.4-fold), but 8 factors (MSX2, PAX6, PIT1, PITX1, PITX2, RPF1, SOX8 and SOX11) singly modulated the *Prop1* expression (Table 3, column B). Notably, only SOX8 repressed *Prop1* expression. Next, co-transfection together with a SOX2 expression vector was performed (Table 3, column C), and expression values were normalized by that of SOX2 alone (Table 3, column D). Although 21 out of 31 factors that were ineffective alone did not affect SOX2 activity on the *Prop1* expression, the other 10 factors modulated SOX2 activity. Four factors, FOXJ1, HES1, HEY1 and HEY2, stimulated SOX2 activity and 6 factors, KLF6, MSX1, RUNX1, TEAD2, YBX2 and ZFP36L1, repressed them. In each group, HES1, HEY1 and HEY2 increased the SOX2 effect remarkably by 2.2- to 3.8-fold, and RUNX1, TEAD2, YBX2 and ZFP36L1 repressed it by 0.2- to 0.4-fold. On the other hand, 8 factors that each had an effect on *Prop1* expression showed almost no effect (MSX2, PAX6, SOX8 and SOX11; 0.8- to 1.1-fold) and/or a weak effect (PIT1, PITX1, PITX2 and RPF1; 1.4- to 1.7-fold) on SOX2 activity. Accordingly, in CHO cells, the reporter assay showed that 19 factors, including SOX2, are potentially able to modulate *Prop1* expression.

Regulation of transcriptional activity of the 5′-upstream region of Prop1 by singly effective factors

Our results showed the presence of responsive regions for SOX2 in the upstream region of *Prop1* (Fig. 3A, Table 4). Similarly, we examined responsive regions for 8 factors, which were each singly effective, by transient transfection of truncated reporter vectors in CHO cells (Table 4). Stimulation was observed in the –2993/–155 b

Table 4. Reporter assay of transcription factors for serially truncated reporter vectors of the *Prop1* 5'-upstream region

Reporter vector	Prop1 (–2993/+21)	Prop1 (–1840/+21)	Prop1 (–1270/+21)	Prop1 (–771/+21)	Prop1 (–443/+21)	Prop1 (–154/+21)	Basic vector[b)]
SOX2	9.4 ± 1.4**	2.8 ± 0.3**	1.7 ± 0.5	1.0 ± 0.2	0.7 ± 0.2	0.6 ± 0.1**	1.0 ± 0.2
MSX2	7.3 ± 0.7**	3.2 ± 0.5**	3.1 ± 0.3**	2.3 ± 0.3**	1.8 ± 0.3**	1.1 ± 0.2	1.0 ± 0.1
PAX6	6.1 ± 0.8**	3.4 ± 0.4**	2.7 ± 0.2**	1.3 ± 0.3	0.9 ± 0.1	0.8 ± 0.2	1.0 ± 0.1
PIT1	3.0 ± 0.3**	1.6 ± 0.2**	0.8 ± 0.1**	0.7 ± 0.3	0.8 ± 0.1	1.0 ± 0.1	1.0 ± 0.0
PITX1	6.9 ± 2.2	3.4 ± 0.7	2.1 ± 0.2**	1.2 ± 0.1	0.9 ± 0.1	0.7 ± 0.2	1.0 ± 0.1
PITX2	17.9 ± 3.7**	6.7 ± 3.0	5.6 ± 0.9**	2.6 ± 0.8	2.0 ± 0.4**	1.0 ± 0.2	1.0 ± 0.1
RPF1	2.9 ± 0.3**	1.5 ± 0.1**	1.3 ± 0.2	0.9 ± 0.1	1.1 ± 0.2	0.6 ± 0.1**	1.0 ± 0.1
SOX8	0.5 ± 0.3	1.0 ± 0.1	0.5 ± 0.1**	0.5 ± 0.0**	0.8 ± 0.2	1.0 ± 0.1	1.0 ± 0.2
SOX11	4.4 ± 1.5	1.2 ± 0.2	1.2 ± 0.4	1.1 ± 0.1	1.8 ± 0.2**	0.6 ± 0.1**	1.0 ± 0.1
Control[a)]	1.0 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1

All values are expressed in means ± SD of quadruplicate reporter assay using the CHO cell line in two to three independent experiments with reproducible data. Representative data are shown. The statistical significance between the values with and without factors was determined by Student's *t*-test. ** $P < 0.01$. [a)] The control was assayed with the pcDNA3.1 vector. [b)] The basic vector was pSEAP2-Basic.

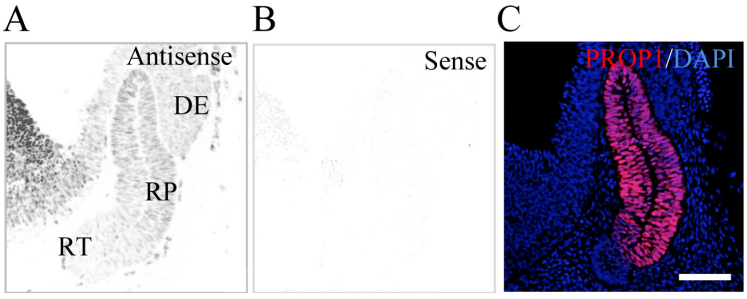

Fig. 5. *In situ* hybridization of *Rpf1* and immunohistochemistry for PROP1. *In situ* hybridization with DIG-labeled anti-sense (A) and sense (B) probes for *Rpf1* was performed using the cephalic part at E13.5. A merged image of immunohistochemistry for PROP1 (red) and nuclear staining with 4, 6'-diamidino-2-phenylindole dihydrochloride (DAPI, blue; Molecular Probes, Vector Laboratories, Burlingame, CA, USA) is shown (C). RP, Rathke's pouch; DE, diencephalon; RT, rostral tip; HT, hypothalamus. Bar 100 µm.

region for MSX2 and PITX2, –2993/–772 b region for PITX1 and PAX6 and –2993/–1271 b region for PIT1 and RPF1, while SOX11 showed stimulation in the –2993/–1841 b and –443/–155 b regions. On the other hand, inhibition was observed in the –154/+21 b region for RPF1 and SOX11, –1270/–772 b region for PIT1 and –1270/–444 b and –2993/–1841 b region for SOX8. Notably, the –2993/–1841 b region showed a remarkable increase in response in comparison with the –1840/–1271 b region. SOX8 did not stimulate any regions, indicating a different role in terms of inhibitory action in comparison with cognate SOX2 and SOX11.

Based on the results of the promoter assay, we were interested in RPF1, which was previously demonstrated to be a novel pituitary transcription factor expressed in Rathke's pouch with a decrease in expression level toward the birth before, and we previously reported that it is a new pituitary transcription factor in the pituitary gland [13]. Hence, we investigated whether RPF1 and PROP1 coexisted in the early pituitary primordium with *in situ* hybridization for *Rpf1* and immunohistochemistry for PROP1. As expected, *Rpf1* transcripts were localized in the rat pituitary primordium (Rathke's pouch) at E13.5 with a definite low-level signal at the rostral tip (Fig. 5A). Low signals were also localized in the diencephalon (the prospective posterior lobe), and strong signals were present in the cells of the primordium of the hypothalamus (Fig. 5A). PROP1 signals were limited to Rathke's pouch and overlapped with *Rpf1* expression (Fig. 5C).

Discussion

PROP1 is a pituitary-specific transcription factor and plays important roles in pituitary organogenesis and differentiation of hormone-producing cells. However, the regulatory mechanism of *Prop1* expression is still poorly understood. In the present study, we examined the transcriptional activity of the 5'-upstream region up to 3 kb and the 1st intron of *Prop1* using several cell lines and examined the effects of pituitary transcription factors on *Prop1* expression. Finally, this study demonstrated for the first time that SOX2, which is always present in PROP1-positive cells [8], is able to modulate *Prop1* expression, and that various transcription factors might participate in the regulation of *Prop1* expression in a SOX2-dependent or SOX2-independent manner. These results help us understand the function of regulatory molecules and the mechanism behind the regulation of *Prop1* expression during pituitary

Table 5. Putative binding site for transcription factors present within 50-base regions of the five putative SOX2-binding sites in the 5'-upstream region of *Prop1*

SOX2-binding site		Binding site within 50-base regions of the SOX2-binding sites				
Region	Sequence	SOX2 dependent		SOX2 independent		Others[1)]
		Stimulation	Repression	Stimulation	Repression	
–2950/–2945	TCAAAG	HES1, HEY1, HEY2				
–2548/–2543	CTTTGT	PITX1 [2)], PITX2 [2)]	MSX1	PITX1 [2)], PITX2 [2)]	MSX2, SOX11	OTX2
–1874/–1869	ACAATG		KLF6			
–1784/–1779	CATTGA	HES1, HEY1, HEY2				
–1137/–1132	TCAAAG					RBP-J

* The putative binding site for transcription factors was analyzed with TRANSFAC (BIOBASE, Waltham, MA, USA). [1)] These factors did not show any effect on *Prop1* expression in CHO cells. [2)] Each of these factors alone stimulated *Prop1* expression and further stimulated SOX2 activity.

organogenesis.

We previously demonstrated that expression of *Prop1* starts in SOX2-positive cells in the rat pituitary primordium at E11.5 and that PROP1/SOX2 double-positive cells account for all cells in the pituitary primordium of Rathke's pouch at E13.5 [8]. Thereafter, PROP1-positive cells decrease in number by the postnatal period but retain their *Sox2* expression [9]. We also demonstrated that PROP1 promptly faded away in PIT1-positive committed cells [8] before their terminal differentiation into ACTH-positive cells [29], suggesting that *Prop1* expression is regulated by SOX2 in rapid stimulation and/or repression by interacting with plural regulatory factors, especially by temporally coexisting with SOX2. Indeed, five putative SOX2-binding sites are present in three 5'-upstream regions of mouse *Prop1*, –2993/–1841 b, –1840/–1271 b and –1270/–771 b. A promoter assay for SOX2 using CHO cells found responsiveness in these three regions and in an additional region, –154/+21 b. Although the most distal region (–2993/–1841 b) containing two putative binding sites showed remarkable stimulation, the most proximal region (–154/+21 b) showed a repressive effect despite the absence of a putative SOX2 binding site. This regulatory activity of SOX2 on *Prop1* expression indicated that some interacting partners of SOX2 exist in CHO cells. Notably, it is known that SOX2 alone does not have transcriptional activity but requires a transcription factor to recognize a particular DNA structure [30] and to create transcriptional activity [31]. Nevertheless, this study demonstrated for the first time that *Prop1* expression might function under the modulation of SOX2, providing us with important knowledge for understanding the regulation of *Prop1* expression in pituitary stem/progenitor cells and for understanding pituitary organogenesis.

It is interesting to discover regulatory factors for *Prop1* expression other than RBP-J, which is the only factor known as a regulator [16]. In the present study, we observed a small effect of RBP-J in four pituitary-derived cell lines and CHO cells. The discrepancy between our data and those of Zhu *et al*. is probably due to cell type-dependent milieus. In the present study, we performed reporter assays using CHO cells for 39 factors and revealed that 18 factors in addition to SOX2 may participate in the regulation of *Prop1* expression in the milieus of CHO cells. These factors are known to be involved in the maintenance of stem/progenitor cells, progress of pituitary organogenesis, cell and tissue specification and differentiation (Table 3). In the present study, they were classified into three groups: Group 1, consisting of FOXJ1, HES1, HEY1 and HEY2, which showed SOX2-dependent stimulation of *Prop1* expression; Group 2, consisting of KLF6, MSX1, RUNX1, TEAD2, YBX2 and ZFP36L1, which showed SOX2-dependent suppression; and Group 3, consisting of MSX2, PAX6, PIT1, PITX1, PITX2, RPF1, SOX8 and SOX11, which were singly effective and/or cooperative with SOX2. Since factors classified in Groups 1 and 2 did not have a remarkable effect on *Prop1* expression by themselves, they might require interaction with SOX2, which would act as a regulator. SOX2 is known to interact with many transcription factors [32, 33], and its DNA binding ability is remarkably enhanced by interaction with other transcription factors that have a binding site close to the SOX2 binding site [27]. Indeed, we found putative binding sites for some factors examined in this study within 50-base length regions of the SOX2 binding site (Table 5). Additionally, SOX2 is reported to recognize the highly characteristic structure of the four-way DNA junction [30], but the presence of the junction has not been confirmed in mouse *Prop1*.

Zhu *et al*. reported that HES1 has no effect on *Prop1* expression using *Hes1*$^{-/-}$ mice [16]. However, it has been pointed out that HES1 and HEY1/HEY2 exhibit compensatory action [34] and Raetzman *et al*. observed high expression levels of *Hey1* in the mouse pituitary primordium on E11.5-14.5 [35]. The present study showed that HES1, HEY1 and HEY2 have similar effects on *Prop1* expression. Thus, we assume that the HES and HEY families compensate for HES defects in *Hes1*$^{-/-}$ mice.

Factors in Group 3 showed unique SOX2-independent regulation. Reporter assays for the responsive region of Group 3 revealed that these factors regulate *Prop1* expression through the most distal region, –2993/–1841 b, possessing two SOX2-binding sites. Notably, we demonstrated that RPF1, which was recently characterized in the pituitary [13], plays a role in PROP1-positive cells in the early pituitary primordium. On the other hand, PITX2 and SOX11 show unique responses in the –1270/–772 b and –443/–155 b regions, respectively. It is interesting that RPF1 and SOX11, similar to SOX2, repressed transcriptional activity through the proximal –154/+21 b region. The present study suggests that plural spatiotemporally expressing factors comprehensively regulate *Prop1* expression to support the progress of early pituitary organogenesis.

Ward *et al*. conducted a challenge to elucidate the tissue-specific

control region in *Prop1* using comparative genomics [15]. They focused on the highly conserved regions in the 5'-upstream region and 1st intron (CE-A and CE-B, respectively, shown in Fig. 1) and revealed their enhancer activity and specification of dorsal expression by CE-B, but not their tissue-specific expression [15]. CE-B encompasses a responsive element for RBP-J, a primary mediator of Notch signaling, and is reported to be important for the maintenance of *Prop1* expression [16, 36]. We observed that deletion of +791/+112 b remarkably increased *Prop1* expression, but further deletion of +520/+790 b eliminated this increased activity (Fig. 2B), indicating the presence of an enhancer element in the +791/+112 b region. However, we observed weak RBP-J-dependent modulation through the 1st intron and the 5'-upstream region, which contain a putative RBP-J binding sequence at –1174/–1168 b (5'-GTGGGAAA-3').

Thus, the present study suggests for the first time that SOX2, which consistently coexists in PROP1-positive cells, acts as a transcription factor for *Prop1* expression with or without interaction with various factors. Additionally, many transcription factors involved in early pituitary organogenesis might participate in the modulation of *Prop1* expression. Since Notch signaling is required for *Prop1* expression, [16, 36], the present data demonstrating that downstream factors of Notch, HES1, HEY1 and HEY2 [37–39], show SOX2-dependent regulation provided us with valuable information concerning the regulation of *Prop1* through Notch signaling. At the least, we have confirmed that *Rpf1*, which was identified as a candidate regulator for *Prop1* expression, is expressed in the PROP1-positive cells in the developing pituitary primordium. Further study of the actions of SOX2 and those of other transcription factors on the control of *Prop1* might provide us with clues to elucidating transition mechanisms for differentiation during pituitary organogenesis.

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