

Lhx9: A Novel LIM-Homeodomain Gene Expressed in the Developing Forebrain

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A novel LIM-homeodomain gene, *Lhx9*, was isolated by degenerate RT-PCR followed by mouse embryonic library screening. *Lhx9* cDNA encodes a protein that is most closely related to *Drosophila* apterous and rodent *Lhx2* proteins. The *Lhx9* spatiotemporal pattern of expression during embryogenesis was similar but distinct from *Lhx2*. Highest expression levels were found in the diencephalon, telencephalic vesicles, and dorsal mesencephalon. Domains of expression respected the proposed neuromeric boundaries (Puelles and Rubenstein, 1993). *Lhx9* was also expressed in the spinal cord, forelimb and hindlimb mesenchyme, and urogenital system. Although *Lhx9* expression was sustained in diencephalon and mesencephalon from embryonic day 10.5 (E10.5) to postnatal stages, it was transient in the future cerebral cortex, where it was turned off

between E14.5 and E16.5. *Lhx9* expression was highest if not exclusively located (depending on the region of interest) in the intermediate and mantle zones, as opposed to the mitotic ventricular zone. *Lhx9* protein was tested for interaction with the recently discovered cofactors of LIM-homeodomain proteins and was found to interact strongly both with CLIM1 and CLIM2. The expression pattern and structural characteristics of *Lhx9* suggest that it encodes a transcription factor that might be involved in the control of cell differentiation of several neural cell types. Furthermore, *Lhx9* protein could act in a combinatorial manner with other LIM-homeodomain factors expressed in overlapping pattern.

Key words: LIM-homeodomain; *Lhx9*; *Lhx2*; forebrain; development; neuromeres

A major issue in developmental neurobiology is the understanding of forebrain development and patterning. During embryogenesis, the anterior neural tube generates complex and highly organized structures, such as the cerebral cortex, the basal ganglia, and the thalamus. In adults, these structures are interconnected, almost always topographically.

Homeodomain genes play decisive roles in the establishment of cerebral structures and/or the generation of cell types. Among them, LIM-homeodomain (LIM-hd) genes encode developmentally expressed transcription factors that contain a homeodomain and two cysteine-rich LIM domains involved in protein-protein interactions (Sanchez-Garcia and Rabbitts, 1994; Dawid et al., 1995). In the current view, the LIM domains inhibit DNA binding by the homeodomain. After fixation of cofactors, transcription is activated, and synergism with other transcription factors is promoted. Indeed, cofactors for LIM-hd proteins have been isolated: NLI/Ldb1/CLIM2 and CLIM1 interact with LIM domains of LIM homeoproteins to potentiate transactivation of downstream genes (Agulnick et al., 1996; Jurata et al., 1996; Bach et al., 1997). These cofactors probably confer time and space specificity to the regulatory action of LIM-hd proteins.

LIM-hd genes were first implicated in cell fate determination in invertebrates; *Caenorhabditis elegans mec-3* and *lin-11* (Way and

Chalfie, 1988; Freyd et al., 1990) and *Drosophila apterous* (Lundgren et al., 1995; Thor and Thomas, 1997) determine cell types and axonal pathfinding. In vertebrates, the spinal cord motor neurons give an example for similar roles of LIM-hd genes: motor neurons express a set of four LIM-hd genes, in a combinatorial manner that is correlated to the motor neuron position in the spinal cord and to the type of peripheral innervated target (Tsuchida et al., 1994). Thus, a LIM-hd combinatorial code might define pathfinding phenotypes. If similar combinations of LIM-hd genes were expressed in the forebrain, they could participate in the establishment of its highly organized circuits. Moreover, *Drosophila islet* governs aminergic phenotypes (Thor and Thomas, 1997). Forebrain LIM-hd genes could govern neurotransmitter expression in neurons as well.

Homeodomain genes expression patterns in the developing CNS have led to the theory of the prosomeric forebrain (Puelles and Rubenstein, 1993; Rubenstein et al., 1994; Puelles, 1995). In this model, regions and boundaries of expression of developmental factors are seen as landmarks for the determination of cerebral areas according to longitudinal and transverse subdivisions that form segmented structures. LIM-hd genes integrate this model well. *Lhx1/lim-1* (Barnes et al., 1994), *Lhx2/LH-2* (Xu et al., 1993), *Lhx3/pLIM* (Seidah et al., 1994; Bach et al., 1995), *L3/Lhx8* (Matsumoto et al., 1996), *Lhx4/Gsh4* (Li et al., 1994), *Lhx5* (Sheng et al., 1997), *Lhx6*, and *Lhx7* (Grigoriou et al., 1998) are all expressed in the developing rodent CNS, and their expression patterns respect neuromeric boundaries (present paper). The role of LIM-hd genes in cellular specification, combined with their apparent function in the coding of positional information, makes them important factors to be studied during brain regionalization and wiring.

In a search to isolate more LIM-hd involved in forebrain

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development, we hypothesized the existence of a subfamily of *Lhx2*-related LIM-hd genes. An RT-PCR cloning strategy allowed the isolation of mouse *Lhx9*, which is similar to but distinct from *Lhx2* in sequence and expression pattern, the two genes thereby forming a new subfamily of forebrain LIM-hd genes.

MATERIALS AND METHODS

RT-PCR cloning. Total RNA from embryonic day 15 (E15) or E18 forebrain were reverse-transcribed to cDNA with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Mannheim, Germany) and used as templates for PCR reactions (Qiagen, Hilden, Germany) using the following degenerate oligonucleotide primers: 51, ATGCGIACITCITTYAARCAVCAYCARCT; 52, ATGAGRACITCITTYAARCAVCAYCARCT; 31, MAYTTIGCIGCIGCRTTYTGRAACCA; and 32, MAYTTIGCYCTIGCRTTYTGRAACCA, where I is an inosine residue. 5' (51 and 52) and 3' (31 and 32) primers were designed to isolate genes of the *Lhx2* subfamily and were chosen to amplify a stretch of DNA in the homeodomain. Any combination of 3' and 5' primers led to the amplification of a single band of 160 bp as expected. After cloning of this 160 bp fragment into pGEM-T (Promega, Madison, WI), sequencing of 80 independent clones revealed the presence of three different fragments. One was mouse *Lhx2*, and the two others were unknown. We concentrated on one that we named *Lhx9*. The 160 bp DNA fragment was used to screen a λ gt11 E14.5 mouse head cDNA library under high stringency. The *Lhx9* cDNA was isolated using standard molecular biology techniques and sequenced on both strands with Amersham (Les Ulis, France) thermosequencing. The 1.016 kb PCR product of *Lhx9* cDNA was subcloned into pGEM-T (Promega) for subsequent experiments.

Tissue preparation. Timed pregnant C57/Black6 mice were anesthetized with 0.1 ml/100 gm body weight pentobarbital. Embryos were harvested, fixed in 0.1 M 4-morpholinepropanesulfonic acid, pH 7.4, 2 mM EGTA, 1 mM MgSO₄, and 3.7% formaldehyde at room temperature, cryoprotected in 30% sucrose, and frozen by immersion in isopentane on dry ice. Noon on the day after the night of mating was considered as E0.5. Postnatal day 1 (P1)–P7 brains were processed identically, except that sucrose was 20%. Whole embryos or dissected brains were cryostat-sectioned at 20 μ m, and sections were stored at -80°C until use.

In situ hybridization and image processing. The pGEM-*Lhx9* plasmid was linearized with *NdeI* or *NcoI* and used as template for RNA synthesis with T7 or SP6 polymerase in the presence of [³⁵S]UTP (10 mCi/mmol; ICN Biochemicals, Costa Mesa, CA) for antisense and sense control probes, respectively. The probe thus included the 5' noncoding region, the two LIM domains, the linker region, and the beginning of the homeodomain. The Bluescript-*Lhx2* plasmid including the 1.1 kb *Lhx2* insert was linearized with *EcoRI*, and T3 polymerase was used for antisense probe synthesis as described (Bach et al., 1997). The Bluescript-*En2* 3' untranslated region plasmid was linearized with *ClaI*, and T7 was used to generate an 800 bp *En-2* probe. Restriction enzymes were purchased from Appligene (Heidelberg, Germany) or Promega, and RNA polymerases were from Stratagene (La Jolla, CA). Briefly, slides were acetylated, dehydrated progressively, and hybridized 16 hr at 60°C in the presence of 5×10^5 – 10^6 cpm of probe/slide. They were then washed under high stringency, treated 1 hr with RNase A (20 μ g/ml), and dehydrated. Sections were exposed 2–3 d to Eastman Kodak (Rochester, NY) Biomax MR film for autoradiography and then dipped in Kodak NTB-2 emulsion. Emulsions were developed after 10–12 d of exposure, and sections were counterstained with methylene blue. Photographs were taken on a Leica (Nussloch, Germany) DMBL microscope, scanned on a Canon (Tokyo, Japan) 2700 scanner, and mounted for figures with Adobe (Mountain View, CA) Photoshop. Images were corrected for color balance, contrast, brightness, or cropping, but no other corrections were made.

In vitro protein–protein interaction assays. PCR products of full-length CLIM1a and CLIM2 were ligated in frame into the *XhoI*–*XbaI* sites of the bacterial expression vector pGEX-KG to yield a glutathione *S*-transferase (GST) fusion protein. The PCR product of the 1.016 kb *Lhx9* cDNA was subcloned into the pGEM-T vector (Promega), and [³⁵S]methionine-labeled *Lhx9* protein was produced using the Promega *in vitro* transcription–translation kit, SP6 RNA polymerase, and [³⁵S]methionine. The *in vitro* protein–protein interaction assays were performed as described previously (Bach et al., 1995).

RESULTS

Cloning of *Lhx9*, a novel LIM homeodomain gene

The RT-PCR strategy for cloning new LIM-hd genes expressed in the forebrain was based on the observation that the *Lhx2* homeodomain was slightly different from other LIM-hd genes expressed in more posterior parts of the brain. An SFKHH amino acid sequence was found in the N terminus part of its homeodomain, instead of the often encountered TITAK motif. We hypothesized the existence of other genes of the same subfamily that could be specific for the forebrain. Degenerate PCR primers were thus designed to amplify a theoretical 160 bp DNA fragment spanning almost the entire homeodomain (with a 5' primer at the level of the SFKHH motif and a 3' primer at the level of the highly conserved VWFQN motif close to the C terminus of the homeodomain). Sequencing of the 160 bp RT-PCR fragment revealed a new sequence (19 nucleotides of 102 were different from *Lhx2* but were conservative at the amino acid level). This PCR fragment was used to screen an E14.5 mouse head λ gt11 cDNA library and allowed the isolation of *Lhx9*. The \sim 1 kb cDNA contained 5' noncoding sequences and, in the open reading frame, two LIM domains and a homeodomain (Fig. 1A, shading). The C-terminal part and the stop codon were not included in this cDNA. Additional screens could not identify the 3' end missing sequences. However, the 300 amino acids encoded by the cDNA are largely sufficient to allow for *Lhx9* classification and riboprobe synthesis. There are two in-frame methionine residues (Fig. 1A, boxed) located 10 amino acids apart at the level of the translation initiation site. Based on similarity with *Lhx2*, we postulate that the second methionine will be used for translation start. *Lhx9* shares 69% identity with rat *Lhx2* at the amino acid level and 68% at the nucleotide level. The amino acid identity rises to 95% in the LIM domains (Fig. 1B) and to 100% in the homeodomain. The main differences between the two proteins are located between the second LIM domain and the homeodomain. The linker fragment between LIM2 and the homeobox is shorter in *Lhx9* [79 amino acids (aa) in *Lhx9* and 96 aa in *Lhx2*] and is more closely related in sequence and in length to chick *Lhx2B* [also 79 aa long (G. Tremml and T. M. Jessell, unpublished data; GenBank accession number L35566)] (Nohno et al., 1997). A basic sequence (RPRKRK; Fig. 1A, underlined) that might serve as a nuclear translocation signal is conserved in the middle of the linker region. The sequence similarities between *Lhx9* and *Lhx2* strongly support the notion that they are two closely related family members (Fig. 1B). Indeed, a comparison with other LIM-hd genes in GenBank showed that *Lhx9* belongs to a growing subfamily including vertebrate *Lhx2*, *Drosophila apterous*, and *C. elegans ttx-3* (Hobert et al., 1997). Moreover, the very high (overall 94%) identity between mouse *Lhx9* and chick *Lhx2* suggests that chick *Lhx2* could rather be the avian homolog of rodent *Lhx9* (Fig. 1B).

Expression of *Lhx9* during embryogenesis of the nervous system

Whole-mount *in situ* hybridization showed that the onset of *Lhx9* expression in the nervous system was at approximately E10.5 (data not shown). Thus, from E11.5 onward, the expression pattern was studied by *in situ* hybridization on sections (Figs. 2, 3; Table 1). From E11.5 to E14.5, *Lhx9* expression was relatively widespread throughout the CNS alar neuroepithelium, with an increase between E11.5 and E12.5 (Fig. 2, compare A,B with C,D). Expression remained high in the following stages.

A

CCACCCCTGCCACCTCCTCTCCAAAAACCAAAATTTTGGGAAGAACAGCTGACACCAAGAAGACATCAAGCACAACCCGATTTCACTGCGCGATCCACCCCTCTTGCCGAAAGA 116

ATG	CTG	AAC	GCC	ACC	ACT	CTA	GAG	GCA	GCC	ATG	CTG	TTC	CAC	GGG	ATC	TCC	GGA	GCC	CAC	ATC	CAA	GGT	ATC	ATG	GAG	GAG	ATG	GAG	CGC	206	
M	L	N	G	T	T	L	E	A	A	M	L	F	H	G	I	S	G	G	H	I	Q	G	I	M	E	E	M	E	R	30	
AGA	TCC	AAG	ACT	GAG	GCC	CGT	CTG	ACC	AAA	GGC	ACT	CAG	CTC	AAC	GGC	CGC	GAC	GCG	GGT	ATG	CCC	CCT	CTC	AGC	CCG	GAG	AAG	CCT	GCT	296	
R	S	K	T	E	A	R	L	T	K	G	T	Q	L	N	G	R	D	A	G	M	P	P	L	S	P	E	K	P	A	60	
CTG	TGC	GCC	GGC	TGC	GGG	GGC	AAG	ATC	TCC	GAC	AGG	TAC	TAT	CTG	CTG	GCC	GTA	GAC	AAA	CAG	TGG	CAT	CTT	AGG	TGC	CTG	AAG	TGC	TGT	386	
L	C	A	G	C	G	G	K	I	S	D	R	Y	Y	L	L	A	V	D	K	Q	W	H	L	R	C	L	K	C	C	90	
<i>LIM 1</i>																															
GAA	TGT	AAG	CTG	GCT	CTG	GAA	TCT	GAG	CTC	ACC	TGC	TTT	GCC	AAG	GAC	GGT	AGC	ATT	TAC	TGC	AAG	GAG	GAT	TAT	TAC	AGA	AGG	TTC	TCT	476	
E	C	K	L	A	L	E	S	E	L	T	C	F	A	K	D	G	S	I	Y	C	K	E	D	Y	Y	R	R	F	S	120	
GTG	CAG	AGA	TGT	GCC	CGC	TGC	CAC	CTT	GGC	ATT	TCC	GCC	TCT	GAG	ATG	GTC	ATG	CGC	GCC	CGA	GAC	TCT	GTC	TAC	CAT	CTG	AGC	TGC	TTC	566	
V	Q	R	C	A	R	C	H	L	G	I	S	A	S	E	M	V	M	R	A	R	D	S	V	Y	H	L	S	C	F	150	
<i>LIM 2</i>																															
ACT	TGT	TCC	ACT	TGC	AAC	AAG	ACC	TTG	ACC	ACG	GGC	GAC	CAT	TTC	GGG	ATG	AAG	GAC	AGC	CTG	GTG	TAC	TGC	CGC	GCA	CAC	TTC	GAG	ACC	656	
T	C	S	T	C	N	K	T	L	T	T	G	D	H	F	G	M	K	D	S	L	V	Y	C	R	A	H	F	E	T	180	
CTC	TTG	CAA	GGG	GAA	TAT	CCA	CCT	CAG	CTG	AGC	TAC	ACG	GAG	CTG	GCG	GCC	AAG	AGC	GGC	GGC	TTG	GCT	TTG	CCT	TAC	TTC	AAT	GGC	ACT	746	
L	L	Q	G	E	Y	P	P	Q	L	S	Y	T	E	L	A	A	K	S	G	G	L	A	L	P	Y	F	N	G	T	210	
GGC	ACC	GTG	CAG	AAG	GGG	CGG	CCC	CGG	AAG	CGG	AAG	AGC	CCA	GCT	CTG	GGA	GTG	GAC	ATC	GTG	AAT	TAC	AAC	TCA	GGT	TGT	AAT	GAG	AAC	836	
G	T	V	Q	K	G	R	P	R	K	R	K	S	P	A	L	G	V	D	I	V	N	Y	N	S	G	C	N	E	N	240	
GAG	GCA	GAC	CAC	TTG	GAC	CGG	GAC	CAG	CAG	CCT	TAT	CCA	CCT	TCA	CAG	AAG	ACC	AAA	CGG	ATG	CGA	ACT	TCT	TTC	AAG	CAT	CAC	CAG	CTC	926	
E	A	D	H	L	D	R	D	Q	Q	P	Y	P	P	S	Q	K	T	K	R	M	R	T	S	F	K	H	H	Q	L	270	
<i>hd</i>																															
CGG	ACC	ATG	AAA	TCC	TAC	TTT	GCT	ATC	AAC	CAT	AAC	CCA	GAT	GCC	AAG	GAC	CTC	AAA	CAG	CTT	GCT	CAA	AAA	ACA	GGC	CTG	ACC	AAA	AGA	1016	
R	T	M	K	S	Y	F	A	I	N	H	N	P	D	A	K	D	L	K	Q	L	A	Q	K	T	G	L	T	K	R	300	

B

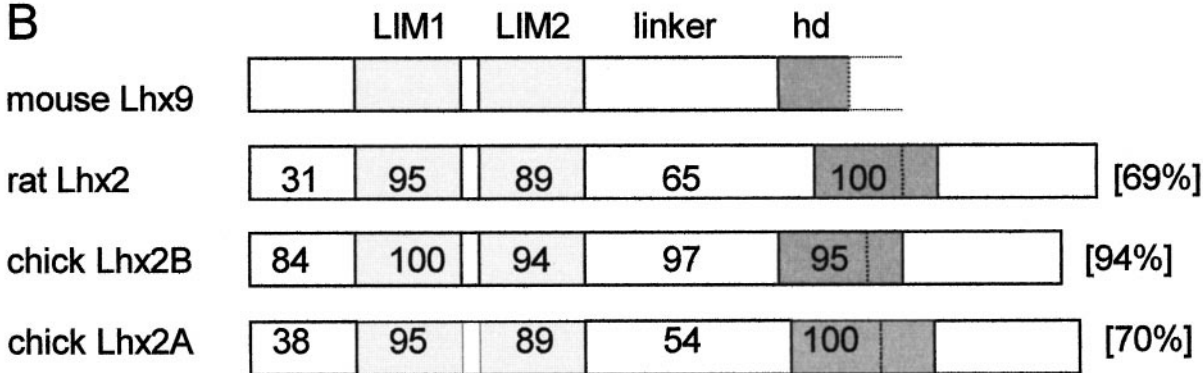


Figure 1. Sequence of murine *Lhx9*. *A*, Nucleotide and deduced amino acid sequence of *Lhx9*. Amino acids are indicated by single-letter code. The two in-frame ATG start codons are boxed. The sequence between the two possible start codons is in italics solely to indicate that based on homology with *Lhx2*, the second one is more likely to be used. LIM1, LIM2, and the hd are indicated by shading. The putative nuclearization signal is underlined. *B*, Comparison of murine *Lhx9* with rat and chick *Lhx2*. The percentage of identity in each domain between gene products is indicated. GenBank/EMBL accession numbers: L06804 (rat *Lhx2*), L35566 (chick *Lhx2B*), and AB005882 (chick *Lhx2A*).

In the telencephalic vesicles *Lhx9* mRNA was expressed in the future cerebral cortex, the hippocampus, the claustrum primordium (where it could correspond to a stream of migratory cells from the striatum to the cortex; see De Carlos et al., 1996), and the caudal ganglionic eminence (amygdaloid complex) (Figs. 2A–C, 3A–C). It was also expressed in the olfactory bulb primordium (Figs. 2C, 3A). Interestingly, levels of expression in the telencephalic walls depended on the region examined: it was relatively weak in the parietal neocortex, high in the frontal neocortex (data not shown), and strongest in the archicortex (hippocampal field; Fig. 3B,C).

The diencephalon showed the highest expression levels (Figs. 2C,D, 3). Moreover, in this region, expression was observed in longitudinal and transverse bands, with sharp boundaries that respect the neuromeric regionalization proposed by Puelles and Rubenstein (1993) (Fig. 3). Indeed, from caudal to rostral (Fig. 3A), *Lhx9* was expressed in the pretectum (p1 prosomere) and in

the dorsal thalamus (except for a thin ventral band close to the alar–basal boundary), the epithalamus, and the epiphysis in prosomere p2. Expression stopped abruptly at the zona limitans intrathalamica, showing no expression in the ventral thalamus (p3 prosomere; Fig. 3C–F). The supraoptic–paraventricular area and the eminentia thalami again formed a band of strong expression in the p4 prosomere that abuts on a negative region around the optic stalk (prosomeres p5 and p6; Fig. 3A,D,E). A narrow band including the retrochiasmatic area and the tuberal hypothalamus expressed *Lhx9* and represented the only domains of expression in the basal plate (Fig. 3A,F; also see Fig. 8A). Interestingly, the boundaries of expression of *Lhx9* at the diencephalic–mesencephalic junction delineated perfectly the borders of the trajectory used by pioneering tracts of the posterior and postoptic commissure (Fig. 2H,I).

Posteriorly, *Lhx9* was expressed throughout the tectum in the mesencephalon (Figs. 2A–D, 3A), in the walls of the hindbrain

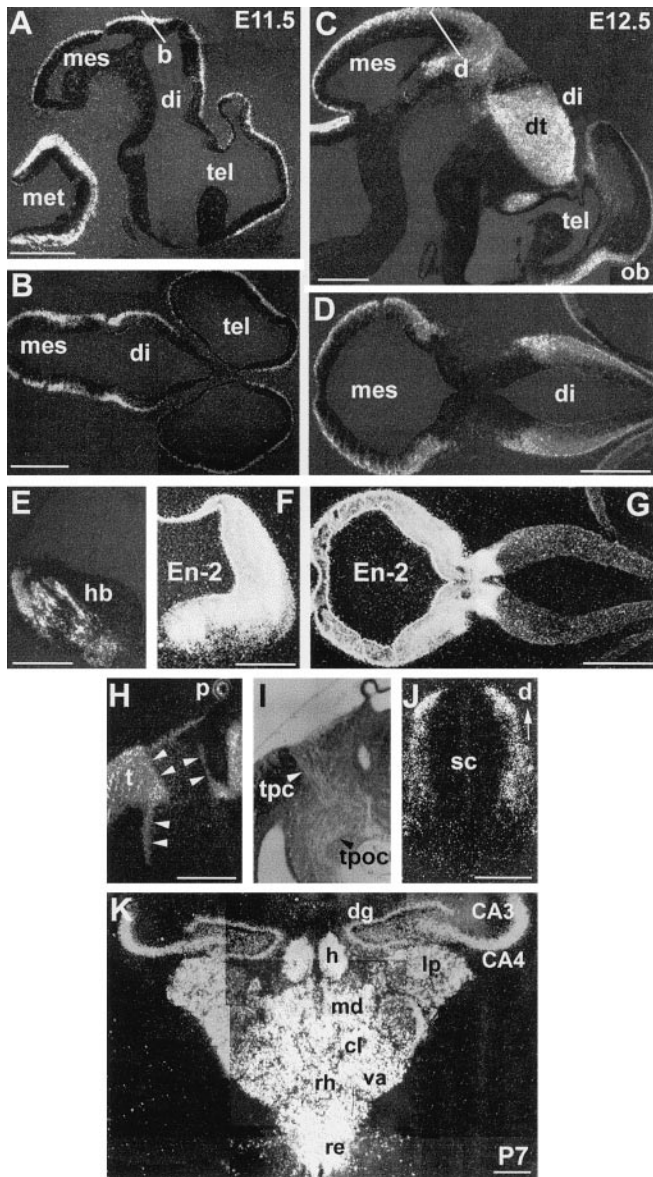


Figure 2. *Lhx9* expression on brain sections from E11.5 (*A, B*), E12.5 (*C–E, J*), E13.5 (*H, I*), and P7 (*K*) embryos and comparison with *En-2* (*F, G*) on dark-field illumination photomicrographs. In this and subsequent figures, pictures are oriented so that anterior is *right* and dorsal is at the *top*. *A, C*, Parasagittal sections through the entire brain at indicated stages. *B, D*, Horizontal sections (level and orientation indicated by *white bars* in *A, C*, respectively). *G*, *En-2* expression on a section adjacent to that shown in *D*. *E, F*, Adjacent coronal hemisections showing the comparison of *Lhx9* (*E*) and *En-2* (*F*) expression in the anterior hindbrain. *H, I*, Bright-field (*I*) and dark-field (*H*) views of the same area showing the correlation between the position of the tpc and the tract of the postoptic commissure (*tpoc*) and *Lhx9* sharp boundaries of expression (*arrowheads*). *J*, Coronal section through the spinal cord. *K*, Coronal section at the level of the hippocampus and thalamus at P7, showing expression in CA3, CA4, and the dentate gyrus (*dg*) and the delineation of various thalamic nuclei by *Lhx9* hybridization signal. *cl*, Central lateral; *hr*, habenula; *lp*, lateral posterior; *md*, mediodorsal; *va*, ventral anterior; *re*, reuniens; *rh*, rhomboid. See Table 1 for anatomical abbreviations. Scale bars: *A–I, K*, 100 μ m; *J*, 50 μ m.

(Fig. 2*A,E*), and in nuclei in the ventral midbrain and hindbrain (Fig. 4*I*). The comparison with *En-2*, a major homeobox gene defining the mesencephalic–metencephalic region, showed that the expression of the two genes was quite different, neither totally

Table 1. List of anatomical abbreviations

acx	Archicortex (hippocampus)
aep	Anterior entopeduncular area
ah	Anterior hypothalamus
cb	Cerebellum
cge	Caudal ganglionic eminence
ci, cs	Colliculus, inferior, superior
cl	Clastrum
cp	Choroid plexus
db	Diagonal band
di	Diencephalon
dt	Dorsal thalamus
e	Eye
emt	Eminentia thalami
et	Epithalamus
h	Habenula
hb	Hindbrain
hcc	Hypothalamic cell cord
is	Isthmus of the mesencephalon
lge	Lateral ganglionic eminence
lv	Lateral ventricle
ma	Mamillary area
mes	Mesencephalon
met	Metencephalon
mge	Medial ganglionic eminence
ncx	Neocortex
ob	Olfactory bulb
os	Optic stalk
p	Pineal gland
p1–p6	Prosomeres 1–6
pep	Posterior entopeduncular area
poa	Anterior preoptic area
pop	Posterior preoptic area
pt	Preteetum
rch	Retrochiasmatic area
rm	Retromammillary area
rp	Rathke's pouch (pituitary)
sch	Suprachiasmatic area
se	Septum
spv	Supraoptic paraventricular area
t	Tectum
tel	Telencephalon
tpc	Tract of the posterior commissure
tu	Tuberal hypothalamus
vt	Ventral thalamus
zli	Zona limitans intrathalamica

overlapping nor strictly complementary (Fig. 2, compare *D* with *G, E* with *F*).

In the spinal cord, *Lhx9* was expressed as a gradient in the dorsal part of the neuroepithelium (Fig. 2*J*, corresponding to the region where commissural and association neurons differentiate) but not in motor neurons.

Between E14.5 and E16.5, a major change in *Lhx9* expression occurred in the neocortical neuroepithelium, because it was turned off (Fig. 5; see below). However, expression persisted in the archicortex (in the dentate gyrus, CA3 and CA4) later on, as well as in the diencephalon, midbrain, and hindbrain. Indeed, at early postnatal stages, (P1–P7), expression was particularly abun-

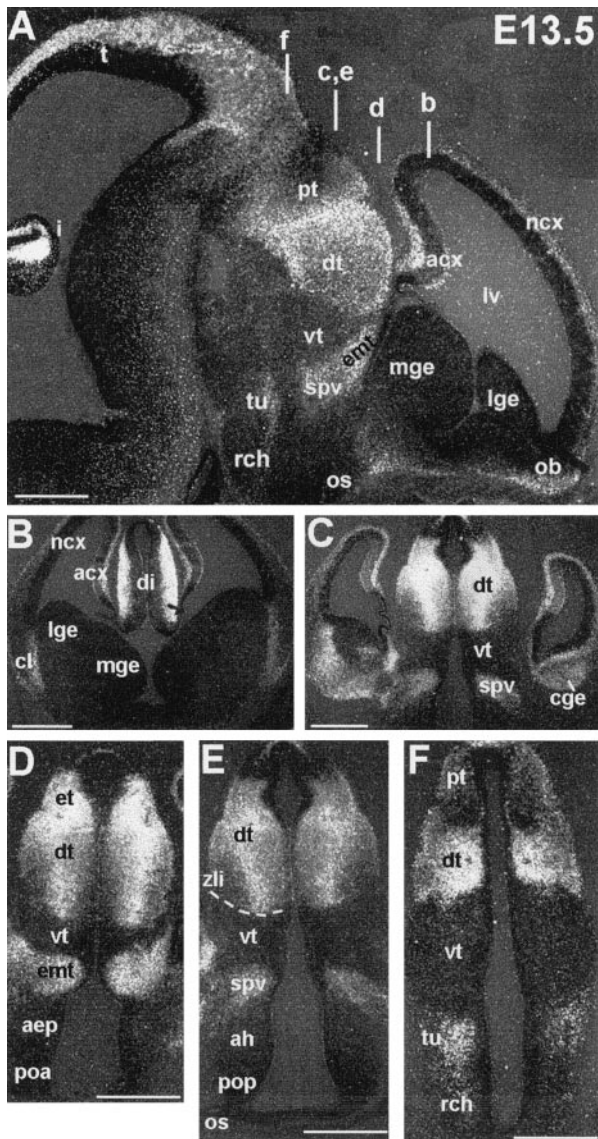


Figure 3. *Lhx9* expression at E13.5. *A*, Sagittal section through the entire brain. *B–F*, coronal sections through the telencephalon and diencephalon (plan of section indicated by white bars in *A*) showing *Lhx9* transverse boundaries in the rostrocaudal extension. The dotted line indicates the zli. Scale bars, 100 μ m. See Table 1 for anatomical abbreviations.

dant in the dorsal thalamus where it allowed the delineation of various nuclei (Fig. 2*K*). *Lhx9* marked a sharp boundary between the very-high-expressing inferior colliculus and the high-expressing superior colliculus (Fig. 4*L*). A number of mesencephalic and pons nuclei, including deep cerebellar nuclei, still expressed *Lhx9* at these postnatal stages (data not shown).

In summary, *Lhx9* expression was relatively widespread, mostly in the alar region of the neuroepithelium. In this respect, it is noteworthy that expression was absent from the large region of the ganglionic eminences in the ventral telencephalon (corresponding to the striatal–pallidal primordium).

***Lhx9* and *Lhx2* are expressed in overlapping but distinct patterns**

Because *Lhx9* and *Lhx2* are closely related family members, we next compared directly the expression of the two genes.

Concerning regional expression, the two genes were expressed

mainly in overlapping patterns, with few noticeable exceptions: *Lhx2* was expressed in the ganglionic eminences, the eyes, the optic stalk, and the neuromeres surrounding the optic stalk, whereas *Lhx9* was not (compare Figs. 4*A,F*, 3*B*, 4*B*). In the prosencephalic region, the two mRNAs were expressed in complementary patterns, showing bands of expression with sharp boundaries, separated by expression-negative zones. More precisely, *Lhx2* was not expressed in the eminentia thalami–supraoptic paraventricular areas, whereas *Lhx9* was, but the *Lhx9*-negative zone at the optic stalk, preoptic area, and anterior hypothalamus was *Lhx2* positive (compare Figs. 4*A,F*, 4*D–G*, 3*E*, 4*C*). Expression of the two genes was perfectly overlapping in the dorsal thalamus and was absent for both genes in the ventral thalamus (Fig. 4, compare *A–F*). Thus, the addition of the two expression domains covered almost the entire diencephalic area with the exception of the ventral thalamus (for summary, see Fig. 8*A*).

In the tectal epithelium, the expression patterns of the *Lhx* genes were overlapping in the marginal layers (Fig. 4*I–J*). In contrast to *engrailed*, *Lhx9* and *Lhx2* were not expressed as a gradient. Their expression domains spanned the mesencephalic–metencephalic junction, slightly thinning out at the isthmus (Fig. 4*I–J*, arrows). Furthermore, when compared with *En-2*, they were not as largely expressed in the ventral mesencephalon (Fig. 4, compare *I–K*).

Interesting differences between the two family members were observed at the cellular level. In the telencephalic vesicles, *Lhx9* mRNA was exclusively located in the differentiating layers of the neuroepithelium, as opposed to the mitotic ventricular zone, whereas *Lhx2* was expressed throughout the depth of the neocortical epithelium, including the ventricular and the differentiating mantle zones (Fig. 5). Concerning the timing of *Lhx9* and *Lhx2* expression, a major difference was again found in the neocortex. Whereas *Lhx9* expression stopped between E14.5 and E16.5, *Lhx2* was still expressed in the neocortex until P1 (Fig. 5) and later (data not shown). Moreover, although *Lhx9* stopped in hippocampal fields CA1 and CA2 around birth (Fig. 3*K*), *Lhx2* was still expressed in the whole hippocampus (data not shown).

Thus, *Lhx9* and *Lhx2* are expressed in patterns that are compatible both for redundant and complementary roles during brain regionalization and neurogenesis.

***Lhx9* expression outside the CNS**

Lhx9 was also expressed in a few developing regions outside the CNS. This included the developing forelimb and hindlimb buds, where a gradient of expression was observed at early stages in the distal mesenchyme (Fig. 6*B,C*). Later on, expression was progressively restricted to the interdigit spaces corresponding to the region where programmed cell death happens for finger formation and to the region surrounding the cartilages (Fig. 6*D,E*). Moreover, *Lhx9* mRNA was also highly expressed in the caelomic cavity at the level of the urogenital ridge, including the gonads and parts of the pancreas and liver epithelium (Fig. 6*A*). As opposed to *Lhx2*, *Lhx9* was expressed neither in the nasal epithelium nor in the pituitary (Fig. 4*A*).

***Lhx9* protein interacts with CLIM1 and CLIM2 cofactors**

Because the *Lhx9* expression pattern is partially overlapping with the expression of the recently isolated cofactors of LIM-hd proteins, we tested whether *Lhx9* protein could physically interact with CLIM1 and CLIM2. GST pull-down assays were performed

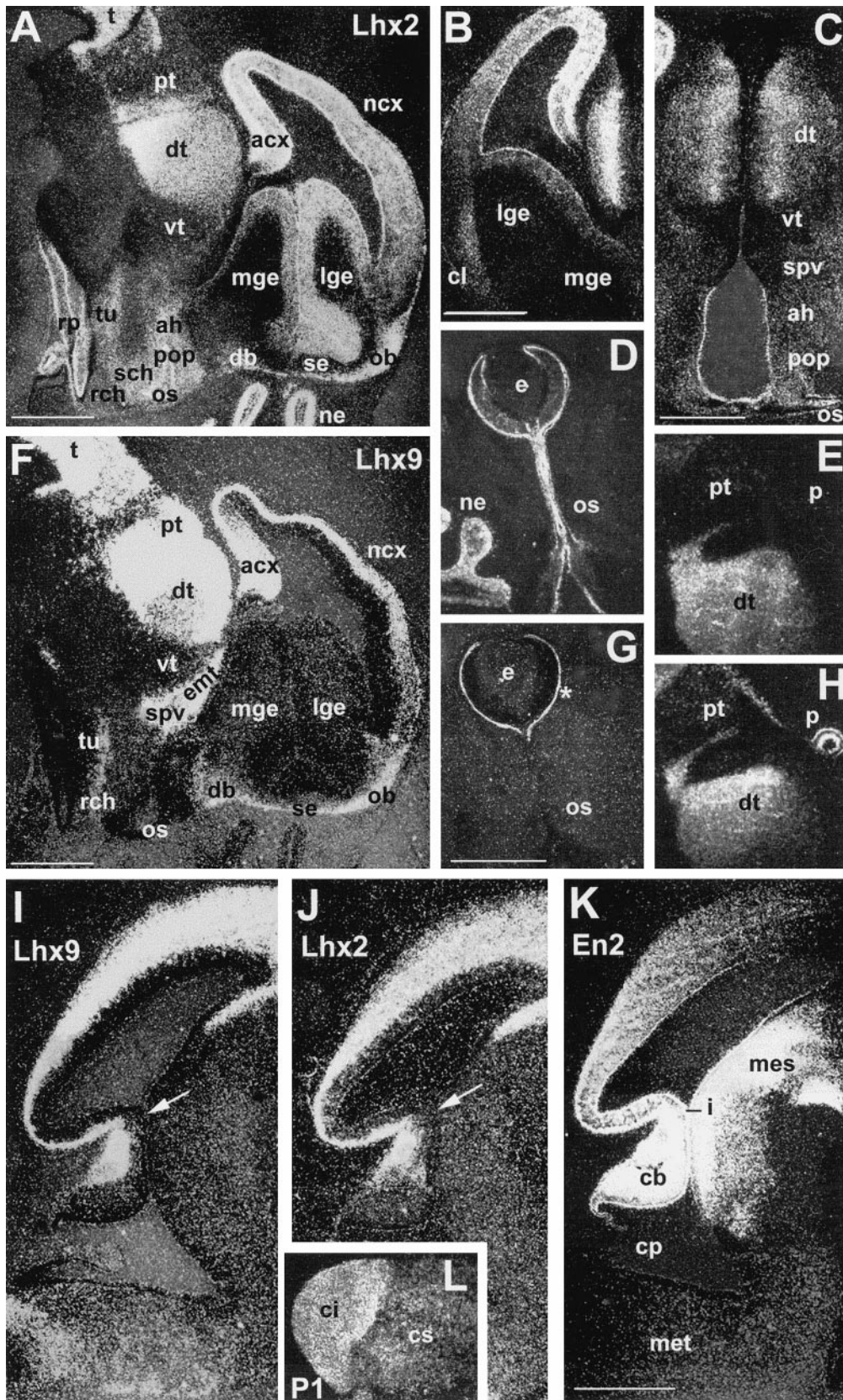


Figure 4. Comparison of *Lhx2* (A–E, J), *Lhx9* (F–I, L), and *En-2* (K) expression on sections. *A, F*, Parasagittal adjacent sections through the entire brain at E14.5, comparing expression of *Lhx2* (*A*) and *Lhx9* (*F*). *B, C*, Coronal sections at E13.5 showing *Lhx2* expression in the telencephalon and diencephalon. Sections are adjacent to those shown in Figure 3, *B* and *E*, respectively, for comparison of transverse boundaries. *D, G*, Horizontal adjacent sections showing *Lhx2* (*D*) and *Lhx9* (*G*) expression in the eye and optic stalk at E14.5. The asterisk in *G* points to the nonspecific signal attributable to the presence of the eye pigmented epithelium. *E, H*, Adjacent parasagittal section showing *Lhx2* (*E*) and *Lhx9* (*H*) in the (Figure legend continues)

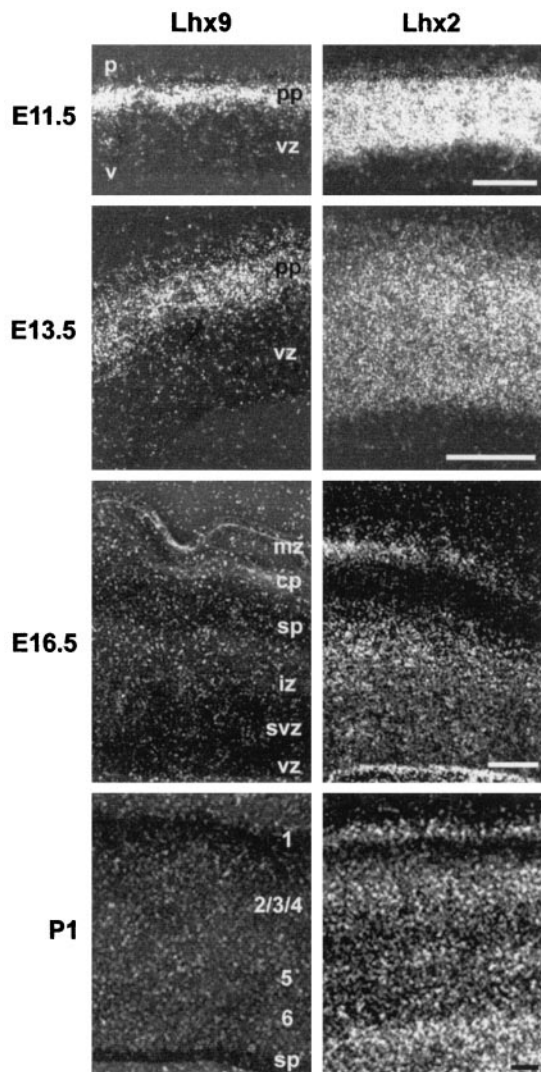


Figure 5. Comparison of *Lhx9* (left) and *Lhx2* (right) expression in the neocortical epithelium at indicated stages. The pial surface (*p*) is at the top, and the ventricular surface (*v*) is at the bottom. Left and right photographs were taken on adjacent sections. *cp*, Cortical plate; *iz*, intermediate zone; *mz*, marginal zone; *pp*, preplate; *sp*, subplate; *svz*, subventricular zone; *vz*, ventricular zone. Scale bars, 100 μ m.

using [³⁵S]Lhx9 and CLIM1 and CLIM2 GST fusion proteins. The results show that Lhx9 strongly interacts with both cofactors *in vitro* (Fig. 7), suggesting possible functional interactions *in vivo*.

DISCUSSION

***Lhx9* and *Lhx2* form a subfamily of LIM-hd genes**

We have isolated a new LIM-hd gene named *Lhx9*, thereby increasing the number of LIM-hd genes expressed in the developing CNS. *Lhx9* is most closely related to rodent *Lhx2* (Xu et al., 1993) and chick *Lh2B-Lh2A* (Nohno et al., 1997), *Drosophila apterous* (Lundgren et al., 1995), and *C. elegans ttx-3* (Hobert et al., 1997). In particular, these proteins share a short but notable

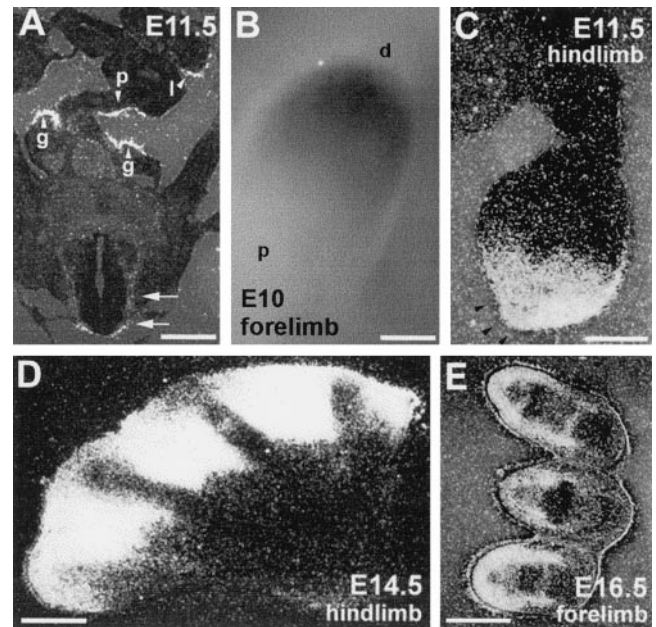


Figure 6. *Lhx9* expression in limbs and urogenital ridge. *A*, Coronal section through the caelomic cavity and the spinal cord (double arrow) at E11.5. Arrowheads point to the developing gonads (*g*), pancreas (*p*), and liver (*l*). Because sex discrimination is not easy at this stage, it should be noted that the same expression pattern was found in all embryos examined. *B*, Expression in E10 forelimb after *in toto in situ* hybridization. *p*, Proximal; *d*, distal. *C–E*, Transverse sections through developing hindlimbs and forelimbs at indicated stages. Arrowheads in *C* indicate absence of expression in the apical ectodermal ridge. See Results for details. Scale bars: *A*, *C–E*, 50 μ m; *B*, 200 μ m.

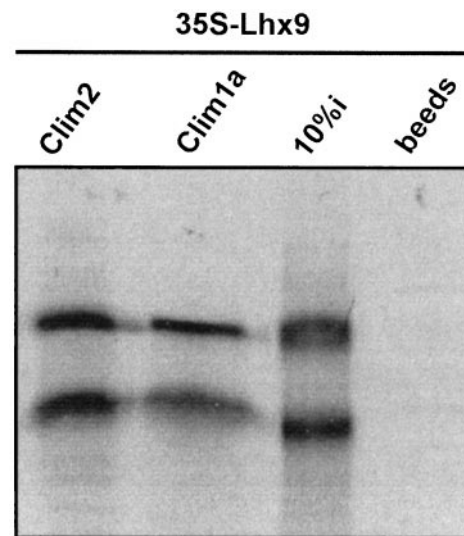


Figure 7. *Lhx9* interacts with CLIM1 and CLIM2. Autoradiogram of a representative GST pull-down assay in which ³⁵S-labeled, *in vitro*-translated *Lhx9* was tested for its ability to bind bacterially expressed GST fusions of CLIM1 and CLIM2. Ten percent (10%i) of the total ³⁵S-labeled protein input and binding to beads alone are shown.

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dorsal diencephalon and pretectum at E13.5. Note the absence of *Lhx2* in the pineal gland. *I–K*, Adjacent parasagittal sections at E13.5 to compare *Lhx9* (*I*), *Lhx2* (*J*), and *En-2* (*K*) expression through the mesencephalon and metencephalon. Arrows in *I* and *J* show the thinning of expression at the isthmus. *L*, Sagittal section through the midbrain of a P1 animal to show the *Lhx9* sharp boundary between the superior and the inferior colliculi. Compare with *I* (earlier stage). Scale bars, 100 μ m. *ne*, Nasal epithelium. See Table 1 for abbreviations.

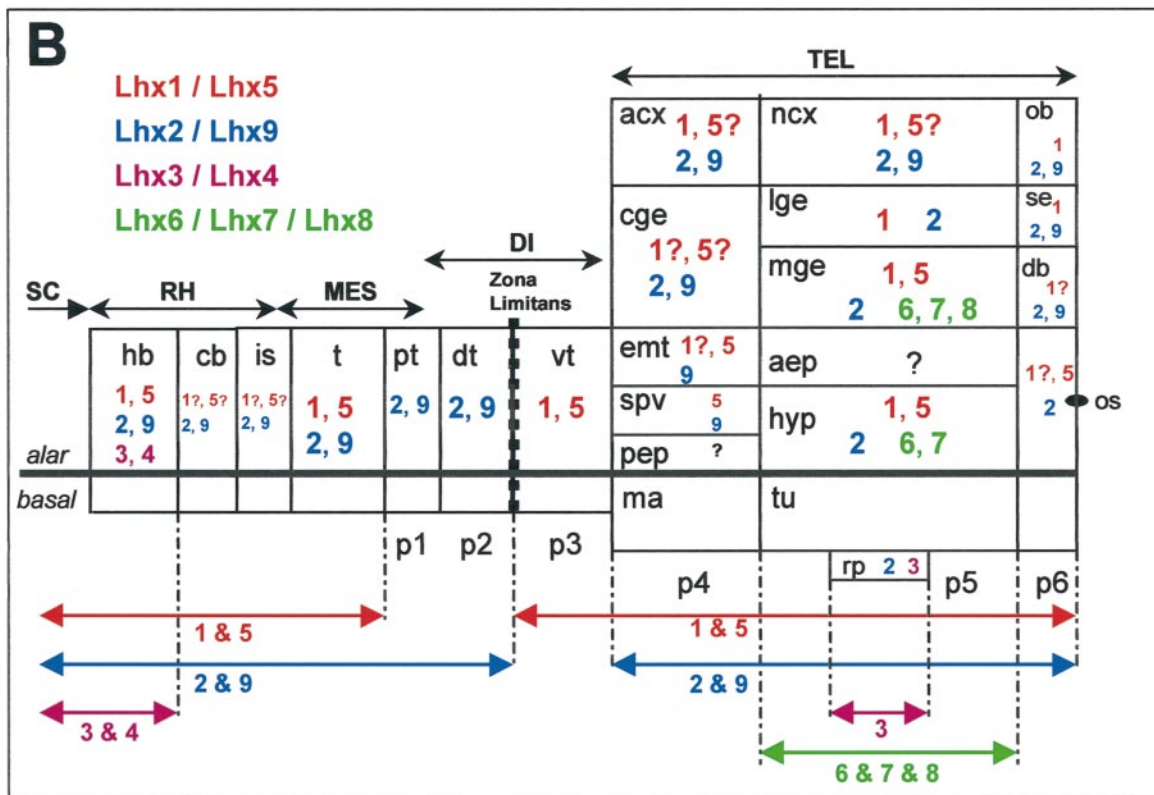
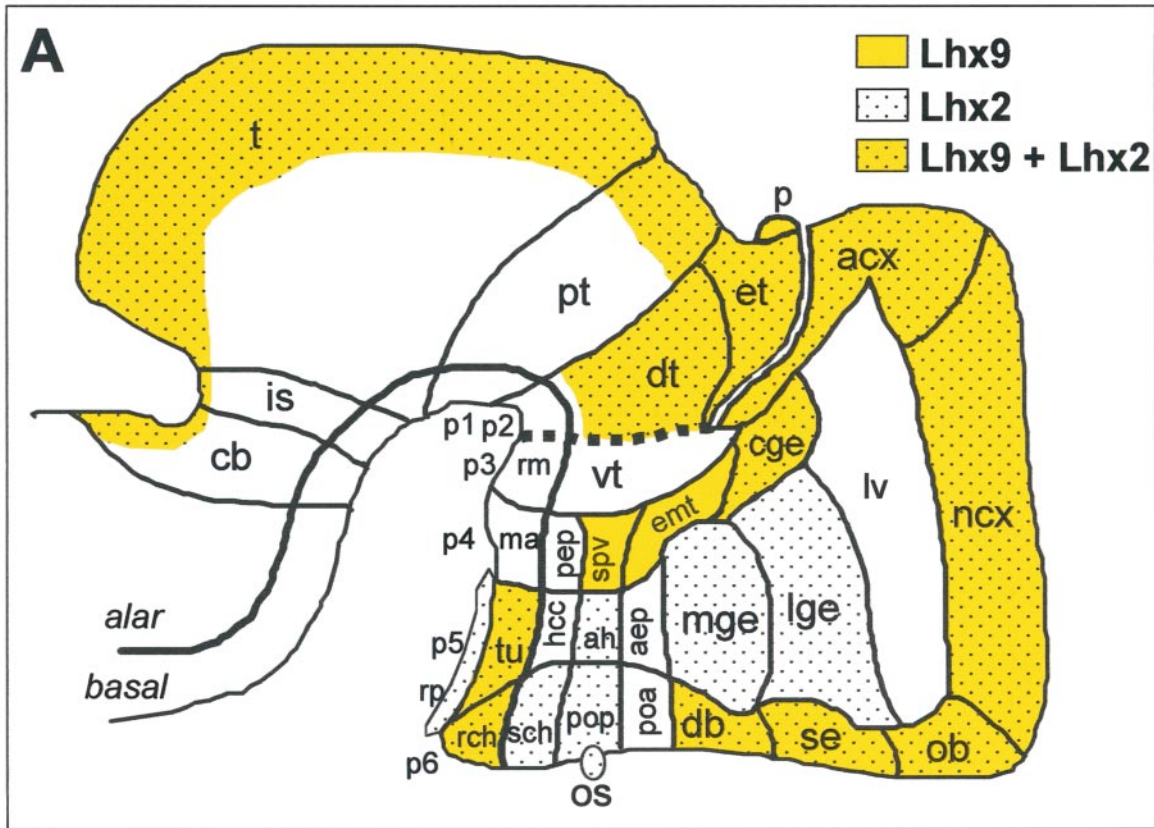


Figure 8. Summary of *Lhx9* and *Lhx2* expression patterns in the context of the neuromeric model (A) and schematic representation of Lhx combinatorial expression in the developing brain (B). Adapted from Puelles and Rubenstein (1993). A, Schematic E13.5 brain shows similarities, differences, and boundaries of expression of *Lhx9* (yellow) and *Lhx2* (dotted). Expression in the ventral mesencephalon and metencephalon is not indicated because of its rather “nuclear-like” pattern. B, Compilation of expression data for *Lhx1/5* (red; Sheng et al., 1997), (Figure legend continues)

difference in their homeodomain when compared with other LIM-hd factors. Surprisingly, although the homology between mouse *Lhx9* and chick *Lh2B* is very high (94%), the *Lhx9* expression pattern is more related to chick *Lh2A*, at least in limbs, in which chick *Lh2A* expression was thoroughly described (Nohno et al., 1997). The high degree of conservation between *Lhx9* and *Lhx2* suggests that they belong to the same subfamily. Interestingly, in vertebrates, LIM-hd genes are often encountered in pairs of closely related homologs, such as *Lhx1/Lhx5*, *Lhx3/Lhx4*, *Lhx6/Lhx7/Lhx8*, and now *Lhx2/Lhx9*. Generally, the two genes of the pair show similar expression patterns (Fig. 8B). This redundancy among the gene family suggests critical and complementary (or synergistic) roles played by these transcription factors during brain formation.

In this respect, it is interesting to rediscuss the phenotype of *Lhx2*^{-/-} embryos with the knowledge of the existence of *Lhx9*. *Lhx2*^{-/-} embryos are anophthalmic, with an aplasic archicortex and hypoplastic neocortex and basal ganglia, but their diencephalon and mesencephalon look normal (Porter et al., 1997). Porter et al. (1997) hypothesized that, depending on the region examined, the more or less defective phenotype could result from functional compensation by an unknown relative gene. *Lhx9* is not expressed in eyes and basal ganglia but is present in the neocortex, archicortex, diencephalon, and mesencephalon. Thus, functional compensation by *Lhx9* might actually happen for the neocortex, diencephalon, and mesencephalon cases. It should be noted that the expression patterns of *Lhx1* and *Lhx5* are also compatible for a partial functional redundancy at these levels, including basal ganglia (Sheng et al., 1997). In contrast, the eyeless phenotype of *Lhx2*^{-/-} embryos suggests that *Lhx2* is a major factor of this family in the eye. Finally, in the case of the hippocampus, it appears that although *Lhx9* is relatively strongly expressed in this area, it is not able to compensate for the lack of *Lhx2* expression. This could mean that *Lhx9* functions in the archicortex and neocortex are different. However, more detailed data on *Lhx1* and *Lhx5* expression would help in understanding the possible *Lhx* combinations for regional specification. Overall, the very high expression levels of *Lhx9* in the diencephalon and mesencephalon, combined with the lack of phenotype in these areas in *Lhx2*^{-/-} embryos, suggests that *Lhx9* plays an important role in the specification of the thalamus and tectum.

***Lhx9* respects neuromeric boundaries**

Transverse and longitudinal subdivisions of the developing brain, corresponding to domains and boundaries of expression of a number of developmental factors, and coincident with morphological structures such as ventricular ridges and external furrows, have allowed the proposal of a neuromeric organization of the forebrain, as described in the model of Puelles and Rubenstein (e.g., Bulfone et al., 1993, 1995; Porteus et al., 1994). The detailed study of *Lhx9* and *Lhx2* expression patterns shows that they respect the proposed neuromeric boundaries. This is summarized in Figure 8A, and we would like to discuss the following points. (1) The expression patterns clearly corroborate the proposed demarcations for p4 prosomere and distinguish nicely both p3–p4 and p4–p5 boundaries, which is important because most gene

markers do not distinguish the p3–p4 limit well. (2) In a more recent variation of their early model, Bulfone et al. (1995) suggest that the entire cerebral cortex (ncx and acx) could be either in p5 (implying that the lge–cortex boundary is longitudinal, and that the cge is the telencephalic portion of p4) or in p4, implying that the lge–cortex boundary is transverse (i.e., neuromeric). The expression patterns observed here are consistent with both views. From the *Lhx9* pattern, the cge clearly belongs to p4. In the telencephalon, however, the *Lhx9* spatiotemporal pattern respects the putative transverse boundary between the archicortex (with strong and persistent expression) and the neocortex (with lower and transient expression), a pattern that is compatible with the early model with acx in p4 and ncx in p5. On the other end, as in many other genes, *Lhx9* also distinguishes the cortex–lge boundary, a feature that is more in favor of the recent model in which this boundary is viewed as neuromeric. (3) In the diencephalon, *Lhx2* and *Lhx9* also give a sharp delineation of the ventral thalamus (in p3) that is free of signal between the adjacent prosomeres p4 and p2 (including the dorsal thalamus with massive expression and the epithalamus), thereby respecting the zona limitans intrathalamica. The other diencephalic subdivisions are well respected, with longitudinal and transverse divisions around the optic stalk region. At this level it is interesting to note that the *Lhx9*-negative optic stalk zone is complementary with *Lhx2*-positive areas. Also, *Lhx9* and *Lhx2* patterns respect a narrow negative band in the ventral part of the dorsal thalamus, close to the alar–basal boundary, that is similar to the *Gbx-2* expression pattern at this level (Bulfone et al., 1993). Interestingly, in the tectum, a sharp boundary appeared between the inferior and superior colliculi around birth and could be relevant to the establishment of optic versus auditory systems. Taken together, these results show that the *Lhx9* pattern integrates well in the neuromeric model and could participate in the establishment of the identity of neuromeres.

The observation that *Lhx9* (and *Lhx2*) expression borders the pathway of pioneering tracts at the dimesencephalic junction is also interesting. Indeed, it has been hypothesized that domains and boundaries of expression of a position-encoding homeodomain gene give landmarks for the establishment of the main axonal tracts in the brain (Figdor and Stern, 1993; Wilson et al., 1993; Macdonald et al., 1994; Rétaux et al., 1996). The two LIM-hd genes studied here might participate as local cues for the guidance of commissural axons at the dimesencephalic junction.

***Lhx9* is transiently expressed in the marginal layers of the neocortical epithelium**

Before E11, the cerebral walls are constituted of a pseudostratified ventricular epithelium in which progenitor proliferation occurs (for review, see Caviness et al., 1995). The onset of mouse cortical neurogenesis has been reported between E10 and E11 (Angevine and Sidman, 1961; Caviness, 1982), a time that corresponds almost exactly to the onset of *Lhx9* expression. Moreover, as opposed to *Lhx2* and *Lhx1* (Sheng et al., 1997), whose expression spans the depth of the cortical neuroepithelium, *Lhx9* expression is restricted to the outer layers. From its expression pattern in the cortical preplate where the first postmitotic cells are

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Lhx2/9 (blue; this paper), *Lhx3/4* (pink; Li et al., 1994; Bach et al., 1995), and *Lhx6/7/8* (green; Matsumoto et al., 1996; Grigoriou et al., 1998) on a “flattened brain” adapted from Bulfone et al. (1993). Gene expression is indicated by color-coded numbers, and a possible *Lhx* combinatorial code clearly emerges. The anteroposterior extension of expression is recapitulated by arrows at the bottom. Basal plate expression is not indicated. The zli is indicated by a dotted line. p1–p6, Prosomeres 1–6. See Table 1 for anatomical abbreviations.

located, and from its absence in the mitotic ventricular zone, *Lhx9* is a good candidate as a factor implicated in differentiation and phenotype acquisition. Moreover, neocortical *Lhx9* expression is switched off between E14.5 and E16.5. The exact time of *Lhx9* decrease in expression will have to be determined precisely, and the correlation with events related to neurogenesis will have to be studied. However, from birth-dating studies, this stage in mice (~E16.5) corresponds approximately to the completion of generation of layer VI–V neurons, to the beginning of layer IV generation, and to the arrival of thalamic afferents (Caviness, 1982; Polleux et al., 1997). Thus, *Lhx9* is expressed during the time deep layer (VI–V) cortical neurons are generated.

Although *Lhx2* was first hypothesized to play a role in cortical neuron differentiation (Xu et al., 1993), it is probably also involved in precursor proliferation (Porter et al., 1997). As discussed above, *Lhx9* is rather involved in differentiation. Other homeobox genes from the *Emx* and *Otx* families are expressed in interesting patterns in the cortical neuroepithelium: *Emx2* is located exclusively in proliferating cells, whereas *Emx1* is found in both proliferating and differentiated neurons (Gulisano et al., 1996). *Otx1* is specifically expressed in dividing and differentiated layer V–VI neurons (Frantz et al., 1994). Genes from other families, such as *T-Brain1*, are expressed exclusively in postmitotic cortical cells (Bulfone et al., 1995). Thus, it seems possible that combinations of all these transcription factors interact to regulate cortical neurogenesis and layer formation (also see next section).

Lhx9 protein interacts with CLIM cofactors

In the pituitary, interaction between CLIM cofactors and LIM domains of the LIM-hd protein P-lim promotes synergistic interactions between P-lim and the homeobox-only protein P-otx on the glycoprotein hormone α subunit promoter (Bach et al., 1997). The CLIMs could physically and functionally interact as well with a number of LIM-hd proteins to confer time and space specificity to the transcriptional control they exert. CLIM1 and CLIM2 are widely expressed in the developing brain (Bach et al., 1997), including regions where *Lhx9* is expressed. Moreover, we show that *Lhx9* strongly interacts with both of them. In the cortical neuroepithelium, for example, *CLIM1* is selectively expressed in the cortical plate, as well as *Lhx9* until approximately E16.5. *Otx1* is expressed in dividing and differentiating layer V–VI neurons (see above; Frantz et al., 1994). Making a parallel with the interactions described in the pituitary between P-lim, P-otx, and CLIMs, it is interesting to hypothesize that a trio of the same protein families, i.e., *Lhx9*, *Otx1*, and *CLIM1*, are candidates for functional interaction involved in the generation of deep layer cortical neurons.

LIM-hd genes acting in a combinatorial manner?

In the spinal cord, the combinatorial expression of the four LIM-hd genes, *Islet-1*, *Islet-2*, *Lim-1*, and *Lim-3*, distinguishes subclasses of motor neurons that select distinct axonal pathways and that occupy different columns in the spinal cord (Tsuchida et al., 1994). Although the complexity of the formation of structures and connections is probably degrees higher in the brain than in the spinal cord, the growing list of LIM-hd genes expressed in the forebrain suggests a possible similar role in the forebrain. In Figure 8B we present a tentative summary of the expression domains of *Lhx1–9*. A combinatorial pattern clearly emerges in the developing brain, with pairs of closely related homologs sharing comparable domains of expression and taking part in the determination of distinct parts of the brain (always respecting the

prosomer limits and particularly the zona limitans). For example, the richest combination is found in the mge, with a unique expression of *Lhx6–7–8* that is restricted to p5. Of interest, *Lhx1*, *Lhx2*, *Lhx5*, and *Lhx9* are all expressed in the diencephalon and in the cortex (Sheng et al., 1997; present work). If they act in a combinatorial mode, they could participate in the establishment of the highly topographic thalamocortical and/or corticothalamic projections (for review, see Molnar and Blakemore, 1995). *Lhx9*, highly expressed in median line and associative thalamic nuclei, could participate in the connections with the *Lhx9*-expressing frontal cortex. Noteworthy is the expression of *Lhx9* in a number of limbic-related structures such as the archicortex, the olfactory bulb, the amygdala, the diagonal band, and the septum, pointing out a possible implication of *Lhx9* in the development of the limbic system.

In summary, in the family of LIM-hd genes that are often expressed in pairs in the developing CNS, *Lhx9* is one of the missing partners for *Lhx2*. Its expression pattern strongly suggests a role in forebrain development, both in the specification of brain subdivisions and in cellular determination.

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