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A Brain Region-Specific Gene Product Lhx6.1 Interacts with Ldb1 through Tandem LIM-Domains[1]

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LIM-homeodomain (LHX) transcription factors play critical roles in cell fate determination during development, in particular, in CNS. The transcriptional activity of several LHX proteins is postulated to be regulated by interaction with an LIM-domain binding protein, Ldb1. We have now identified a novel LHX molecule, termed Lhx6.1, that is closely related to a recently reported Lhx6 molecule. The Lhx6.1 transcript is found in several restricted regions in the developing CNS, mostly within the embryonic forebrain. We further show that Lhx6.1 interacts with Ldb1 through tandem LIM-domains, implying transcriptional regulation of Lhx6.1 by Ldb1.

Key words: developing CNS, Ldb1, Lhx6.1, LIM-homeodomain (LHX) gene, protein-protein interaction.

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The central nervous system (CNS) is comprised of large numbers of cell types, including neuronal cells that are coordinately arranged in numerous regions, each of which is specialized for a distinct function. The homeobox gene families, which can be classified into several subfamilies, are thought to function in establishing positional identity and in regulating the morphogenesis of region-specific neuronal cells (*1*). Such subfamilies include the LIM-homeodomain (LHX) transcription factor family, each member of which shows a mutually exclusive expression pattern within the developing CNS (*2-7*). Studies on gene expression patterns in the developing CNS and the targeting of these genes in mouse have demonstrated the importance of LHX genes in the specification of neuronal cell identity (*8-11*).

The LHX protein family is characterized by two tandem cystein-rich zinc-binding motifs referred to as LIM domains, followed by a DNA-binding homeodomain. LIM domains, which are known to mediate protein-protein interactions, in the LHX molecules have been suggested to play a negative regulatory role in the transcriptional activation of LHX proteins (*12, 13*). The recently isolated protein Ldb1/NLI binds to the LIM domains of several LHX proteins and LMO (LIM-only protein) proteins (*14-18*). Jurata *et al.* reported that the direct interaction of Lhx3-Isl1/2 is disrupted by Ldb1 protein (*19*). In addition, Agulnick *et al.* demonstrated that the trans-activation function of Xlim1 is upregulated by co-expression of Ldb1 (*15*). Based on these reports, the transcriptional activities of LHX proteins are thought to be regulated by a direct interaction with Ldb1 protein that would abrogate the inhibitory role of the LIM-domains.

In the present study, we identified a new LHX gene, termed Lhx6.1, which is expressed predominantly in the developing CNS. We further show that the Lhx6.1 protein is capable of interacting with Ldb1 the protein, suggesting that Lhx6.1 shares a common transcriptional regulatory mechanism with other LHX genes.

MATERIALS AND METHODS

Cloning of Mouse and Human Lhx6.1 cDNAs—mRNA from whole brain of embryonic day 13.5 (E13.5) mouse embryos and adult mice were used to construct an E13.5 brain-specific cDNA library using a PCR-SELECT cDNA subtraction kit (CLONTECH) according to the manufacturer's protocol. cDNA clones representing transcripts expressed at higher levels in E13.5 mouse brain than in adult brain were selected using a dot blot hybridization strategy, and their nucleotide sequences were determined. cDNA fragments containing novel sequences were labeled with α-^{32}P dCTP, and used as probes for screening the E17 mouse cDNA library to isolate full-length cDNAs. One of these clones was found to belong to the LHX gene family as judged by the presence of two LIM domains and one homeodomain, and was designated Lhx6.1 for its closest similarity to the previously reported Lhx6 (see "RESULTS" for details). Human Lhx6.1 was isolated using a PCR cloning method. cDNA fragments encoding the 5′-, central, and 3′-regions of human Lhx6.1 were independently amplified by PCR with the following sets set of primers for mouse Lhx6.1: mLhx6.1-13 (5′-CAGGTGATGGCCCAGCCAGG) and mLhx6.1-11 (5′-TCTCCTTGTTCTTGATGTAGCAG) were used for the 5′-region; mLhx6.1-12 (5′-TCTGGAGATCCTGGACCGG) and mLhx6.1-14L (5′-TTAGTACTGAAAAAGGATGAC) were used for the central region; and mLhx6.1-14 (5′-GCCCTGTCCGACGACATC-

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CA) and mLhx6.1-14S (5′-TCAGCGGCTGAAGGGCAG) were used for the 3′-region of hLhx6.1. Each of the amplified human cDNAs was then sequenced.

Northern Blotting—Twenty micrograms of total RNA was electrophoresed in 1% formaldehyde/agarose gels and transferred to Hybond N+ nylon membrane filters (Amersham). The filters were hybridized with ^{32}P-labeled Lhx6.1, the cDNA fragment containing 3′-UTR, or an Ldb1 cDNA fragment. Hybridization was carried out in ExpressHyb hybridization solution (CLONTECH) at 68°C for 2 h. The filters were washed in 2×SSC containing 0.1% SDS at room temperature with several changes of buffer, then for 40 min at 60°C in 0.1×SSC containing 0.1% SDS. The filters were exposed to X-ray film for 1 day with an intensifying screen.

RT-PCR—First strand cDNAs were synthesized from 1 μg of total RNA using Superscript II (GIBCO BRL). The respective first cDNAs were then used directly for amplification of the Lhx6.1a, Lhx6.1b, and G3PDH genes by PCR. The 5′ primers used were: primer A (GGTACAGTGCGGGCAGGTGC) for Lhx6.1a cDNA, primer B (CACGGCTACATTGAGAGTCATC) for Lhx6.1b cDNA. The 3′ primer used was primer C (CTCAGCGGCTGAGGGGCAG), which is within the common sequence of the Lhx6.1a and Lhx6.1b cDNAs. Reaction products were resolved in 1% agarose gels and stained with ethidium bromide.

In Situ Hybridization—^{35}S-labeled or digoxygenin (DIG)-labeled antisense or sense RNAs corresponding to 3′-UTR of mLhx6.1 cDNA were transcribed *in vitro*, and used as probes for *in situ* hybridization. The radioactive *in situ* hybridization procedure was performed essentially as described previously (*21*). Mouse embryos (E9.5, E14.5, and P1.0) were placed in crushed dry ice. Serial sections 10 μm in thickness were prepared using a Cryostat (Leica) and dried on 3-amino-propyl-triethoxy Silan coated slides. The slides were fixed in 4% paraformaldehyde for 20 min at room temperature and treated with acetic anhydride. Hybridization was done with an ^{35}S-labeled RNA probe for 16 h at 55°C. The slides were washed, treated with RNaseA, dehydrated in ethanol, and air dried. Autoradiography was performed by exposing the sections to film for 1 week. The sections were then counter-stained with crecyl violet. Non-radioactive whole mount *in situ* hybridization experiments were performed as described previously (*21*). The hybridized probe was detected with an anti-DIG antibody by an alkaline phosphatase reaction. No signals were detected in control samples using ^{35}S-labeled and DIG-labeled sense RNA probe.

Plasmid Construction—The cDNA fragments encoding full length Ldb1, Lhx6.1a, and Lhx6.1b, or part of Lhx6.1 (LIM1, LIM2, or LIM1 plus LIM2 domains) were amplified by PCR from E14.5 mouse brain cDNA. After their nucleotide sequences were confirmed, the PCR products were ligated into pEF-BOSE-M or pEF-BOSE-F containing a c-Myc or FLAG epitope tag, respectively (*22*).

Transfection and Immunoblot Analysis—COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and transfected with

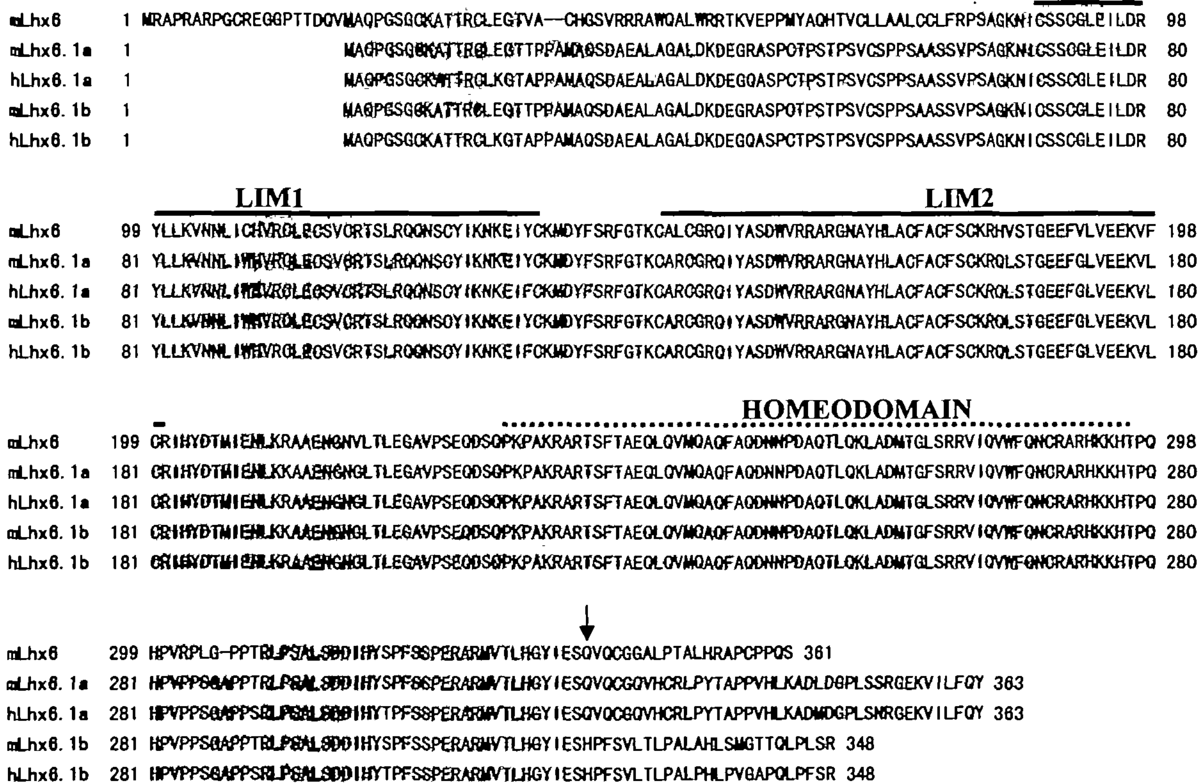

Fig. 1. **Multiple amino acid sequence alignment of mouse and human Lhx6.1, and mouse Lhx6.** Deduced amino acid sequences of mouse (m) and human (h) Lhx6.1 differ in part from that of Lhx6 cloned from mouse by another group (mLhx6) (*20*). Amino acids conserved in at least four of the five aligned clones are shaded. Solid lines indicate two LIM domains. The dashed line represents a homeodomain. The arrow indicates the boundary at which the amino acid sequences of Lhx6.1a and Lhx6.1b differ.

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constructs using a TransIT-LT1 Polyamine Transfection Reagent (Mirus) according to manufacturer's protocol. After 2 days in culture, the cells were solubilized with Nonidet P-40 (NP-40) lysis buffer (0.5% NP40, 10 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM EDTA, 2 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin), and subjected to immunoprecipitation and immunoblotting as described previously (22).

RESULTS

Cloning of Lhx6.1 cDNA—To better understand CNS development, we intended to identify novel genes that are expressed predominantly in the developing brain. For this

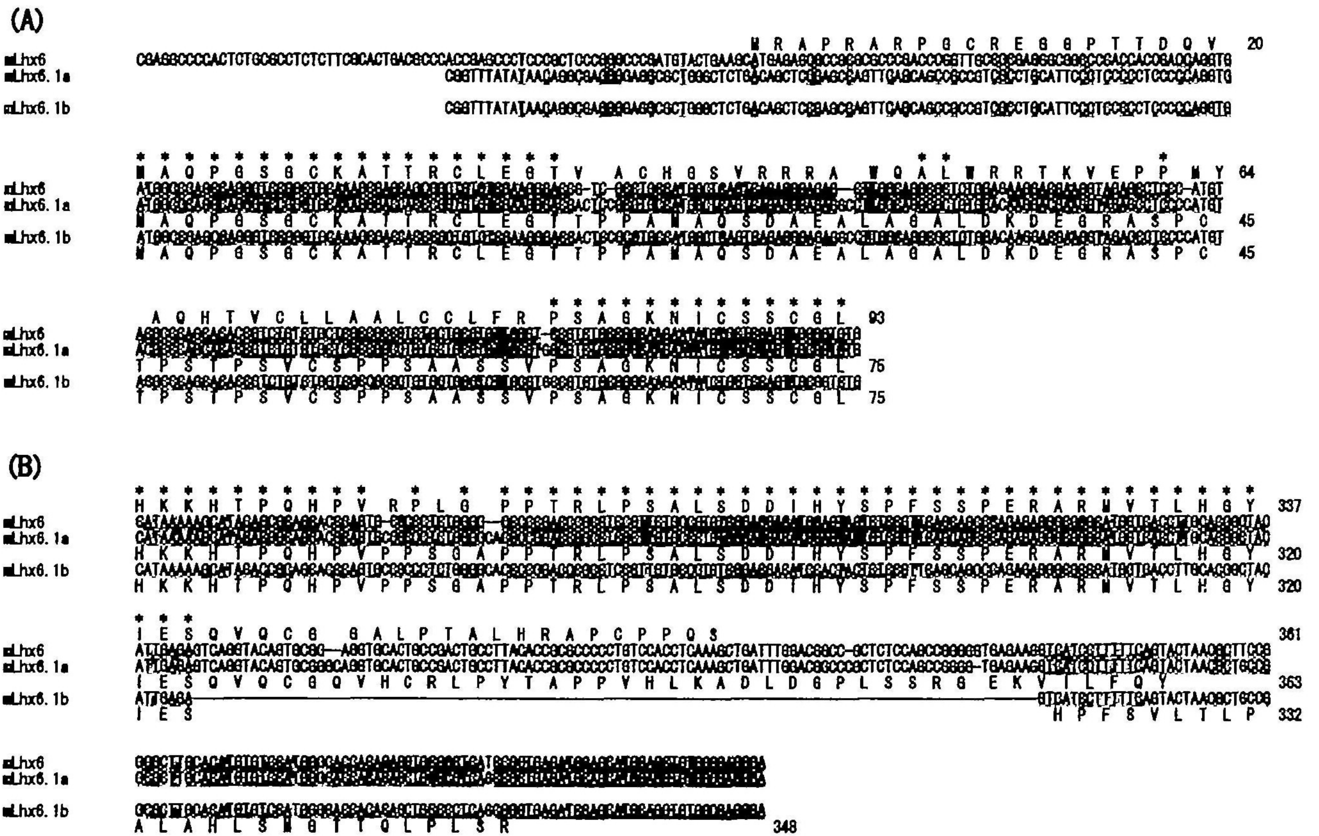

Fig. 2. **Comparisons of nucleotide sequences and deduced amino acid sequences of the terminal regions of mLhx6, mLhx6.1a, and mLhx6.1b.** Asterisks indicate amino acid residues that are identical among all three aligned clones. Nucleotide insertions and deletions in the 5'- (A) and 3'- (B) regions produce shifts in the reading frame of mLhx6 and mLhx6.1. A 103-bp deletion in the 3'-region of the mLhx6.1b sequence results in the amino acid sequence difference in the C-terminal region between mLhx6.1a and mLhx6.1b (B).

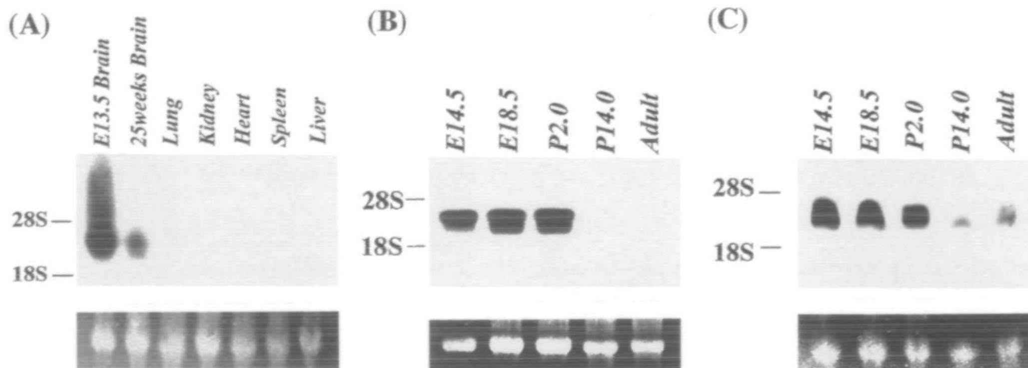

Fig. 3. **Northern blot analysis of the mRNA expression of Lhx6.1 and Ldb1.** (A) Tissue distribution of Lhx6.1 transcripts. Total RNAs extracted from E14.5 mouse brain and several adult tissues including brain were blotted onto a Hybond N+ membrane, and hybridized with ^{32}P-labeled Lhx6.1 cDNA. (B) Lhx6.1 expression in whole brain during mouse embryogenesis. Total RNAs were prepared at the indicated stages and subjected to northern blot analysis as described above. (C) Ldb1 expression in whole brain during mouse embryogenesis was analyzed as described in (B) using ^{32}P-labeled Ldb1 cDNA.

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purpose, cDNA prepared from E13.5 mouse brain was subtracted from adult brain cDNA. One novel clone obtained, ebs-71, encoded two LIM domains and one homeodomain, indicating that this gene belongs to the LHX gene family. Screening a mouse E17.5 cDNA library with this cDNA fragment as a probe yielded a full-length cDNA of ebs-71.

While we were conducting the further characterization of this new molecule, another group independently reported a new member of the LHX gene family, named Lhx6 (*20*), that is clearly related to ebs-71, showing 85% nucleotide sequence similarity. Thus we designated our clone "Lhx6.1." As indicated in Fig. 1, although the overall amino acid sequences of our Lhx6.1 and Lhx6 are very similar, complete divergence was seen in both the N-terminal and C-terminal regions (Fig. 1). When the nucleotide sequences of Lhx6.1 and Lhx6 are compared, they are nearly identical to each other, but the 5′-noncoding regions show almost no homology, and some insertions and deletions are found in the coding region at the 5′- and 3′-endo (Fig. 2, A and B). The nucleotide sequences of the two independently isolated clones Lhx6.1a and Lhx6.1b are 100% identical throughout the cDNA clone except for the region where alternative splicing is postulated to occur (see below and Fig. 2B for the details). Therefore, it was postulated that these deletions and mutations produce a shift in the reading frame of Lhx6, resulting in changes in the amino acid sequences in the N-terminal and C-terminal regions.

Our full-length cDNA screening identified two types of Lhx6.1 transcript, one encoding 363 amino acids (Lhx6.1a) and the other 348 amino acids (Lhx6.1b) (Fig. 1). A comparison of the nucleotide sequences of Lhx6.1a and Lhx6.1b showed a 103-bp deletion in the C-terminal region

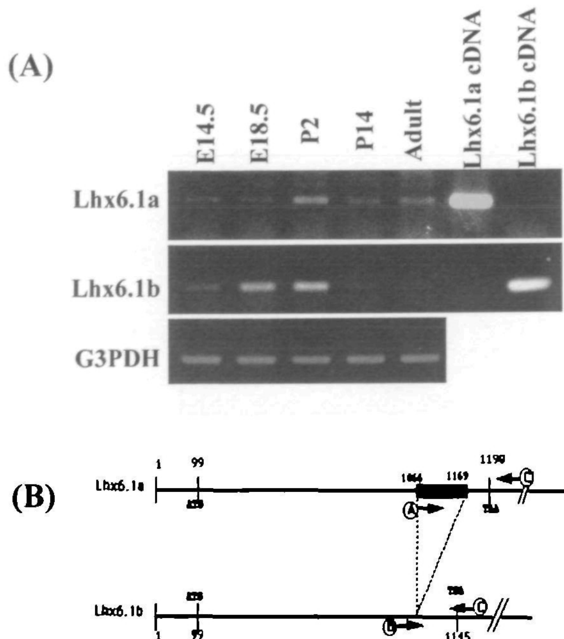

Fig. 4. **Two splicing variants of Lhx6.1 are expressed in the developing brain.** (A) RT-PCR analysis of Lhx6.1a and Lhx6.1b expression in mouse brain between E14.5 and adult. Lhx6.1a and Lhx6.1b cDNAs were used to confirm primer specificity. (B) Schematic representation of structure of Lhx6.1 cDNA and the location of primers used to amplify Lhx6.1a and Lhx6.1b.

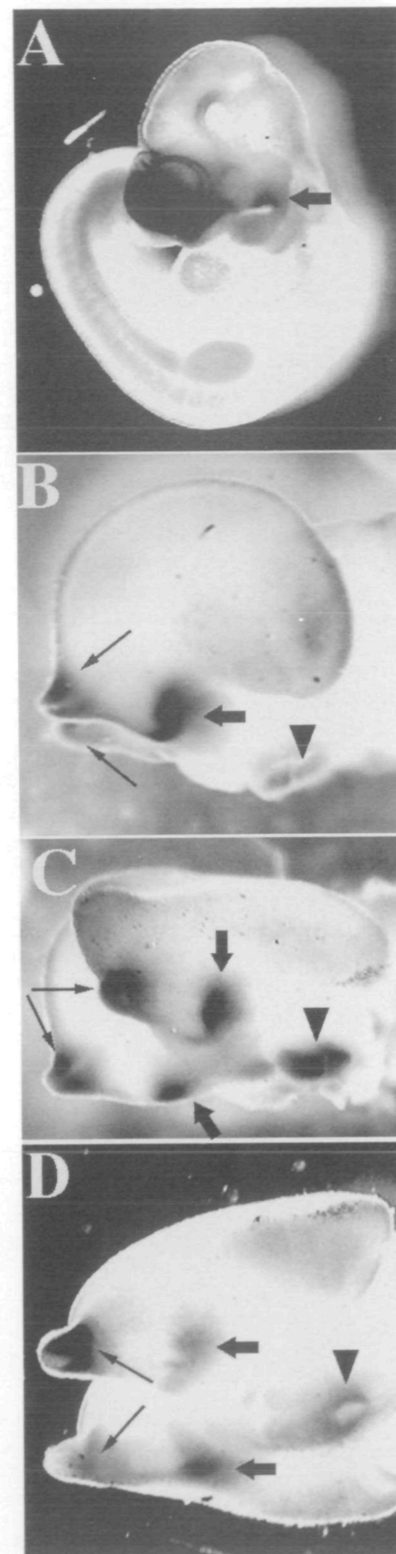

Fig. 5. **Whole mount *in situ* hybridization analysis of Lhx6.1 expression.** A whole embryo at E9.5 (A), and whole brain at E13.5 (B, C) and E14.5 (D) were hybridized with DIG-labeled Lhx6.1 antisense riboprobes. In the E9.5 embryo, Lhx6.1 was expressed in the forebrain and the branchial arch (bold arrow in A). In the developing brain, lateral (B) and ventro-lateral (C, D) views showing restricted expression of Lhx6.1 in the basal forebrain; the thin arrow indicates the olfactory bulb; the arrowhead indicates the arcuate nucleus (arc); the bold arrow indicates the region consisting of the medial ganglionic eminence (MGE) and the preoptic area (POA).

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of Lhx6.1b. The complete dissimilarity of the amino acid sequences of the C-terminal parts of Lhx6.1a and Lhx6.1b, and the reduced number of amino acid residues in the latter, may be due to alternative splicing and a resultant frame shift (Fig. 2B).

We next aimed to isolate a human ortholog of Lhx6.1 by a PCR cloning strategy with various combinations of primers based on the mouse cDNA sequence. Two transcripts encoding 363 and 348 amino acids were obtained. From the sequence similarity, these two are most likely human Lhx6.1a and Lhx6.1b, respectively. A comparison of the amino acid sequences of human and mouse Lhx6.1 revealed more than 95% homology (Fig. 1).

Expression of Lhx6.1 in Embryonic Brain—We analyzed Lhx6.1 expression in embryonic brain and several adult tissues including brain by northern blot analysis (Fig. 3A). Lhx6.1 expression was readily observed in embryonic brain, but barely detectable in adult brain. In other adult tissues examined, no Lhx6.1 expression was detectable. In order to determine Lhx6.1 expression levels during brain maturation, mouse brains at various developmental stages were subjected to northern blot analysis (Fig. 3B). The expressions of two transcripts of Lhx6.1 were abundant from E14.5 to postnatal day 2.0 (P2.0) brain, but were drastically lower in P14.0 and adult mouse brain. These results suggest that Lhx6.1 is closely associated with CNS development. To compare the temporal expression pattern of Lhx6.1 with LIM-domain binding protein (Ldb1) genes, we analyzed the Ldb1 expression pattern during brain development. The Ldb1 expression pattern was almost identical to that of Lhx6.1 during brain maturation (Fig. 3C).

Expression Ratio of Lhx6.1a and Lhx6.1b during Brain Maturation—To determine the expression of Lhx6.1 variants, we designed primers that specifically detect transcripts of Lhx6.1a (primer A shown in Fig. 4B; see "MATERIALS AND METHODS" for the actual sequence) and Lhx6.1b (primer B), and performed RT-PCR analysis. Lhx6.1a and Lhx6.1b cDNAs were first used as positive control templates. It was confirmed that these primers distinguish the two differentially spliced forms of Lhx6.1 (Fig. 4A). As shown in Fig. 4A, the expression of Lhx6.1a peaked in P2.0 brain and remained high in adulthood, whereas Lhx6.1b expression was abundant from E18.5 to P2.0 and then decreased at P14.0 through adulthood (Fig. 4A).

Restricted Expression of Lhx6.1 Gene in the Brain—We studied the distribution of Lhx6.1 mRNA during mouse embryogenesis by two strategies, whole mount *in situ* hybridization and *in situ* hybridization on serial sections. Whole mount *in situ* hybridization analysis showed the Lhx6.1 mRNA to be expressed in part of the forebrain and the first branchial arch at E9.5 (Fig. 5A). *In situ* hybridization on sections from E9.5 embryos clearly indicated that the regions expressing Lhx6.1 in the forebrain of E9.5 mouse are restricted to the preoptic area (POA) and the hypothalamic region (Fig. 6, A and A′).

Next, in order to determine the distribution of Lhx6.1 mRNA in the developing brain at later stages, E13.5 and E14.5 brains were dissected and used for whole mount *in situ* hybridization. Lhx6.1 transcripts were detected mainly in the basal forebrain at these two developmental stages where they were restricted to three regions (Fig. 5, B, C, and D). We examined the expression in these areas in more detail by *in situ* hybridization on serial sections. In the hybridized sections, Lhx6.1 expression was detectable in the olfactory bulb (OB), arcuate nucleus (arc), medial ganglioninc eminence (MGE), and preoptic area (POA) (Fig. 6, B and C).

In addition to these regions, Lhx6.1 expression was seen to have spread in the cortex and hippocampus in the P1.0 brain (Fig. 6D). In non-neuronal tissues, Lhx6.1 mRNA

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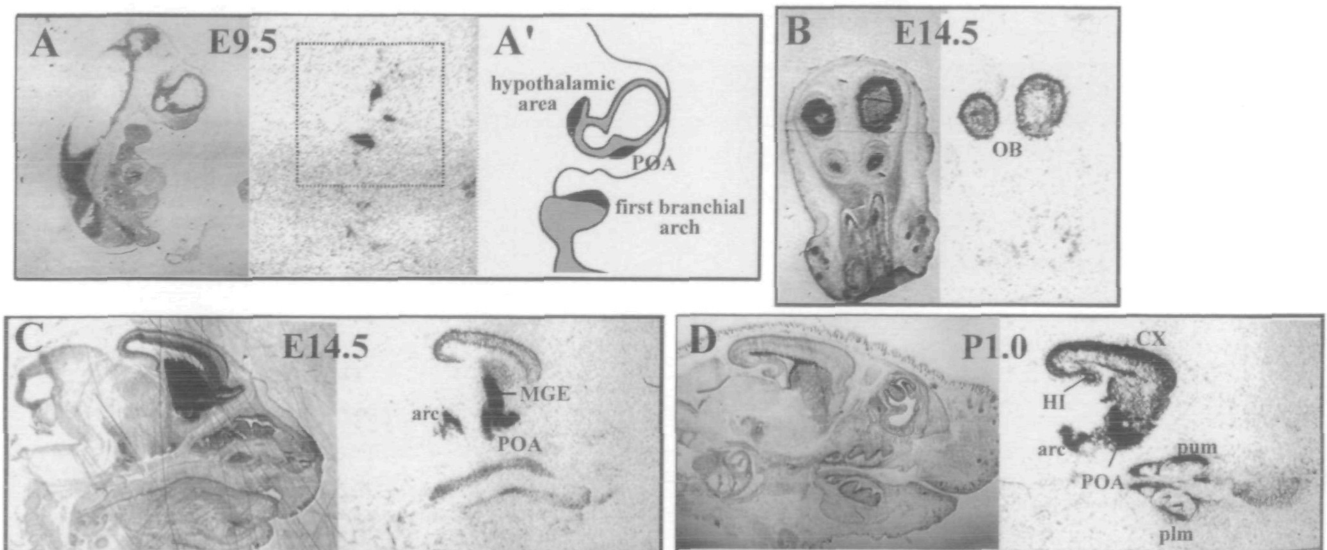

Fig. 6. **In situ hybridization analysis of Lhx6.1 expression in serial sections from E9.5, E14.5, and P1.0 mice.** Sagital (A, C, D) and coronal (B) sections from mice at E9.5 (A), E14.5 (B and C), and P1.0 (D) were hybridized with Lhx6.1 antisense probe (right panels) and then counterstained with crecyl violet (left panels). (A′) shows a schematic representation of the boxed region in (A). POA: preoptic area; arc: arcuate nucleus; OB: olfactory bulb; MGE: medial ganglionic eminence; CX: cortex; pum: primordium of upper molar tooth; plm: primordium of lower molar tooth; HI: hippocampus.

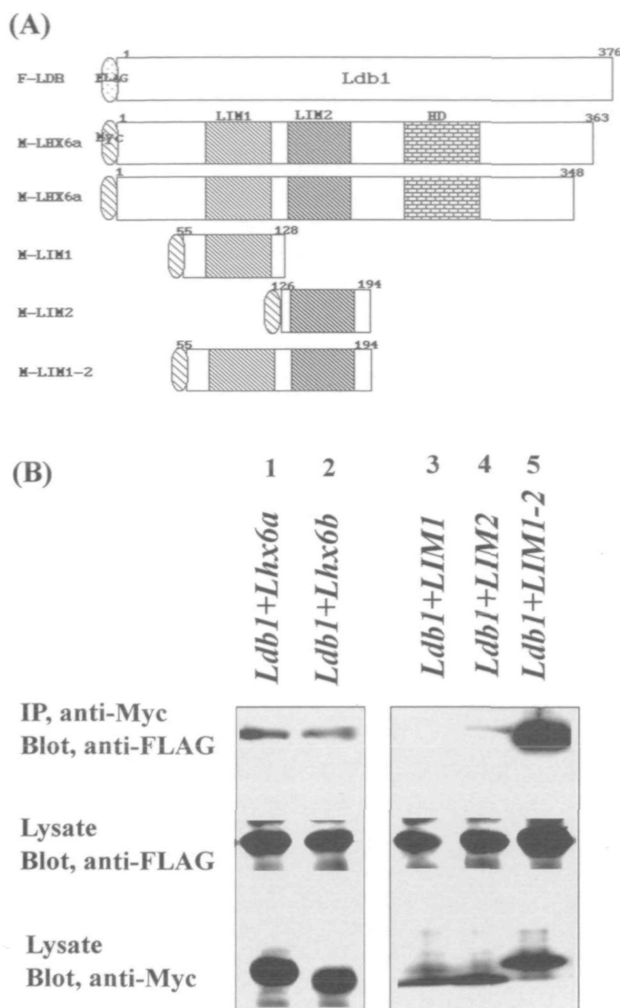

Fig. 7. **Interaction of Lhx6.1 with Ldb1.** COS7 cells were transiently co-transfected with expression vectors encoding FLAG-Ldb1 and Myc-tagged Lhx6.1 constructs as indicated in (A). Cell extracts were subjected to immunoprecipitation with anti-Myc antibody. Precipitates were resolved by SDS-PAGE, transferred to a PVDF membrane, and subjected to immunoblot analysis with anti-FLAG antibody (upper panel in B). Cell extracts were also subjected directly to immunoblot analysis by anti-FLAG or anti-Myc antibody (middle and bottom panels, respectively).

was localized to the primordial upper and lower molar teeth of P1.0 mouse (Fig. 6D).

Specific Association of Lhx6.1 to Ldb1—The similar temporal expression patterns of Lhx6.1 and Ldb1 as shown in Fig. 3, B and C, led us to consider the possibility that these two proteins may function in coordination in the developing CNS. To determine whether Lhx6.1 is capable of interacting with Ldb1 as in the case of other several LHX proteins, an *in vivo* association assay was performed. In this experiment, FLAG-tagged Ldb1 (F-LDB) and Myc-tagged Lhx6.1a or Lhx6.1b (M-LHX6a or M-LHX6b) expression vectors were constructed (Fig. 7A) and used to investigate whether Ldb1 and Lhx6.1 are complexed. F-LDB was co-transfected with M-LHX6a or M-LHX6b into COS7 cells, and cell lysates were prepared and subjected to immunoprecipitation and immunoblotting. Myc-tagged Lhx6.1 proteins were immunoprecipitated with anti-Myc antibody, and detected with anti-FLAG antibody. As shown in Fig. 7B (lanes 1 and 2), FLAG-tagged Ldb1 protein was detected in the immunoprecipitates of Myc-tagged Lhx6.1a and Lhx6.1b, clearly indicating that both Lhx6.1 proteins interact with Ldb1. In addition, the amino acid differences between Lhx6.1a and Lhx6.1b in the C-terminal sequence do not affect their ability to bind to Ldb1. Next we tested whether the two tandem LIM domains are both required for the interaction with Ldb1. For this purpose, we made several Myc-tagged LIM domain constructs of the Lhx6.1 protein containing either a single LIM domain (M-LIM1 or M-LIM2) or tandem LIM domains (M-LIM1-2) (Fig. 7A), and used them to test the *in vivo* binding efficiency to the Ldb1 protein. As shown in Fig. 7 (lanes 3 and 4), constructs with a single LIM domain alone (M-LIM1, M-LIM2) bound only slightly to Ldb1, whereas constructs with intact tandem LIM domains (M-LIM1-2) bound to Ldb1 with much higher affinity (lane 5). These results are in agreement with those of Breen *et al.* and Jurata *et al.*, in which tandem LIM domains of Xlim-1 or Lmx1 were suggested to be required for optimal binding to Ldb1 (*18, 16*).

DISCUSSION

A LIM-homeodomain (LHX) transcription factor, Lhx6.1, was isolated by a subtraction strategy to clone genes expressed predominantly in the developing CNS. Lhx6.1 is closely related to Lhx6, which was recently reported by Grigoriou *et al.* (*20*). The amino acid sequences of Lhx6.1 and Lhx6 appear totally different in the N- and C-termini. A comparison of the nucleotide sequences of both genes shows them to be almost identical, with some nucleotide insertions and deletions observed in the 5′- and 3′-terminal regions. This suggests that the amino acid sequence differences in the N- and C-terminal regions are due to frame shifts caused by nucleotide insertions and deletions. Although it is not clear why such nucleotide sequence differences were generated in the two genes, the sequences of Lhx6.1 obtained from mouse and human are much more closely related to each other than to the Lhx6 sequence, suggesting that the Lhx6.1 sequence reported here may be correct.

Members of the murine LHX gene family have been shown to be expressed in a mutually-exclusive manner in most parts of the embryonic brain, and it is presumed that their combinatorial expressions in the CNS may define the neuronal cell fate or regional specification. The newly isolated LHX gene, Lhx6.1, is expressed at high levels in the developing brain, with its expression restricted to the MGE and the branchial arch. The expression of Lhx6.1 in these two regions is in agreement with the expression of the Lhx6 gene as reported by Grigoriou *et al.* (*20*). In addition, we observed Lhx6.1 transcripts in two other regions of the basal forebrain, the olfactory bulb and the arcuate nucleus. Although L3, an LHX gene member, is also reported to be expressed in the MGE (*23*), the Lhx6.1 expression pattern, which is restricted to three regions in the developing forebrain, is unique among other LHX gene members. In the early stages of mouse development (E9.5), the Lhx6.1 mRNA transcript appears in three distinct regions, the hypothalamic region, the preoptic area, and the first branchial arch. It is interesting to note that these regions

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possess the potential to differentiate into the arcuate nucleus, MGE, and molar tooth. Therefore, Lhx6.1 might play a role in determining cell fate in restricted domains from the earliest stages of formation.

The regions expressing Lhx6.1 overlap with those expressing Ldb1, which are widely distributed in the embryonic brain (*17*). Furthermore, the results of our northern blot experiments showing that the temporal expression of the Lhx6.1 gene during development is roughly the same as that of the Ldb1 gene suggests a close relationship between Lhx6.1 and Ldb1.

Our present study shows that Lhx6.1 binds the Ldb1 protein. Interestingly, a partial Lhx6.1 consisting of only the tandem LIM domains interacts with Ldb1 with much higher affinity than the complete Lhx6.1 including the homeodomain. A similar result was obtained in our preliminary experiments: A mutant form of Lhx6.1 in which half of the homeodomain was disrupted showed higher binding affinity to Ldb1 than intact Lhx6.1 (data not shown). These results support the idea that the LIM domains interact with the intramolecular homeodomain either directly or indirectly, thereby inhibiting the binding of the homeodomain to DNA. It is postulated that a protein capable of interacting with the LIM domains could allow the homeodomain to be free to bind to DNA and thus activate the transcriptional activity of LHX proteins (*24*). Most members of the LHX protein family are thought to interact with Ldb1. Our results showing the direct binding of Ldb1 to wild type Lhx6.1 through tandem LIM domains support the possibility that Lhx6.1 is also functionally regulated by Ldb1. Lhx6.1 is postulated to become functionally active upon interaction with Ldb1. In this way, Lhx6.1 may play a role in neuronal cell type determination in a manner similar to that suggested for other members of the LHX gene family.

Lhx6.1 has two differentially spliced forms, Lhx6.1a and Lhx6.1b, which have different C-terminal amino acid sequences. As demonstrated in this study, the efficiency of the direct interaction of Ldb1 with Lhx6.1a or Lhx6.1b is almost the same. This suggests that the C-terminal regions of Lhx6.1 and Lhx6.1b do not affect their ability to interact with Ldb1. A recent study demonstrated that the C-terminal domain of Lmx1.1 is required for general transcriptional activation (*25*). Therefore, the two isoforms of the Lhx6.1 gene might possess different transcription activation properties although they interact equally with Ldb1. Taken together with our RT-PCR observation that the expression patterns of the Lhx6.1a and Lhx6.1b genes are different during brain development, we can hypothesize that the transcription of Lhx6.1 target genes might be modulated not only by the interaction with Ldb1, but also by the difference in the ratio of Lhx6.1a and Lhx6.1b expression at each stage. Identification of the target genes of Lhx6.1, analysis of their promoter regions, and identification of Lhx6.1 binding DNA sequences will provide clues to understand the transcriptional regulatory mechanism of Lhx6.1.

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