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# Specificity protein 1 (Sp1) plays role in regulating LIM homeodomain transcription factor Lhx4 gene expression

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**Abstract**

Both Sp-family factor Specificity protein 1 (Sp1) and LIM homeodomain transcription factor Lhx4 are involved in regulating the development of pituitary gland and nervous system in mammals. Sp1 gene mutation results in death of mouse embryo around day 11 of gestation, and mouse anterior pituitary development is severely hypoplastic after Lhx4 mutation. While Sp1 interacts with the related Lhx3 gene it is unclear whether Sp1 and Lhx4 also interact to regulate their physiological functions. The present study demonstrates that Lhx4 promoter is TATA-less and GC-rich and these sequences are conserved in different species. We have shown using site-directed mutagenesis and the Dual-Glo™ Luciferase Assay System that within the −515 to +36 bp basic activity regions of hLhx4 promoter the GC boxes were important for Sp1 regulation of the hLhx4 promoter. The electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) experiments confirmed that Sp1 interacted with Lhx4 by directly binding to GC boxes located in Lhx4 promoter. We conclude Sp1 directly regulates Lhx4 gene expression.


*Keywords:* Sp1; Interacts; Regulate; Lhx4; Promoter; GC box

Lhx4 is a member of the LIM homeodomain (LIM-HD) gene family. During embryonic and postnatal development, the Lhx4 gene is expressed in the pituitary and nervous system of mammals [1–3]. Humans with Lhx4 gene defects exhibit combined pituitary hormone deficiency (CHPD) [4,5] and several reports indicate that inappropriate expression of Lhx4 contributes to some leukemias [6,7]. Mutation of mouse Lhx4 gene causes neonatal death, the embryo anterior pituitary is severely hypoplastic, and the cell numbers of all five pituitary cell lineages are much reduced [8]. Lhx4 is essential for Rathke's pouch precursor cells and the survival of adult pituitary cell side population containing pituitary stem cells, which differentiate into specific pituitary hormone-producing cells [9].

The human Lhx4 (hLhx4) gene consists of six coding exons spanning 45 kb of q25 region of chromosome 1 [3] and the human Lhx4 protein has great homology with other mammals [10]. These proteins have identical LIM domains, and a central DNA-binding homeodomain, which are conserved in different LIM family factors [11]. Lhx4 activates gene transcription by binding to TATA box [12]. However, we still do not know the factors and the related molecular mechanism regulating Lhx4 gene expression.

Sp1 is a member of Sp-family and its glutamine rich region can act as strong activation domains [13,14]. Sp1 is ubiquitously expressed in different cell types [15] and regulates the expression of many genes, such as *TCL1* [16], gacilitative glucose transporter isoform-3 [17], and neuronal nitric oxide synthase [18]. It has been reported that Sp1 can bind to and act through the GC box [19], and Lhx4 promoter is conserved, TATA-less, GC-rich. These observations led us to

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propose that Sp1 might be involved in the regulation of Lhx4 expression and function. The present study was thus designed to elucidate the molecular interaction between Sp1 and Lhx4, and to demonstrate whether Sp1 is a regulator of Lhx4 promoter through interaction with GC box.

## Materials and methods

*Sequence analysis/plasmid constructions.* Human (Hu), rhesus monkey (Rh), bovine (Bo), and mouse (Mo) genome sequence data were accessed through GenBank at National Center for Biological Information (NCBI). The Accession Nos. were AF179849 (Hu), XM_001115069 (Rh), XM_613195 (Bo), and AC121314 (Mo). The sequences of these promoter region analysis and alignment were performed using MegAlign of DNAstar software (DNASTAR, WI), and Clustal W Method was selected to align sequences from the Align Menu [20]. The DNA fragment of the Lhx4 upstream sequence was generated by PCR (standard reaction) using normal human genomic DNA as substrates. The primers were designed based on human genome sequence data from NCBI. The different upstream hLhx4 promoter regions were amplified using an antisense primer with HindIII restriction site: *hLhx4anti* 5′-aagcttcagtctgtttccagtcc-3′, the sense primer with *hLhx4pro199* 5′-gctcctgcagtgtgaatgaa-3′ (199 bp), *hLhx4pro360* 5′-ttgcgaggccccgccccgc-3′ (360 bp), *hLhx4pro515* 5′-ct tggaacccacgaaacc-3′ (515 bp), *hLhx4pro1556* 5′-aggtgagcaggtgtcccttgttt-3′ (1556 bp), *hLhx4pro1924* 5′-ccctttcgccagtcctgaactct-3′ (1924 bp), and *hLhx4pro2721* 5′-gttcataccgcttcatattctttt-3′ (2721 bp). PCR was performed with Pfu polymerase. The PCR products were cloned into the pMD18-T vector (Takara), which were then transferred into the luciferase vector pGL3-basic vector (Promega) by restriction site KpnI and HindIII.

*Site-directed mutagenesis.* The −360mutation and −195mutation reporter vectors were constructed as the following: 360 bp hLhx4 cDNA sequence from −360 hLhx4 promoter vector was used as substrates. PCRs were performed using *mutA* 5′-ttgcgaggccccgccccgctccctgccgccccgccc-3′ and *O* 5′-aagcttc agtctgtttccagtcc-3′. −195mutation reporter vector was prepared using the SOE PCR technique [21]. Firstly, 360 bp hLhx4 cDNA sequence from −360 hLhx4 promoter vector was amplified into two separate fragments, each fragment was produced with a forward anchor primer *hLhx4pro360* 5′-ttgcgaggccccgccccgc-3′ and a reverse mutagenic primer *mutB*′ 5′-gtgcc gaaggttgggcctccct-3′, and a reverse anchor primer *O* and a forward mutagenic primer *mutB*′ 5′-agggaggcccaaccttcggcac-3′. Secondly, two separate purified fragments were used as substrates, and the primers were anchor primer *hLhx4pro360* and the reverse primer *O*. The PCR products were then transferred into pGL3-basic vector by using standard cloning techniques.

*Cell culture and reporter assay.* Human embryonic kidney HEK293T, and mouse pituitary LβT2 and αT3 gonadotrope cells were cultured in DMEM with 10% FBS at 37 °C and 5% $CO_2$. The transfections were performed using the Fugene kit (Roche) according to the manufacturer's recommendations. Briefly, $1 \times 10^5$ cells were plated in 35 mm plates in each experiment, the total quantity of DNA per well was standardized to 0.5 μg of expression vector and 1 μg of luciferase reporter gene vector, and 0.8 μg pTK-*Renilla* vector (Promega) per well was cotransfected to serve as an internal standard for transfection efficiency. DNA and 3 μl Fugene were mixed in 97 μl DMEM (Invitrogen). The mixture was then incubated for 15 min at room temperature and added to the cells in culture. The cells were then lysed after 48 h culture and reporter gene activities were measured using Dual-Luciferase Assay Kit (Vigorous).

*Electrophoretic mobility shift assays (EMSA).* Nuclear extracts were prepared as follows: $5 \times 10^6$ αT3 cells were resuspended and incubated for 15 min at 4 °C in 400 μl buffer A [10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA (pH 8.0), 0.1 mM EGTA, 1 mM dithiothreitol (DTT), and protease inhibitors]. Twenty-five microliters of 10% Nonidet P-40 was then added and all αT3 cell extracts were centrifuged at 12,000 rpm for 30 s. The pellets containing nuclear fractions were then resuspended in 40 μl buffer B [20 mm Hepes (pH 7.9), 400 mM NaCl, 1 mM EDTA (pH 8), 1 mM DTT, and protease inhibitors], vortexed for 15 min at 4 °C, and centrifuged at 12,000 rpm for 5 min, the supernatants correspond to nuclear extracts. Sp1 consensus-synthetic double-stranded oligonucleotides, the sense sequences *−68hLhx4s* wt5′-gggggaaggagcgg**gc**ggggg agggaagag-3′, *−195hLhx4s* wt5′-ggatgtgccgaagg**gc**gggcctccctgcgg-3′, *−292hLhx4s* wt5′-gctccctgccgccc**cg**ccccctccgcccc-3′, *−312hLhx4s* wt5′-ggcttgcgaggccc**cg**ccccgctccctgcc-3′, and *−181hLhx3s* wt5′-ccgggaggtggg**cg** ggcgcgcgggcgggg-3′ were designed and labeled at 5′ termini with [γ32P]deoxy-ATP using the T4 Polynucleotide Kinase, then purified on a Sephadex G25 fine column. Nuclear extracts (8 μg) were incubated for 15 min at 4 °C in binding buffer [20 mM Hepes (pH 7.9), 60 mM KCl, 1 mM EDTA, 300 μg/ml BSA, and 12% (vol/vol) glycerol] containing 1 μg poly dI-dC (homopolymer of deoxyinosine and deoxycytidine residues). Labeled probes were then added and incubated for an additional 30 min at room temperature. The antibody against transcription factor Sp1 (sc-59, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added to the binding reactions as a positive control. The DNA–protein complexes were resolved on a 6% nondenaturing polyacrylamide gel in 1× Tris–borate–EDTA buffer, respectively. Gels were dried and subjected to autoradiography.

*RT-PCR.* For analysis of mouse Lhx4 (mLhx4) expression in pituitary αT3 cell lines, cDNA was synthesized from total RNA using oligo-d(T) (Promega) as a primer. PCR then was performed using primers mLhx4s 5′-ctatgagacagccaagcaa-3′ and *mLhx4a* 5′-atggagaaactcccattca-3′. The cycling parameters were 94 °C, 30 s; 60 °C, 30 s; 72 °C, 1 min; for 30 cycles. Reaction products were analyzed on 1% agarose gel electrophoresis.

*Chromatin immunoprecipitation (ChIP).* ChIP was performed using the Active Motif ChIP Assay Kit (Active Motidf, USA). In brief, αT3 and LβT2 cells were grown to 80% confluency on two 10 cm plates, and proteins were cross-linked to DNA by the addition of 1% formaldehyde directly to the cell medium for 10 min at room temperature. The cells were then collected by scraping in 1× PBS and pelleted by centrifugation. The cells were then resuspended in 1 ml ice-cold Lysis Buffer supplemented with 5 μl PIC + 5 μl PMSF. After cooling on ice for 30 min, the cells were transferred into a 1.5 ml conical tube and centrifuged at 5000 rpm for 10 min at 4 °C to pellet the nuclei. Then the pellet was resuspended in 1.0 ml shearing buffer supplemented with 5 μl PIC, and the chromatin was subsequently sheared by using a Sonics Vibracell VC 130 sonicator. The cellular DNA was sonicated to fragments of 200–1000 bp and the lysate was diluted with ChIP IP buffer and precleared with 100 μl Protein G beads. Protein–DNA complexes were incubated overnight with the Sp1 antibody as used in EMSA. Nonspecific IgG antibody provided in Kit was used as negative control. A fraction of the protein DNA was not precipitated but set aside for the total chromatin examination (termed input). Protein–DNA complexes were eluted with elution buffer, and cross-links were reversed by using 8 μl of 5 M NaCl at 65 °C for 6 h. DNA was purified using mini-columns and then collected in 50 μl nuclease-free water. Simultaneously the input fraction was also collected using mini-columns. The aimed sequence, spanning from the −326 to −90 bp region of the mouse Lhx4 gene (Accession No. AC121314), was amplified by PCR using primers *mLhx4spro* 5′-agcccgccgcagtattta-3′ and *Lhx4apro* 5′-atggag aaactcccattca-3′. A single 237 bp band was generated and electrophoresed on a 1% agarose, stained with ethidium bromide. PCR conditions were as follows: 30 s at 95 °C, 20 s at 56 °C, and 20 s at 72 °C repeated 35 cycles followed by an extension of 10 min at 72 °C. The input was used as a positive control. The experiments were repeated three times.

*Statistical analysis.* To normalize transfection efficiency, all promoter luciferase values were divided by pTK-*Renilla* luciferase, all values are presented as means ± SEM. Statistical differences were assessed by a one-way analysis of variance (ANOVA) followed by Student's *t* test; $P < 0.05$ was considered significant.

## Results

### *Four GC boxes are conserved in Lhx4 promoter of mammals*

Different species of Lhx4 promoter sequence analysis and alignment (Fig. 1) were performed using DNAstar

```
                                                                                -312
hu... CCCGCCACGG CTGGGCGGAG GCGGAGGCGG AGGCGGGGAG GGGGGGGGGG TGCCGGCTTG CGAGGCCCCG
rh... CCCGCAA-GG CTGGGCGGAG GC-------- ---------- GGTGGGGGGG TGCCTGCCTG CGAGGCCCCG
bo... CGCGCA---- --C--CGCGG GC--A----- ---------- -GCGGGTGGG GGAAGGCCAG TGAGGCCCCG
mo... CCCGCA---- --------CG GC-------- ---------- --AGGGTGGG GGAGGGGCTG C-AGGCCCCG
                           -292
hu... CCCCGCTCCC TGCCGCCCCG CCCCCCTCCG CCCCGCCGGC CTCACTCGGG GCGCCGGGAC ----------
rh... CCCCGCTCCC TGCCGCCCCG CCCCCCTCCG CCCCGCCGGC CTCACTCGGG GCGCCGGGAC ----------
bo... CCCCGCTCCA TGCAGTCCCG CCCTCCT--G CCTGGCCGGA CTCCCTCGGG GCGCTGGGAC ----------
mo... CCCCCTATCA GGCGGCCCCG CCCTCCC--G CACCTCCGAC CAGATTCGGG GCTACCGAAC ----CCCCCC

hu... ---------- -CCCCACTCC CTCC-----G GGGTCGGCTG CCCGCCGCCG GGTTGGGGG- ATGTGCCGAA
rh... ---------- -CCCCACTCC CTCC-----G GGGTCGGCTG CCCGCCGCCG GGTTGGGGG- ATGTGCCGAA
bo... ---------- -CCCCACTCC GGCC-----T GGGTTCGCCG CTTGCTGCCC GGTGGGGGG- CTTGGCTGAA
mo... TACTTCTCGC CCCCTACTCT GGCTAGCCGG GGGTCTGCAG CGGGCTGCCC GGTTGGCAGC ACTAGCTGGA
        -195
hu... GGGCGGGCCT CCCTGCGGAA AGCGACGTCA CGGCCGCTCC TGCAGTGTGA ATGAATCAAA ACGCCCGGGT
rh... GGGCGGGCCT CCCTGCGGAA AGCGACGTCA CGGCCGCTCC TGCAGTGTGA ATGAATCAAA ACGCCTGGGT
bo... GGGCGGGCCT GCCTGCGGAA AGCGACGTCA CGGCCGTTCC TGCAGTGTGA ATGAATCAAA ATGCCTGGGT
mo... GGGCGGGCTT CCCTGCGGAA AGCGACGTCA CGGCCGCTCC TGCAGTGTGA ATGAATCAAA ATGCCTGGGT
                                                                        -68
hu... GACCGCGGCC TCCCGGGCGG CCGGCACGCG AAGGGTGGAG GGGGAGGGGG AAGGA-GCGG GCGGGGGAGG
rh... GACCGCGGCC TCCCGGGCGG CCGGCACGCG AAGGGTGGAG GGGGAGGGGG AAGGA-GCGG GCGGGGGAGG
bo... GACCGCGGCC TCCCCAGCGG CCGGCACGCC AAGGGTGGAG GGGGAGGGGG AGGGAAGCGG GCGGGGGAGG
mo... GACCGCGGCC TCCAGGGCAG CCGGCACGCG AAGGGTGGAG GGGGAGGGGG AGAG--GAGG GCGGGGAAGG

hu... GA--AGAGGA AAAAA--GCC AGAGCTGCAG CAACAGCGTC TC---AACCT GGGATGTGCA CCAACCccgg
rh... GAGGAGAGGA AAAAA--GCC AGAGCTGCAG CAACAGCGTC TC---AACCT GGGATGTGCA CCAACCccgg
bo... GAGGAGGGGA AAAAAAAGCC AGAGCTGCAG CAACAGCGTC TCCTCAACCC GGGATGTGCA CCAACCcggg
mo... GAAGGGGGG- -G--A--GCC AGAGCTGCAG CAACAGTGTC TCCTCAACCC GGGATGTGCA CCAGCCcggg

hu... agagcgagat CAAAGGGACT GGAAACAGAC TGGGGACTGG CGGGGGGAGG GGGCCGGCCA GCCTGTGGAG
rh... agagcgagat CAAAGGGACT GGAAACAGAC TGGGGACTGG TGGGGGGAGG GGGCCGGCCA GCCTGTGGAG
bo... agagggagat CAAAGGGATT TGAAACAGAC CGGGGACTGG CAGGGGGAGG GGGCCAGGCA GCCTGTGGAA
mo... agagcgagat CAAAGGGATT TGAAACAGCC TGAGGACTGG CGGGGG-AGG GGGCCAGCCA GCTTGTGGAG
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Fig. 1. Comparative sequence analysis of Lhx4 proximal promoter regions. Lhx4 genes feature GC-rich, TATA-less promoters, have four conserved GC boxes [notable GC boxes are shown in bold and their position in nucleotides relative to the hLhx4 start codon (+1) are indicated. Transcription start site positions are shown in lowercase bold italics with an arrow above]. (The GenBank Accession Nos. for the Lhx4 nucleotide sequence Human AF179849; Rhesus monkey XM_001115069; Bos taurus XM_613195; Mouse AC121314.) bo, Bos taurus; hu, human; mo, mouse; rh, rhesus monkey.

software. The results show that four GC boxes (bold) of Lhx4 promoters are greatly conserved in human, rhesus monkey, bovine, and mouse, all of which have the character of TATA-less and GC-rich.

### *360 hLhx4 and −515 hLhx4 promoters have the basal activity*

To determine the basal activity region of Lhx4 promoter, we constructed a series of deleted reporter genes containing different lengths of the upstream region. The series of 5′ deleted fragments ending at positions −2721,−1924, −1556, −515, −360, −292, and −199 were, respectively, generated by PCR and then linked to pGL3-basic vector. The 2701 hLhx3 promoter was used as a positive control. These vectors were transiently transfected into 293T, LβT2, and αT3 cell lines, and the luciferase activities were measured after 48 h, respectively. The results are consistent among three cell lines, and the data show that the activities of hLhx4 promoter gene spanning −360 bp to +36 and −515 bp to +36 are significantly stronger than control vector ($P < 0.05$) (Fig. 2), which suggests that this region mediates the basic activity of hLhx4 promoter.

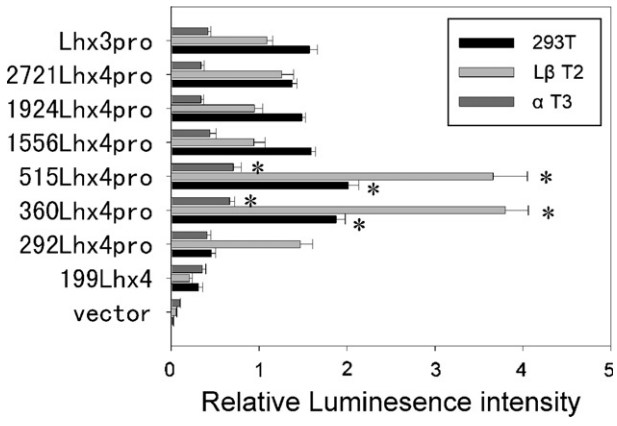


Fig. 2. Different activity of 5′ flanking regions of the hLhx4 promoters. Molar equivalents of luciferase reporter genes with the indicated 5′ flanking regions of the hLhx4 promoters were transiently transfected into mouse pituitary gonadotrope LβT2 cell lines and the basal activities were determined. Similar data were obtained using mouse αT3 pituitary cells and 293T cell. pTK-*Renilla* vector was cotransfected to serve as an internal standard for transfection efficiency. Promoter activity is expressed as relative fold luciferase units over vector control (pTK-*Renilla*). *Values that differ significantly from empty vector ($P < 0.05$). All results represent means ± SEM of six independent experiments.

### *Sp1 up-regulates the hLhx4 promoter activity*

To characterize the effects of the Sp1 transcription factor, the −360 hLhx4 promoter luciferase reporter gene with Sp1 or with Lhx3a expression vectors was cotransfected into 293T cells. We observed that Sp1 greatly activates the −360 hLhx4 promoter luciferase reporter gene ($P < 0.05$). However, Lhx3a had modest effects on the −360

hLhx4 promoter luciferase reporter gene. Similar results were obtained from αT3 and LβT2 cells (Fig. 3A).

*GC box mutagenesis reduces the activity of hLhx4 promoter*

In order to determine whether −195, −292, and −312 GC boxes are essential to hLhx4 promoter activity, the site-directed mutagenesis was performed. The results showed that Lhx4 promoter activity was significantly ($P < 0.05$) reduced compared with the wild type constructs after any of these GC boxes had been muted. All the results were similar between 293T, αT3, and LβT2 cell lines (Fig. 3B).

*Sp1 binds to Lhx4 promoter in vitro and in vivo*

EMSA experiments were performed to test the interacting properties of Sp1 to GC box-containing sequences in the hLhx4 promoters. The oligonucleotides of hLhx4 promoters −68, −192, −292, and −312 GC boxes were radiolabeled and incubated with nuclear extracts from αT3 cells. The −181hLhx3 GC box sequence was used as positive control for the binding of Sp1, as it has been shown to bind −181GC box of the mouse human Lhx3 [22]. The results showed that Sp1 can bind to −192, −292, and −312 GC box site of hLhx4 promoters, and no complex was detected in the −68 GC box site. But the above interacting

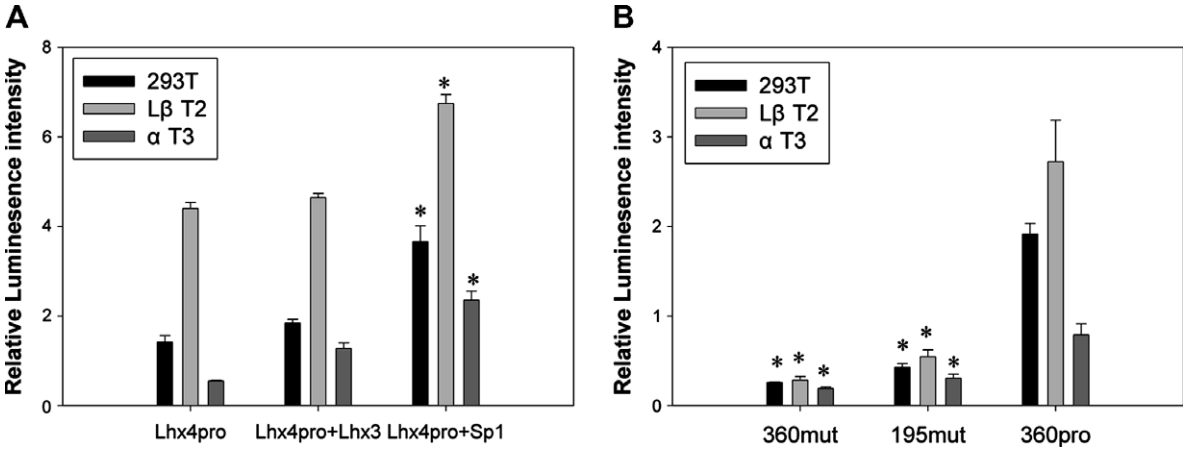


Fig. 3. (A) Sp1 up-regulates the activity of Lhx4 promoter. Luciferase reporter genes −360 bp to +36 bp hLhx4 promoter were transiently cotransfected into LβT2, αT3, and 293T cell lines with different expression vectors for the indicated transcription factor cDNA. *Values that differ significantly from only 360 bp hLhx4 promoter ($P < 0.05$). (B) GC boxes are important to the basal activities of Lhx4 promoter. Wild type −360 to +36 bp hLhx4 promoter luciferase reporter gene or equivalent constructs with mutations of the indicated Sp1-binding GC boxes were transiently transfected into pituitary LβT2 cell lines, similar data were obtained using αT3 and293T, and their activities were determined. Promoter activity is expressed as relative fold luciferase units over vector control (pTK-*Renilla*). *Values that differ significantly from wild type 360 bp hLhx4 promoter ($P < 0.05$). All results represent means ± SEM of three independent experiments.

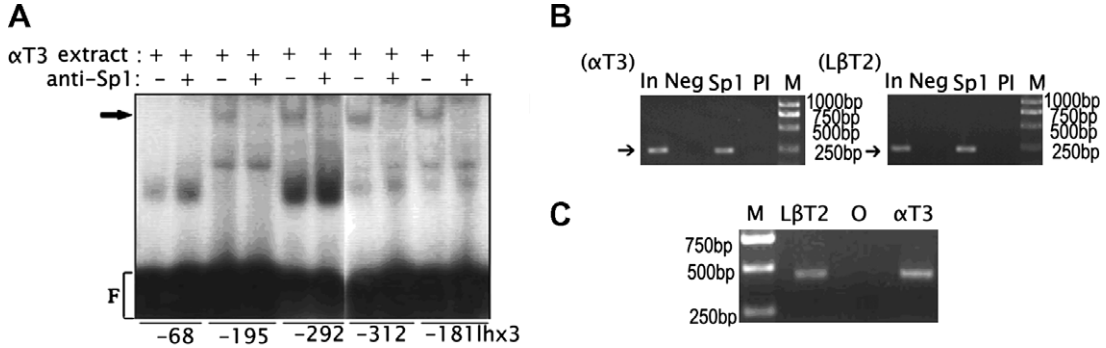


Fig. 4. Sp1-binding sites in the hLhx4 proximal promoters. (A) EMSA experiments were performed. Radiolabeled DNA probes representing Lhx4 promoter −68 bp, −195 bp, −292 bp, −312 bp and Lhx3 promoter −181 bp regions were incubated with protein extracts from pituitary gonadotrope αT3 cells (left panel black arrow) and the resulting complexes were separated by electrophoresis. Anti-Sp1 antibodies were used to disrupt complexes containing Sp1 (right panel black arrow). F, free, unbound DNA. (B) ChIP experiments demonstrate Sp1 occupation of the proximal region of the endogenous mouse Lhx4 promoter in αT3 and LβT2 pituitary cells. In, input positive control; M, molecular markers (in base pairs); Neg, negative control (no substrate); PI, preimmune negative control; Black arrow head express 237 bp Lhx4 promoter. This experiment was repeated at least three times. (C) Expression of Lhx4 in pituitary cell lines αT3 and LβT2. RT-PCR was used to amplify specific regions of Lhx4 (447 bp product) using cDNA from the indicated cell lines. Negative control reactions were performed in parallel in the absence of reverse transcriptase. M, molecular marker; O, negative control (no substrate); αT3, αT3 gonadotrope cells; LβT2, LβT2 gonadotrope cells.

complexes were blocked by the addition of anti-Sp1 antibody (Fig. 4A).

ChIP experiments were conducted to determine whether Sp1 can bind to the regulatory region of the endogenous Lhx4 gene in the αT3 and LβT2 gonadotrope cell lines using Sp1 specific antibody. The primers encompassing −312, −292, and −195 GC box sites were used to detect precipitation of genomic DNA. As expected, these primers amplified a 237 bp region of mouse Lhx4 promoter from input chromatin (Fig. 4B, (αT3) lane 1 and (LβT2) lane 1), and Sp1 was immunoprecipitated (Fig. 4B, (αT3) lane 3 and (LβT2) lane3). No immunoprecipitation was detected when Sp1 antibody was replaced by a nonspecific IgG control antibody (Fig. 4B, (αT3) lane 4 and (LβT2) lane 4) and negative control (Fig. 4B, (αT3) lane 2 and (LβT2) lane2). The ChIP results show that the Sp1 takes part in the promoter regulation in vivo.

RT-PCR was used to analyze the expression of Lhx4 in αT3 and LβT2 cells (Fig. 4C). Negative control reactions (O) were performed in parallel in the absence of reverse transcriptase. This confirmed that Lhx4 is expressed in αT3 and LβT2 cells.

## Discussion

Studies of the LIM family factor Lhx4 have focused on its function and effects on other factors, such as Pit-1 [13], Lhx3, and Islet-1 [2]. However, little is known about the factors influencing Lhx4 expression. The present study provides the first evidence that the activity of Lhx4 is regulated by Sp1.

In this study, we initially analyzed the activity of Lhx4 promoter fragments spanning from −2710 to +36 bp in 293T, αT3, and LβT2 cell lines. The results showed that −360 to +36 bp and −515 to +36 bp regions have the highest basal activity of hLhx4, and deletion studies confirmed that the regions from −360 to +36 bp of the proximal Lhx4 promoter are critical for transcriptional activity. These results offer us the baseline information on the Lhx4 promoter to allow further study the molecular mechanism of other factors regulating Lhx4.

The Lhx4 promoter region is conserved in different mammals and sequence analysis showed that these promoters have the character of TATA-less and GC-rich. Our present study has shown for the first time that Lhx4 expression and function are regulated by Sp1. Firstly, EMSA results show that Sp1 is involved in regulating Lhx4 activity through −312, −292, and −195 GC boxes. Secondly, ChIP results show that Sp1 participates in regulating the Lhx4 promoter by interaction with the GC box in cells. Finally, the GC mutation experiments showed that (−312, −292, and −195) GC boxes are essential for full transcriptional activity of the Lhx4 minimal promoter. The Lhx4 and Lhx3 promoters have great homology, in particular both being GC box rich. A previous study has shown that Sp1 participates in Lhx3 gene regulation [22]. Given the great homology between Lhx3 and Lhx4, in particular the GC box rich nature of the promoters it is perhaps not too surprising that Sp1 had effects in regulating Sp1 expression. We have confirmed that in a gonadotrope precursor cell line Sp1 interacts with the Lhx4 promoter leading to up-regulation of gene expression. Mutations of Sp1 causes mouse embryo death at day 11 of gestation [19]. Given that Lhx4 expression is detected in the pituitary gland of mouse embryo about at day 10 of gestation [8] and Lhx4 mutations result in reduced pituitary development [2,4,5], our present results suggest that Sp1 may play an important role in regulating Lhx4 as an essential factor involved in pituitary development.

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