

# Expression and regulation of *Lhx6* and *Lhx7*, a novel subfamily of LIM homeodomain encoding genes, suggests a role in mammalian head development

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## SUMMARY

LIM-homeobox containing (*Lhx*) genes encode transcriptional regulators which play critical roles in a variety of developmental processes. We have identified two genes belonging to a novel subfamily of mammalian *Lhx* genes, designated *Lhx6* and *Lhx7*. Whole-mount in situ hybridisation showed that *Lhx6* and *Lhx7* were expressed during mouse embryogenesis in overlapping domains of the first branchial arch and the basal forebrain. More specifically, expression of *Lhx6* and *Lhx7* was detected prior to initiation of tooth formation in the presumptive oral and odontogenic mesenchyme of the maxillary and mandibular processes. During tooth formation, expression was restricted to the mesenchyme of individual teeth. Using explant cultures, we have shown that expression of *Lhx6* and *Lhx7* in mandibular mesenchyme was under the control of signals derived from the overlying epithelium; such signals were

absent from the epithelium of the non-odontogenic second branchial arch. Furthermore, expression studies and bead implantation experiments in vitro have provided strong evidence that *Fgf8* is primarily responsible for the restricted expression of *Lhx6* and *Lhx7* in the oral aspect of the maxillary and mandibular processes. In the telencephalon, expression of both genes was predominantly localised in the developing medial ganglionic eminences, flanking a *Fgf8*-positive midline region. We suggest that *Fgf8* and *Lhx6* and *Lhx7* are key components of signalling cascades which determine morphogenesis and differentiation in the first branchial arch and the basal forebrain.

Key words: LIM homeodomain, *Lhx6*, *Lhx7*, *Fgf8*, branchial arches, tooth development, epithelial-mesenchymal interactions, medial ganglionic eminence

## INTRODUCTION

Homeobox-containing genes constitute a class of transcriptional regulators that play critical roles in the control of pattern formation and cell type specification. For example, the Antennapedia-like HOM-C class of homeobox genes in *Drosophila melanogaster* and their vertebrate homologues (*Hox* genes), have been shown to be involved in the establishment of cell identity along the anteroposterior axis of the embryo (Krumlauf, 1994). This class of homeobox genes are clustered in distinct complexes, an organisational feature that has been conserved during evolution, from *Caenorhabditis elegans* to mammals, and is associated with the overlapping patterns of expression of individual genes. Many homeobox-containing genes are, however, found dispersed outside the HOM-C/*Hox* clusters. Such genes often encode polypeptides which, in addition to the homeodomain, contain additional domains (such as paired, POU or LIM domains; Burglin, 1994) which can regulate the transcriptional activity of the protein.

LIM homeodomain proteins (*Lhx*; for nomenclature see Taira

et al., 1995) are characterised by the association of two LIM domains (cysteine-rich motifs involved in intra- and intermolecular interactions) with a homeodomain and constitute a family of transcriptional regulators that were originally identified by sequence homology between the *C. elegans* genes *lin-11* and *mec-3* and the mammalian transcription factor *islet-1* (Freyd et al., 1990; Karlsson et al., 1990; Way and Chalfie, 1988). Genetic studies in both invertebrates and vertebrates have shown that LIM homeodomain encoding genes are required for the control of cell fate specification and differentiation. In *C. elegans*, *mec-3* and *lin-11* control the differentiation of mechanosensory neurons and the asymmetric division of a variety of cell types respectively (Freyd et al., 1990; Way and Chalfie, 1988). In *D. melanogaster*, *islet* (homologue of the vertebrate *islet-1* and *-2* genes) is required for normal axonal pathfinding and neurotransmitter synthesis by a subset of CNS motor neurons and interneurons (Thor and Thomas, 1997). In the same organism, the *apterous* gene establishes the dorsal fate of cells in the wing imaginal disc and is required for the fasciculation and pathfinding of subsets of interneurons as well

as for the development of a subset of embryonic muscles (Blair et al., 1994; Bourgouin et al., 1992; Cohen et al., 1992; Lundgren et al., 1995). In vertebrates, targeted mutagenesis of *Lhx* genes has established that they are necessary for the differentiation of specific cell types, such as subclasses of CNS neurons (*isl-1*) or subpopulations of endocrine cells of the anterior pituitary (Pfaff et al., 1996; Sheng et al., 1997). Finally, misexpression studies have indicated that the *Lmx1* gene is a critical component of the molecular mechanisms which lead to establishment of the dorsal phenotype of the vertebrate limb bud (Riddle et al., 1995; Vogel et al., 1995).

We describe here the identification and expression pattern during embryogenesis of two novel LIM homeobox genes, *Lhx6* and *Lhx7*, from the mouse. We demonstrate that *Lhx6* and *Lhx7* are expressed in overlapping subdomains of the first branchial arch and the basal forebrain and that expression in the first arch primordia is under the control of signals from the overlying oral epithelium. One of these signals is likely to be Fgf8, which is expressed in the oral epithelium and is capable of inducing expression of both *Lhx6* and *Lhx7* in mandibular mesenchyme in vitro. Overall, our studies have identified a novel molecular cascade that is likely to play a critical role in the patterning and differentiation of characteristic orofacial structures as well as in the development of the forebrain of mammalian embryos.

## MATERIALS AND METHODS

### Isolation of *Lhx6* cDNA

Oligo-dT primed cDNA template prepared from E13.5 mouse ENS precursors was subjected to 40 cycles of PCR (1 minute at 94°C, 1 minute at 40°C and 1 minute at 72°C). The 5' primer, GCCGGATCCTA(C/T)TG(C/T)AA(A/G)(A/G/C)(A/G)IGA(C/T)T was derived from the amino acid sequence YCK(R/N/D/E)D(Y/F) of the second LIM domain, while the 3' primer, GCCGTCGAC(A/G)TT(C/T)TG(A/G)AACCAIACI(C/T)(G/T)IAIIAC, was based on the highly conserved amino acid sequence RV(L/I/V)(Q/R)VWFQN of the third helix of the homeodomain. The sequences used for primers were based on the comparison of all the vertebrate Lhx proteins. The 453 bp PCR product was subsequently cloned into pBluescript SK+ (Stratagene). 100 different recombinant clones were sequenced.

### cDNA library construction and screening

A cDNA library was constructed from poly(A)<sup>+</sup> RNA isolated from head of E14.5 embryos using the ZAPExpress cDNA synthesis kit (Stratagene). 1.2×10<sup>6</sup> individual plaques were screened with the 453 bp PCR fragment corresponding to *Lhx6* and positive clones were analysed by restriction mapping and sequencing.

### In situ hybridisation

Radioactive in situ hybridisation procedures were carried out as described by Wilkinson (1995), with the following modifications. Embryonic heads were sectioned at 8 µm and floated onto TESPA (3-aminopropyltriethoxysilane) coated slides. The slides were pre-treated with 5 mg/ml Proteinase K and 0.25% (v/v) acetic anhydride and hybridisation was carried out overnight in a humidified chamber at 55°C. The slides were then washed twice at high stringency (20 minutes at 65°C in 2× SSC, 50% formamide, 10 mM DTT) and treated with 40 mg/ml RNase A for 30 minutes at 37°C. The high stringency washes (at 65°C in 2× SSC, 50% formamide, 10 mM DTT) were repeated, followed by a further wash at 65°C in 0.1× SSC, 10 mM DTT. The sections were then washed in 0.1× SSC at room temperature, and dehydrated through 300 mM ammonium acetate in 70% ethanol, 95% ethanol and absolute ethanol. The slides were air dried and dipped in Ilford K.5 photographic emulsion. Autoradiography was performed by exposing the sections in a

light-tight box at 4°C for 10-14 days. Slides were developed using Kodak D19, fixed in Kodak UNIFIX and counter stained with malachite green.

Non-radioactive in situ hybridisation on fresh frozen sections of mouse embryos was performed as previously described (Schaeren-Wiemers and Gerfin-Moser, 1993). Whole-mount in situ hybridisations were performed as described by Wilkinson (1992). For sections, hybridised embryos were postfixed for several hours in 4% paraformaldehyde in phosphate-buffered saline (PBS), washed 3× in PBS, cleared in PBS-1% Triton X-100 for several hours, equilibrated in 30% sucrose and embedded in OCT compound (BDH). Cryosections were cut at 25 µm. Antisense riboprobes were generated using the following templates: for *Lhx6*: 1.5 kb *Bam*HI-*Eco*RI fragment of the 3'UTR; for *Lhx7*: 0.96 kb *Eco*RI-*Sph*I fragment spanning the 5'UTR and the LIM domains (this probe identifies both *Lhx7* and *Lhx7a* mRNAs); for *Gbx-2* the 1.8 kb fragment generated by PCR using the following primers: 5' primer CCGGATCCGTGGGAGACGGACTG, and 3' primer CCGTCGACCTGCACTCAACTCAAAAAGCC based on the *Gbx-2* cDNA sequence published by Chapman and Rathjen (1995). For analysis of expression of *Dlx-2*, *Fgf8*, *Bmp-4* and *Shh* antisense riboprobes were generated as previously published.

### RNase protection analysis

RNA was extracted from embryos by the LiCl/urea method. 40 µg of total RNA was hybridised O/N at 54°C with 10<sup>5</sup> cpm of [<sup>32</sup>P]UTP (800 Ci/mmol, Amersham)-labelled antisense riboprobe in 30 µl of 40 mM Pipes (pH 6.4), 400 mM NaCl, 1 mM EDTA, 80% formamide. The unprotected probe was digested with a mixture of RNase A (40 mg/ml) and RNase H (3500 U/ml) and the protected products were resolved in a 6% denaturing polyacrylamide gel. The probes used were: (a) for *Lhx6*, a 410 bp fragment (nt 699-1110); (b) for *Lhx7* and *Lhx7a*, a 163 bp fragment (nt 1022-1185). Part of this probe (63 bp) is within the common sequence of *Lhx7* and *Lhx7a* cDNAs and therefore detects both transcripts.

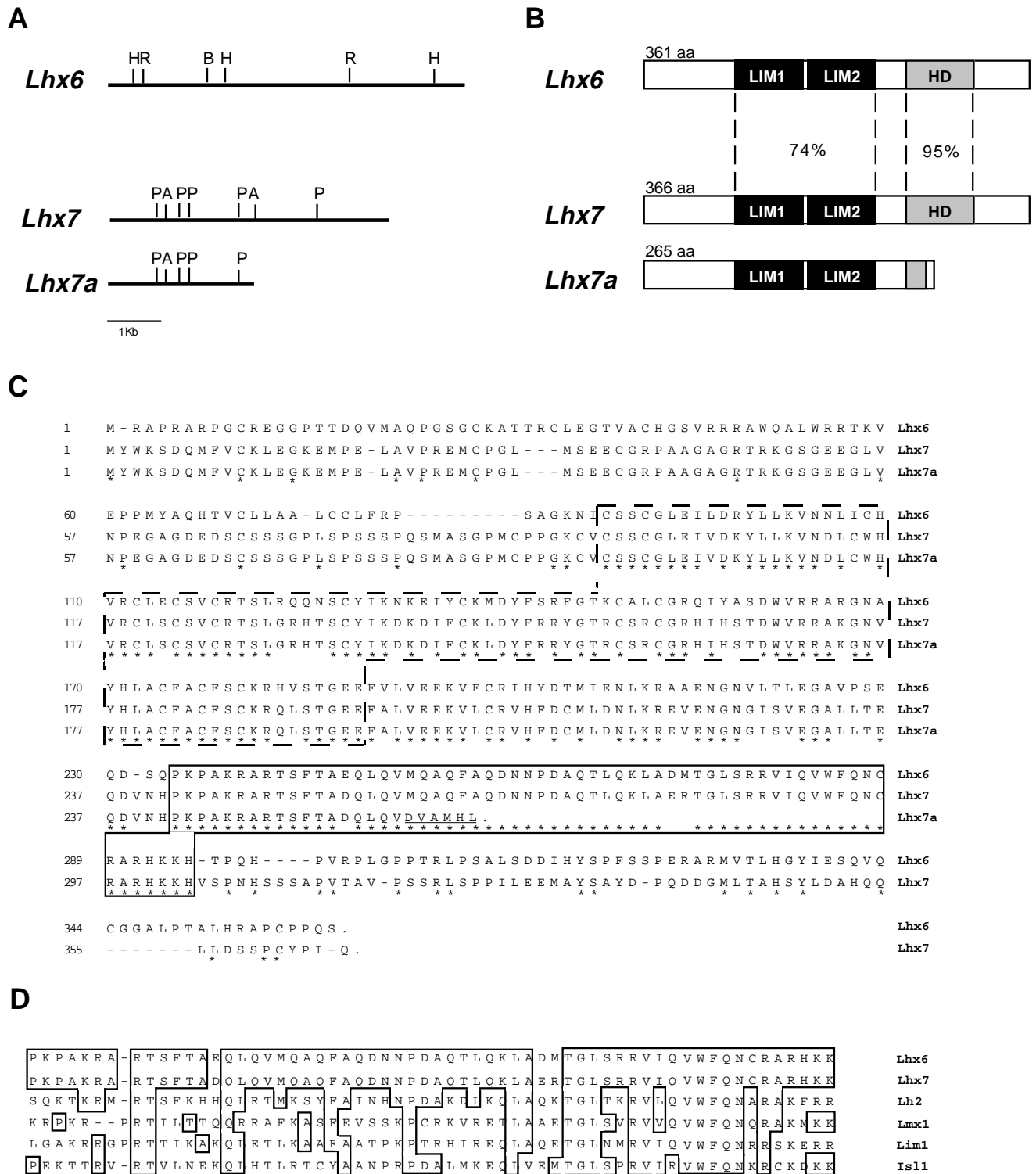
### Recombination and bead experiments

E10.5 and E11.5 mandibles and E10.5 second branchial arches were dissected in D-MEM and the epithelium was removed using dispase (2 U/ml) in PBS (Ca<sup>2+</sup> and Mg<sup>2+</sup> free). The mandibles were incubated for 9 minutes at 37°C, the second branchial arches for 7 minutes at 37°C. Following incubation, the tissues were washed in D-MEM with 10% foetal calf serum (FCS), and the epithelium was dissected off using electrolytically sharpened tungsten needles. The mesenchyme was placed on membrane filters supported by metal grids according to the Trowell technique as modified by Saxen (Saxen, 1966; Trowell, 1959). For the recombinations the epithelium was then placed on the top of the mesenchyme. For delivery of Fgf8 and BSA proteins heparin acrylic beads (Sigma) were used. These were washed thoroughly in PBS, added to the protein and incubated overnight at 4°C. The Fgf8 and BSA were used at a concentration of 1 mg/ml. For the BMP4 and Shh-N protein Affi-Gel-blue beads (Bio Rad) were used. These were washed and dried out before being placed in the protein for 1 hour at 37°C. BMP4 was used at a concentration of 100 µg/µl, and Shh-N was used at 1.25 µg/µl which were able to induce *Msx-1* and *Ptc* respectively (data not shown). The recombinant tissue explants were cultured at 37°C for 24 hours in D-MEM with 10% FCS in a humidified atmosphere of 5% CO<sub>2</sub>. All solutions used contained penicillin and streptomycin at 20 IU/ml. At the end of the culture period, the explants were washed in ice-cold methanol for 1 minute then fixed in fresh 4% paraformaldehyde (in 1× PBS) for 1 hour at RT. Cultures were then processed for whole-mount in situ hybridisation (Wilkinson, 1992).

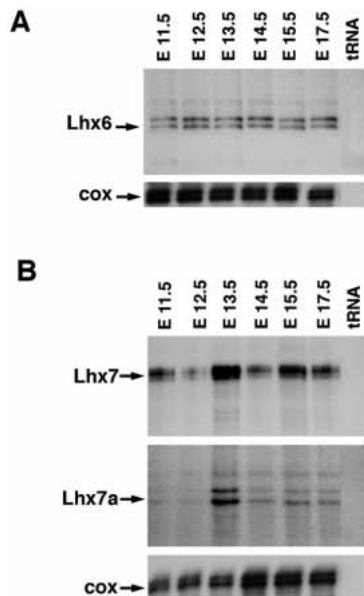
## RESULTS

### Isolation of cDNAs encoding two novel LIM homeodomain proteins

In order to identify genes which encode novel members of the LIM homeodomain family of transcription factors involved in



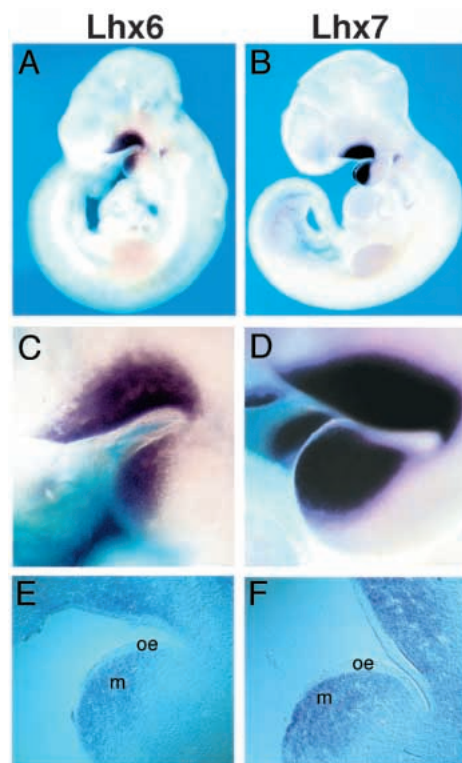
**Fig. 1.** *Lhx6* and *Lhx7* encode two novel members of the LIM homeodomain family of transcription factors. (A) Schematic presentation and partial restriction enzyme map of the *Lhx6*, *Lhx7* and *Lhx7a* cDNAs. Restriction enzymes: E, *EcoRI*; B, *BamHI*; H, *HincII*; P, *PstI*; A, *ApaI*. (B) Schematic representation of the predicted *Lhx6*, *Lhx7* and *Lhx7a* proteins. LIM1 and LIM2 indicate the two LIM domains present on all three proteins and HD indicates the homeodomain present in *Lhx6* and *Lhx7*. (C) Alignment of the predicted amino acid sequences of *Lhx6*, *Lhx7* and *Lhx7a*. Asterisks indicate identical residues. The LIM domains and the homeodomain are boxed with a dashed line and a solid line, respectively. The amino acids which are unique to the *Lhx7a* protein are underlined. (D) Comparison of amino acid sequence of the *Lhx6* and *Lhx7* homeodomains to those of other LIM homeodomain proteins. Boxed residues indicate amino acid identity. The homeodomain of *Lhx6* is 95% identical to that of *Lhx7* but only 62% identical to that of the next closest member (*Lhx2*). The EMBL accession numbers of the sequence of *Lhx6* and *Lhx7* cDNAs are AJ000337 (MMLHX6) and AJ000338 (MMLHX7) respectively.



**Fig. 2.** *Lhx6*, *Lhx7* and *Lhx7a* are expressed during mouse embryogenesis. RNase protection analysis of *Lhx6* (A) and *Lhx7* and *Lhx7a* (B) expression between E11.5 and E17.5. tRNA indicates a negative control lane.

the development of the mammalian peripheral nervous system, we combined reverse transcription and polymerase chain reaction (RT-PCR) using degenerate primers specific for Lhx-encoding sequences and mRNA from mouse embryo enteric neuroblasts. Using this approach, 9 identical clones were isolated which corresponded to an apparently novel LIM homeodomain encoding gene, called here *Lhx6* (in naming these genes, we have adopted the nomenclature originally suggested by Taira et al., 1995). Since preliminary northern blot analysis indicated that *Lhx6* transcripts were enriched in embryo head mRNA preparations, an E14.5 mouse embryo head cDNA library was constructed and screened with the original *Lhx6* PCR probe. Restriction enzyme mapping and sequence analysis of the positive cDNAs revealed that, in addition to the *Lhx6*-related clones, our screen yielded a number of additional cDNAs which appeared to be derived from a novel but closely related gene, called *Lhx7*. Compilation of all *Lhx6*- and *Lhx7*-related clones allowed us to generate cDNAs capable of encoding two novel proteins, comprising 361 and 366 amino acids respectively (Fig. 1). Both proteins contain two LIM domains (LIM-1 and LIM-2) followed by a homeodomain (HD; Fig. 1B,C). The overall similarity between *Lhx6* and *Lhx7* is 75%, with the homeodomain and the LIM domains being the most conserved regions (95% and 74% identity respectively; Fig. 1B). Moreover, sequence comparison of the homeodomains of *Lhx6* and *Lhx7* to those of other LIM homeodomain proteins, showed that they are more similar to each other than to other members of this family, indicating that they constitute a novel subfamily of the Lhx protein family (Fig. 1D).

In addition to the clones described above, our cDNA library screen identified a single clone which, upon further characterisation, appeared to be partly identical to *Lhx7*. This clone, called here *Lhx7a*, was capable of encoding a truncated



**Fig. 3.** Whole-mount in situ hybridisation analysis of *Lhx6* and *Lhx7* expression in E10.5 mouse embryos. Embryos were hybridised with *Lhx6* (A) or *Lhx7* (B) riboprobes. Expression of both genes is restricted to subdomains of the first branchial arch. (C,D) High magnifications of the first branchial arch of the embryos shown in A and B respectively. Expression of *Lhx6* and *Lhx7* is detected in the oral (future odontogenic) aspect of the mandibular process and in at least part of the maxillary process. (E,F) Sagittal sections of the first branchial arch region of the embryos shown in A and B. Expression of *Lhx6* and *Lhx7* is confined in the neural crest-derived mesenchyme of the maxillary and the mandibular primordia. oe, oral epithelium; m, mesenchyme.

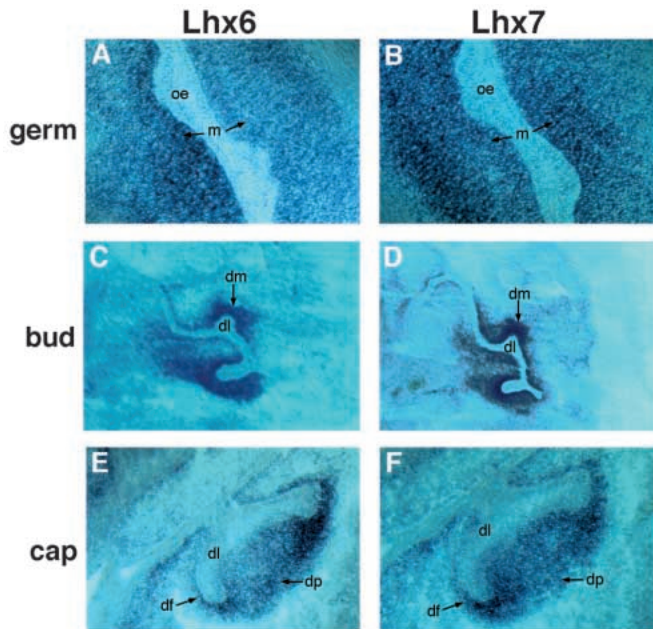
version of *Lhx7*, containing the two LIM domains, the first 18 amino acids of the homeodomain and 6 unrelated amino acids (Fig. 1A-C).

#### Expression of *Lhx6* and *Lhx7*: RNase protection and RT-PCR analysis

To determine the expression of *Lhx6* and *Lhx7* during mammalian embryogenesis, we performed a series of RNase protection experiments using total mouse embryo RNA representing various stages of embryogenesis and riboprobes specific for *Lhx6* or *Lhx7* (for probe details, see Materials and Methods). As shown in Fig. 2A,B, mRNAs encoding *Lhx6*, *Lhx7* and *Lhx7a* were detected at all embryonic stages analysed (i.e. E11.5-17.5). Furthermore, these data show that the *Lhx7a* cDNA clone represents a genuine product of the *Lhx7* locus, a conclusion that was also supported by RT-PCR analysis with *Lhx7a* mRNA specific primers (data not shown).

#### Expression of *Lhx6* and *Lhx7*: in situ hybridisation analysis

To gain insight into the developmental role of *Lhx6* and *Lhx7*,



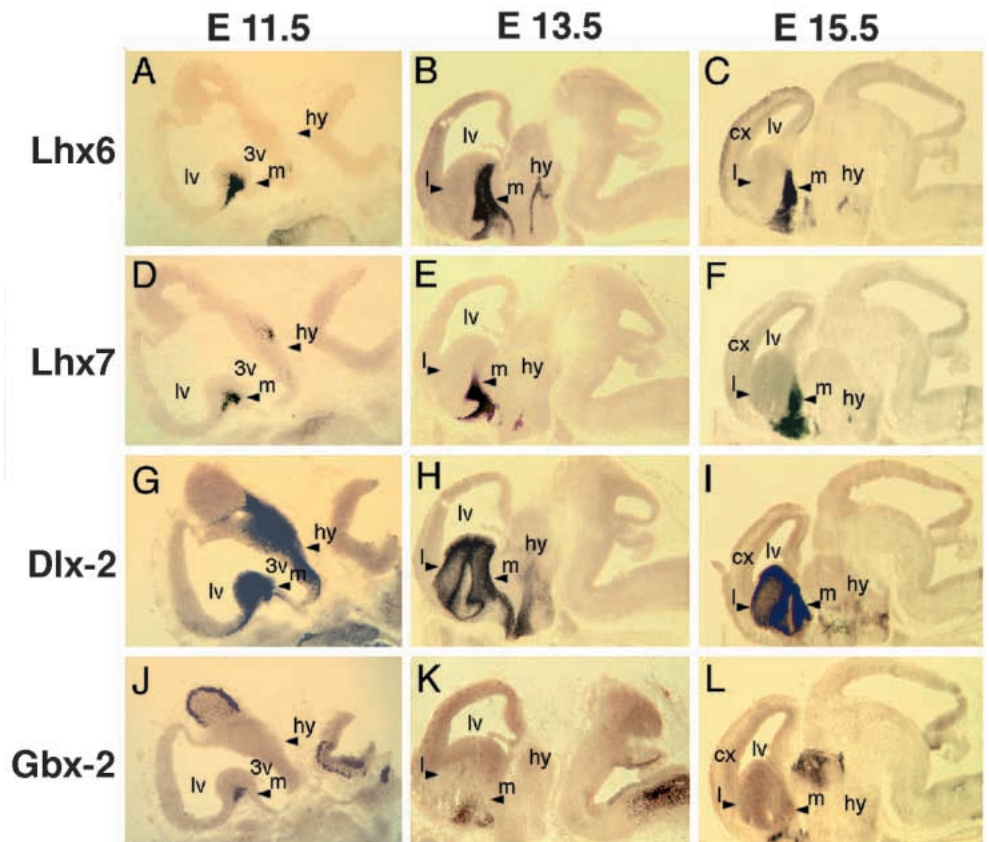
**Fig. 4.** In situ hybridisation analysis of *Lhx6* (A,C,E) and *Lhx7* (B,D,F) expression during mouse tooth development. Expression of both genes is restricted to the neural crest-derived mesenchyme throughout the period of odontogenesis. Germ, bud and cap indicate three characteristic stages of tooth development. oe., oral epithelium; m, neural crest-derived mesoderm; df, dental follicle; dl, dental lamina; dm, dental mesenchyme; dp, dental papilla.

Despite the original identification of *Lhx6* in enteric neuroblasts, we were unable to detect its transcripts in foetal gut using in situ hybridisation, indicating that the gene is expressed in the developing enteric nervous system at levels that are below the limits of detection of this technique.

**Expression of *Lhx6* and *Lhx7* in the first branchial arch and its derivatives**

Low levels of *Lhx6* and *Lhx7* transcripts were first detected by whole-mount in situ hybridisation in a subdomain of the first branchial arch of E9.5 embryos (data not shown). At E10.5, both genes were expressed at high levels, and with an overlapping pattern, in the maxillary and mandibular processes of the first branchial arch. However, expression was not uniform, but instead was restricted to the subdomains bordering the cleft that separates the two facial primordia (Fig. 3). Also, sections of hybridised embryos showed that *Lhx6* and *Lhx7* transcripts were restricted to the neural crest-derived ectomesenchyme and were excluded from the overlying oral epithelium (Fig. 3E,F). At E11.5, both genes were expressed in a pattern similar to that of the previous stage (data not shown). Significantly, no expression was detectable in other

we used in situ hybridisation analysis to determine the temporal and spatial distribution of their transcripts during mouse embryogenesis. Our data (presented below) show that expression of *Lhx6* and *Lhx7* was temporally and spatially regulated during mouse embryogenesis, with both genes expressed at high levels in overlapping domains of the first branchial arch and its derivatives and the basal forebrain.



**Fig. 5.** Comparison of the expression of *Lhx6*, *Lhx7*, *Dlx-2* and *Gbx-2* on serial sagittal sections of the developing CNS at E11.5, E13.5 and E15.5. *Lhx6* and *Lhx7* are expressed in overlapping pattern in the MGE. *Lhx6* is mainly expressed in the subventricular and the submantle zones of the MGE, while *Lhx7* transcripts are detected mainly in the submantle and mantle zones (compare B and E to H). (A-C) *Lhx6*, (D-F) *Lhx7*, (G-I) *Dlx-2* and (J-L) *Gbx-2*. 3v: third ventricle; l: lateral ganglionic eminence; m: medial ganglionic eminence; cx, cortex; lv, lateral ventricle; hy, hypothalamus.

branchial arches. It appears, therefore, that the expression domains of *Lhx6* and *Lhx7* coincide with the regions of the first arch mesenchyme which acquire odontogenic potential and contribute to the formation of individual teeth. Consistent with this, expression of *Lhx6* and *Lhx7* in the branchial region at subsequent developmental stages was primarily associated with developing teeth.

Mammalian teeth develop through a series of reciprocal interactions between the ectodermally derived oral epithelium and the juxtaposed neural crest-derived mesenchyme of the first branchial arch. (Thesleff and Nieminen, 1996). The first morphological sign of tooth development in mammalian embryos is the appearance of localised thickenings of the stomodeal epithelium (around E11.5-12.5; tooth germ stage. Fig. 4A,B). Further thickening of the dental epithelium and invagination of the underlying mesenchyme leads to the formation of the dental laminae (E13.5; tooth bud stage Fig. 4C,D). At E14.5, the condensed mesenchyme surrounding the bud invades the base of the dental lamina, forming a hillock-shaped mesenchymal dental papilla (cap stage; Fig. 4E,F). At subsequent stages, the dental papilla has deeply invaginated the dental lamina and constitutes the core of the developing teeth (bell stage; not shown). Expression of *Lhx6* and *Lhx7* during mouse odontogenesis was analysed by in situ hybridisation on sections of embryos ranging from E12.5-P2. At the tooth germ stage (E12.5), expression of both genes was identical and distributed uniformly in the ectomesenchyme adjoining the oral epithelium (Fig. 4A,B). By the tooth bud stage (E13.5), expression was downregulated from most regions of the mandibular and maxillary mesenchyme, but was maintained (and indeed upregulated) in the mesenchyme adjacent to the epithelial thickenings which constitute the tooth primordia (Fig. 4C,D). Similarly, at subsequent stages of tooth cap (Fig. 4E,F) and bell (not shown), high levels of *Lhx6* and *Lhx7* expression were restricted to the mesenchymal component of the developing tooth. Finally, by postnatal day 2, expression of both *Lhx6* and *Lhx7* genes was downregulated in the developing teeth (data not shown). Overall, our data indicate that prior to initiation of tooth development, *Lhx6* and *Lhx7* are expressed uniformly in the odontogenic mesenchyme of the first branchial arch, whereas during odontogenesis, expression is restricted to the mesenchyme participating in the formation of individual teeth.

#### Expression of *Lhx6* and *Lhx7* in the developing central nervous system

In the developing CNS, expression of *Lhx6* and *Lhx7* was detected mainly in the basal telencephalon. In mammalian embryos, the telencephalic hemispheres appear at E9.5, as lateral evaginations of the wall of the prosencephalon. At this stage, the ventricular walls form a continuous cellular sheet without obvious signs of regional specialisation. The first evidence of differentiation along the dorsoventral axis of the forebrain is the appearance, at E10.5, of thickenings of the basolateral walls of the telencephalic ventricles which lead to the formation of a set of bilateral intraventricular elevations, the medial and lateral ganglionic eminence (MGE and LGE). MGE and LGE give rise to different components of the basal ganglia, the pallidum and striatum respectively (Bayer, 1984; Deacon et al., 1994; Lammers et al., 1980; Marchand et al., 1986; Olsson et al., 1995). Several molecular markers have

been described for the basal telencephalon of mammalian embryos, such as *Dlx-2*, which is expressed in the LGE and MGE, and *Gbx-2*, which is expressed specifically in a subdomain of MGE (Bulfone et al., 1993a,b). Expression of these genes and additional molecular markers (such as MASH-1 and MAP2), has indicated that the MGE of E12.5-13.5 mouse embryo, can be subdivided into four molecularly distinct cell layers: the ventricular, subventricular, submantle and mantle zones (Porteus et al., 1994). *Dlx-2* is detected predominantly in the subventricular and submantle layers of the MGE, while expression of *Gbx-2* is restricted to the mantle layer of the MGE (Fig. 5G-L; for details see: Bulfone et al., 1993b; Porteus et al., 1994). Precursor cells undergoing mitosis are present primarily in the ventricular and subventricular zones, while postmitotic differentiating cells are localised mainly in the submantle and mantle zones (Porteus et al., 1994). We have used in situ hybridisation on serial sagittal sections of mouse embryos ranging from E11.5 to E17.5 to analyse the expression of *Lhx6* and *Lhx7* in the developing mammalian forebrain and compare it to that of *Dlx-2* and *Gbx-2*. As shown in Fig. 5A-F, neither of the two genes was expressed in the LGE at any of the stages analysed. However, both *Lhx6* and *Lhx7* were expressed from the early stages of development of the MGE in a characteristic overlapping pattern: *Lhx6* transcripts were localised to the subventricular and submantle zones of the MGE, whilst expression of *Lhx7* was mainly restricted to the submantle and mantle zones. The relative expression of *Lhx6* and *Lhx7* and the independent markers used (*Dlx-2* and *Gbx-2*) are shown clearly in Fig. 5B,E,H and K. Overall, our data indicate that *Lhx6* and *Lhx7* are expressed in the MGE from the earliest stages of its formation, in overlapping cellular populations which include dividing neuroblasts as well as postmitotic neurons. In addition to the MGE, both genes are also expressed in distinct domains of the hypothalamus (Fig. 5B-F).

#### Epithelial mesenchymal interactions

Explant cultures of the first branchial arch have been used as a model system to study the role of epithelial-mesenchymal interactions in gene expression associated with tooth induction (Lumsden, 1988). Using such a system, it has been shown that the oral epithelium is required for the early induction of expression of several genes coding for signalling molecules and transcription factors that are critical for normal odontogenesis (e.g. *Bmp-4*, *Msx1*; Chen et al., 1996; Jowett et al., 1993; Vainio et al., 1993). To determine whether expression of *Lhx6* and *Lhx7* in the ectomesenchyme of the first branchial arch requires signals from the overlying oral epithelium, explant cultures of E10.5 mouse embryo mandibles were established in which the mesenchyme was grown in the presence or absence of the oral epithelium. As shown in Fig. 6A,B, coculture of the mesenchymal and epithelial components of mandibles resulted in relatively high levels of expression of *Lhx6* and *Lhx7*. However, no expression of *Lhx6* or *Lhx7* was observed in the mesenchyme of mandibular explants in which the oral epithelium had been removed (Fig. 6C,D). Time course experiments indicated that the downregulation of *Lhx6* and *Lhx7* expression was detected within 3 hours following removal of the mandibular epithelium. These data suggest that the oral epithelium is

required for the initial induction and maintenance of *Lhx6* and *Lhx7* expression in the mesenchyme of the first branchial arch.

There are two potential explanations for the restricted expression of *Lhx6* and *Lhx7* in the first branchial arch. One possibility is that expression of these genes is an intrinsic property of the neural crest cells populating the first branchial arch. Alternatively, expression may be induced in the first arch mesenchyme by signals unique to the oral epithelium of this arch, which are absent from the epithelium of the other branchial arches. To distinguish between these possibilities, recombination experiments were performed between the first and second branchial arch epithelial and mesenchymal components. As shown in Fig. 6E,F, recombination of (first arch) oral epithelium with second arch mesenchyme resulted in induction of *Lhx6* and *Lhx7* expression in the latter. However, the reverse recombination (i.e. second arch epithelium and first arch mesenchyme), resulted in significant downregulation of both genes in the ectomesenchyme (Fig. 6G,H). These data (summarised schematically in Fig. 6I-M) strongly suggest that signals from the oral epithelium of the mandible are responsible for the restricted expression of *Lhx6* and *Lhx7* in the mesenchyme of the first branchial arch.

#### **Fgf8 regulates expression of *Lhx6* and *Lhx7***

The requirement of oral epithelium for expression of *Lhx6* and *Lhx7* in the mesenchyme, prompted us to examine in detail the distribution of various signalling molecules in this tissue as a means of identifying the endogenous inducer(s) of *Lhx6* and *Lhx7*. Potential candidates for oral epithelial signals include members of the fibroblast growth factor family (such as Fgf8), members of the bone morphogenetic protein family (such as Bmp-4) and sonic hedgehog (Shh), all of which have been localised in oral epithelium (Bitgood and McMahon 1995; Vainio et al., 1993; Neubüser et al., 1997). We therefore compared the expression domains of *Fgf8*, *Bmp-4* and *Shh* in oral epithelium to that of *Lhx6* and *Lhx7* in underlying mesenchyme using in situ hybridisation at E10.5-11.5. As shown in Fig. 7C,D, and consistent with previous reports (Heikinheimo et al., 1994; Crossley and Martin, 1995; Mahmood et al., 1995), expression of *Fgf8* in the branchial region was restricted to the oral epithelium of the first branchial arch and extended in a broad mediolateral domain which matched very closely the underlying domain of expression of *Lhx7* (Fig. 7A,B) and *Lhx6* (not shown). No expression of *Fgf8* was detected in the equivalent region of the second branchial arch, which is also negative for *Lhx6* and *Lhx7* transcripts. *Bmp-4* was also expressed in the oral epithelium of the first branchial arch, but its expression domain was small and restricted to the medial aspect of the maxilla and the mandible (Fig. 7E,F). Shh was only visible in the oral epithelium of the maxilla at this stage (Fig. 7G,H). In addition to the first branchial arch, correlation between *Lhx6/7* and *Fgf8* expression was also observed in the basal forebrain. At the earliest stage at which significant levels of expression of *Lhx6* and *Lhx7* can be detected, the domain of expression of these genes, which includes the MGE and the preoptic area, extends right up to the *Fgf8*-expressing group of cells in the midline of the basal forebrain (Fig. 7I,J and data not shown). Overall, our data show that in the mammalian embryo, the *Lhx6*- and *Lhx7*-expressing domains are closely associated with *Fgf8*-producing cell groups and suggest that members of this growth

factor family are involved in the regulation of expression of these LIM-homeobox genes.

To test this hypothesis, beads soaked in recombinant Fgf8, BMP4 and Shh were implanted into cultured explants of epithelium-free mandibular mesenchyme isolated from E10.5 and E11.5 mouse embryos. Fgf8-releasing beads produced a clear induction of expression of both *Lhx6* and *Lhx7*, while control beads (i.e. soaked in bovine serum albumin-BSA), or beads soaked in BMP4 or Shh were unable, under similar culture conditions, to induce either of these genes (Fig. 8A-F and data not shown). Overall, our data indicate that Fgf8 is capable of regulating the expression of *Lhx6* and *Lhx7* in the first branchial arch mesenchyme.

## **DISCUSSION**

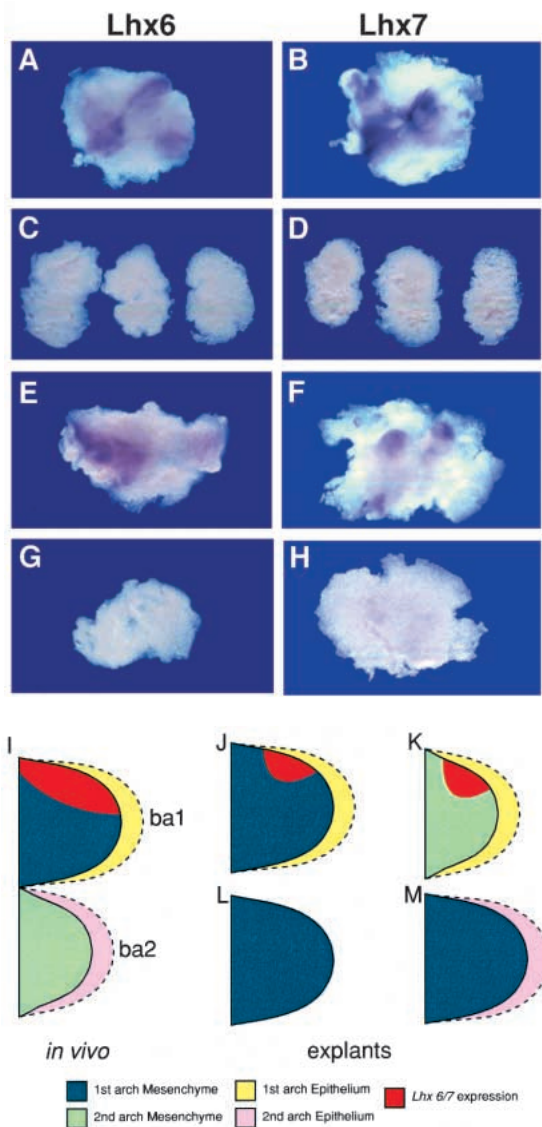
### ***Lhx6* and *Lhx7*: two novel LIM homeodomain encoding genes**

By employing a RT-PCR approach to identify LIM homeodomain encoding genes expressed in enteric neuroblasts, and screening a mouse embryo head cDNA library, we have identified two genes, *Lhx6* and *Lhx7*, which appear to encode novel members of the LIM homeodomain family of transcription factors. Despite the original identification of *Lhx6* in mRNA preparations of enteric neuroblasts, the highest levels of expression during mouse embryogenesis were observed outside the developing gastrointestinal tract. More specifically, abundant mRNAs encoding *Lhx6* and *Lhx7* were observed in the first branchial arch and the MGE of the basal forebrain, suggesting that these transcriptional regulators have a critical role in the morphogenesis and differentiation of specific domains of the mammalian head.

Lhx proteins constitute a distinct subclass of homeodomain proteins characterised by the association of two cysteine-rich motifs, the LIM domains, with a DNA-binding homeodomain (Dawid et al., 1995). Sequence comparison has indicated that the homeodomains of the Lhx proteins are more homologous to each other than to non LIM-associated homeodomains, although the extent of similarity varies significantly between various members of the family. Interestingly, vertebrate *Lhx* genes can be grouped into small subfamilies (2-3 members) which have highly similar homeodomains and a high degree of overall amino acid homology (Dawid et al., 1995; Taira et al., 1995). For example, *islet-1* and *islet-2* and *lim-1* and *lim-2* belong to such subfamilies, with almost identical homeodomains and 72-76% overall homology (Dawid et al., 1995). The sequence data and the (almost identical) pattern of expression of *Lhx6* and *Lhx7* reported here, indicate that they constitute a novel subfamily of LIM homeodomain encoding genes which are likely to play a critical role in the development and patterning of the first branchial arch and the basal forebrain.

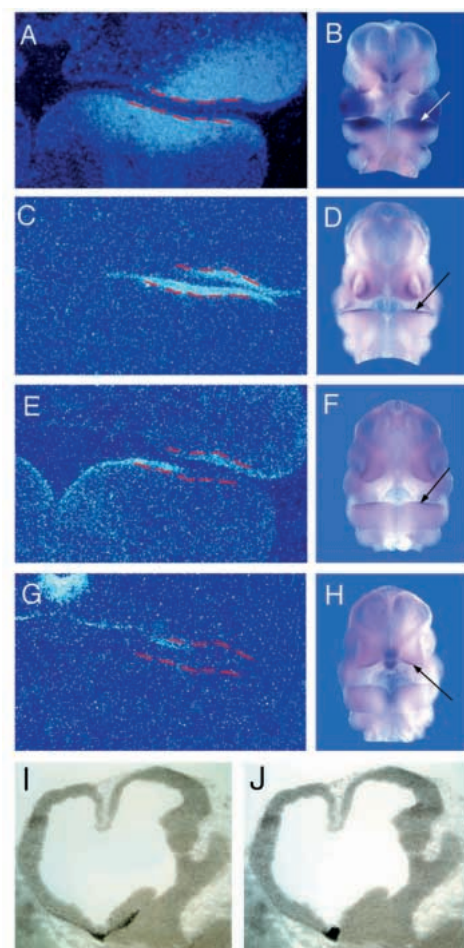
It has been proposed that the LIM domains of the Lhx proteins function as protein-protein interaction motifs which regulate the binding of the homeodomain to DNA and thus the transcriptional activity of the holoprotein (Arber and Caroni, 1996; Feuerstein et al., 1994; Sanchez Garcia and Rabbitts, 1993; Schmeichel and Beckerle, 1994). This suggestion is consistent with mutational analysis which shows that deletion of the LIM domains results in constitutive activation of LIM

homeodomain transcription factors (Sanchez Garcia and Rabbitts, 1993; Taira et al., 1994; Xue et al., 1993). Conversely, overexpression of the LIM domains of a particular class of Lhx proteins results in their inactivation and phenocopies loss-of-function mutations in the corresponding genes (Kikuchi et al., 1997). Based on these findings, a model of negative regulation of Lhx proteins by the resident LIM domains has been



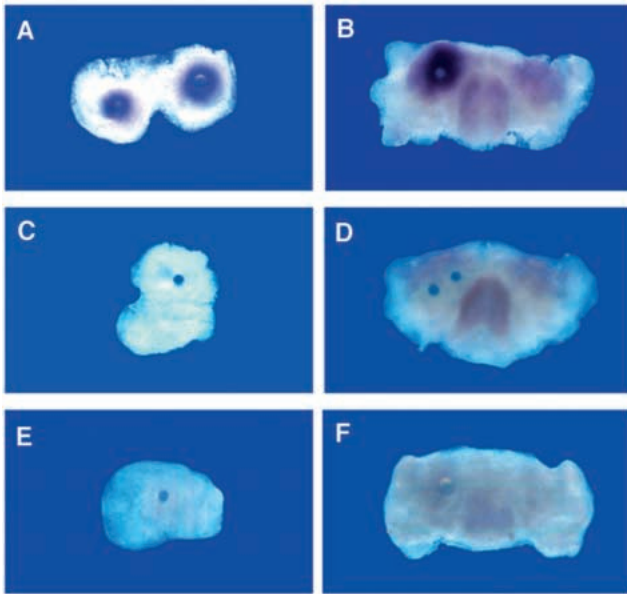
**Fig. 6.** Whole-mount in situ hybridisation analysis of *Lhx6* (A,C,E,G) and *Lhx7* (B,D,F,H) expression in recombinant explants. (A,B) Mandibular mesenchyme that was dissociated and subsequently reassociated with oral epithelium. (C,D) Mandibular mesenchyme that was cultured in the absence of oral epithelium. (E,F) Second arch mesenchyme that was associated and cultured with oral epithelium from the mandibular process. (G,H) Mandibular mesenchyme that was associated and cultured with second branchial arch epithelium. I-M are a schematic presentation of the recombination experiments described in A-H; I shows the in vivo situation. Recombination of first (J) or second arch (K) mesenchyme with first arch epithelium, results in induction of *Lhx6* and *Lhx7* expression. No expression was detected in mesenchyme cultured on its own (L) or recombined with second arch epithelium (M).

suggested, according to which the LIM domains normally interact with and inhibit the binding of the homeodomain to DNA. However, in the presence of an 'activator', which is capable of binding to the LIM domains, the homeodomain is free to bind to DNA and transcriptional activation of the target genes ensues (Kikuchi et al., 1997). The recent isolation of LIM domain binding factors specific for distinct subclasses of Lhx proteins provides further support to this model (Agulnick et al., 1996; Jurata et al., 1996). The identification of a cDNA (*Lhx7a*) encoding a truncated form of the Lhx7 protein suggests a unique feature of this gene, as yet not reported for other Lhx loci, i.e. its ability to encode a transcription factor (*Lhx7*) along with a potential regulator of its activity (*Lhx7a*). It is possible that the product of *Lhx7a* mRNA, when coexpressed with *Lhx7*, functions in a dominant negative



**Fig. 7.** Comparative analysis of expression of *Lhx6*, *Lhx7* and *Fgf8* in the head of E11.5 mouse embryos. In situ hybridisation (with  $^{35}\text{S}$ -labeled riboprobes) on serial sections of the maxillary and mandibular processes of E10.5 mouse embryos (A,C,E,G) and whole-mount in situ hybridisation (with digoxigenin-labeled riboprobes) on the head of similar stage embryos (B,D,F,H). The probes used were specific for: *Lhx7* (A,B), *Fgf8* (C,D), *Bmp-4* (E,F) and *Shh* (G,H). I and J show two serial sections from the brain of E11.5 mouse embryos hybridised with digoxigenin-labeled riboprobes specific for *Lhx7* and *Fgf8*. Similarly to *Lhx7*, the domain of expression of *Lhx6* in the basal forebrain of E11.5 embryos also extends right up to the *Fgf8*-expressing groups of cells (not shown).



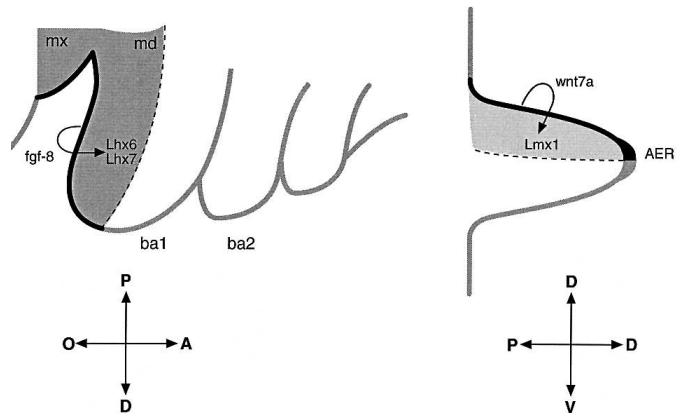


**Fig. 8.** Fgf8 induces expression of *Lhx6* and *Lhx7* in mandibular mesenchyme. Beads soaked in Fgf8 (A,B), BMP-4 (C,D) or BSA (E,F) were implanted in the mesenchyme of mandibles from which the epithelium had previously been removed. The mandibles had been derived from E10.5 (A,C,E) or E11.5 (B,D,F). Induction of *Lhx7* (shown here) or *Lhx6* (not shown) was assayed at the end of the culture period (24 hours) by whole-mount in situ hybridisation. ( $n=10$  for BMP-4 and  $n=15$  for Fgf8 and BSA).

fashion, titrating away potential activators which would otherwise bind to the LIM domains of the full-length protein, thus allowing full activity of its homeodomain.

#### **Lhx6 and Lhx7 and patterning of the first branchial arch**

Several regulatory genes have been described in vertebrate embryos which are expressed in the neural crest-derived mesenchyme of the branchial arches (e.g. *Msx-1*, *Msx-2* and *Dlx* genes) (Bulfone et al., 1993a; Dolle et al., 1992; MacKenzie et al., 1992, 1991; Robinson and Mahon, 1994). However, expression of such genes is usually not restricted to a particular branchial arch, but instead extends throughout the branchial region. In this respect, the expression of *Lhx6* and *Lhx7* is unique in that it is restricted to the mesenchyme of the first branchial arch and its derivatives, the maxilla and mandible. The mesenchyme of the branchial arches is mainly derived from neural crest emigrating from the caudal midbrain and the rostral rhombomeres of the hindbrain (Fukiishi and Morris-Kay, 1992; Serbedzija et al., 1992; Imai et al., 1996; Osumi Yamashita et al., 1994). Heterotopic transplantation experiments in avian embryos have suggested that distinct groups of neural crest cells derived from different axial levels of the hindbrain are specified to develop into characteristic structures prior to their emigration from the neural axis (Noden, 1983). Therefore, the restricted expression of *Lhx6* and *Lhx7* could indicate that the subpopulation of neural crest cells migrating into the first branchial arch is preprogrammed to express these genes. However, our experiments suggest that this is not likely to be the case. First, expression of both *Lhx6* and *Lhx7* in the first branchial arch mesenchyme is not cell



**Fig. 9.** Schematic presentation of the cellular and molecular interactions which lead to patterning of the mandible along the oral-aboral (O-A) axis (left) and dorsoventral patterning of the limb bud (right). In the vertebrate limb bud, expression of *Wnt7a* by the dorsal ectoderm induces expression of *Lmx1* in the dorsal mesoderm (shaded area) which is sufficient for generating dorsal identity. We propose that, in a similar fashion, Fgf8 produced by the overlying oral epithelium of the mandibular process (md), induces expression of *Lhx6* and *Lhx7* which is sufficient for establishment of polarity along the O-A axis of the mandible and tooth formation. Fgf8 is also likely to be responsible for patterning of the maxillary process (mx).

autonomous, but instead is dependent on signals originating in the overlying oral epithelium. Second, in addition to the first arch mesenchyme, the ectomesenchyme of the second branchial arch is capable of expressing *Lhx6* and *Lhx7* when recombined with first arch epithelium. Overall, our data indicate that the molecular events leading up to the expression of *Lhx6* and *Lhx7* in the branchial region of mammalian embryos, do not reflect the unfolding of a developmental programme inherent in the neural crest, but instead result from local inductive interactions taking place subsequently to the migration of the neural crest into the first branchial arch.

In situ hybridisation has identified three possible candidate signalling molecules expressed in oral epithelium between E9.0 and E12.5. Fgf8 is first expressed at E9.0 in oral epithelium of the mandible and maxilla (Heikinheimo et al., 1995; Crossley and Martin, 1995; Mahmood et al., 1995). This expression is followed by *Shh* and *Bmp-4* which are expressed in more localised domains, medial to Fgf8 (Bitgood and McMahon, 1995; Iseki et al., 1996; Aberg et al., 1997) (Fig. 7). We tested the ability of these factors to affect *Lhx6* and *Lhx7* gene expression in mandibular mesenchyme and found potent activation with Fgf8 but no effects with BMP4 or *Shh*. These in vitro studies, combined with the close apposition of the domains of expression of *Lhx6* and *Lhx7* and *Fgf8* in the first branchial arch in vivo, suggest that Fgf8 is the endogenous epithelial signal that induces expression of these genes. Increasing evidence indicates that Fgf8 functions as an organising molecule in a variety of developmental processes. For example in the midbrain/hindbrain boundary, Fgf8 is produced by the isthmic organising centre and is capable of inducing and patterning the midbrain. Thus, implantation of beads soaked in Fgf8 in the caudal diencephalon repatterns the surrounding tissue and leads to formation of ectopic midbrain (Crossley et al., 1996a). Fgf8 also appears to have a critical

role in induction of limb bud and its subsequent patterning. Fgf8 beads placed in the interlimbic region of chicken embryos induce the formation of ectopic limbs from the lateral plate mesoderm (Cohn et al., 1995; Crossley et al., 1996b; Vogel et al., 1995; Ohuchi et al., 1997). Finally, recent experiments have provided evidence that Fgf8 has a critical role in tooth development. It is thought that this growth factor, produced by the oral ectoderm can activate expression of *Pax-9* (a gene necessary for tooth induction) in a wide domain of mandibular arch mesenchyme, while the more medially expressed BMP-4 antagonises this inducing effect, thus restricting *Pax-9* transcripts to the sites of prospective molar tooth mesenchyme (Neubüser et al., 1997). The capacity of Fgf8 to induce several transcription factors, such as *Lhx6*, *Lhx7* and *Pax-9*, indicates that this is an important signalling molecule acting on the mandibular mesenchyme and suggests that it mediates the organising and patterning effects of the oral ectoderm on this tissue.

The development of branchial arches in vertebrate embryos shows many similarities to that of the limb bud. For example, epithelial-mesenchymal interactions are critical for growth and patterning of both structures along their proximodistal axes. Moreover, such interactions are mediated by signals which, to a large extent, are interchangeable between branchial arches and limb buds (Richman and Tickle, 1989, 1992). In addition to the proximodistal axis, limbs have a characteristic dorsoventral (D-V) axis, reflected, for example, in the characteristic pattern of dorsal and ventral muscle groups. A series of studies have shown that the D-V pattern of the mature limb is the result of a D-V axis established in the limb bud during early embryogenesis (for review see: Tickle, 1995). Furthermore, some of the cellular and molecular events that are important in establishing the D-V axis of the limb bud have been identified. It is thought that signalling by the dorsal limb ectoderm, mediated by *Wnt7a*, induces the expression of the LIM homeobox gene *Lmx1* in the underlying dorsal mesoderm, which is sufficient to specify dorsal cell fate during limb development (Riddle et al., 1995; Vogel et al., 1995; and Fig. 7). Branchial arches also develop along a characteristic oral-aboral (O-A) axis which is evident by the anatomical landmarks of the derivative head structures. For example, the mandibular process of the first branchial arch gives rise to the lower jaw, with the O-A axis defined by the presence of teeth along its oral aspect. Despite significant progress in identifying some of the molecules which play a critical role in the development of individual teeth, the molecular mechanisms underlying the patterning of dentition in general and the establishment of the O-A axis of the mandible in particular, are only beginning to be elucidated (Thomas et al., 1997). Based on the data presented here, showing that *Lhx6* and *Lhx7* define the O-A axis of the mandibular process prior to the appearance of tooth primordia (i.e. E10.5), we suggest that these genes have a critical role in development of the mammalian dentition by conferring odontogenic potential to the oral mesenchyme of the mandible. A similar role of *Lhx6* and *Lhx7* can be envisaged for tooth development in the maxillary process. We therefore suggest, that *Lhx6* and *Lhx7* have a role in specifying the ectomesenchymal cells of the first branchial arch that will be competent to form teeth. Furthermore, the demonstration that expression of *Lhx6* and *Lhx7* is under the control of Fgf8 produced by the oral epithelium, suggests a cascade of cellular

and molecular interactions, leading to the establishment of the O-A axis and tooth development, which are analogous to those operating in the establishment of the D-V axis of the vertebrate limb bud. According to such a model, the epithelium covering the oral aspect of the mandibular process produces signalling molecules which are responsible for the induction of *Lhx6* and *Lhx7* in the underlying mesenchyme, leading to the acquisition of oral character and hence tooth formation. The potentially analogous roles played by *Lmx-1* and *Lhx6/7* in the establishment of D-V polarity in the limb bud and the O-A polarity in the mandibular process respectively are indicated schematically in Fig. 9.

### Expression of *Lhx6* and *Lhx7* in odontogenic mesenchyme suggests a role in tooth development

Throughout the late period of mouse odontogenesis, expression of *Lhx6* and *Lhx7* is restricted to the neural crest-derived mesenchyme of the mandible and maxilla. In this respect, these genes resemble several others, such as *Msx-1*, *Pax-9* and *Lef-1*, the expression of which is also restricted to the mesenchyme from the bud stage of tooth development. Knock-out experiments have shown that these transcriptional regulators have a crucial role in the reciprocal inductive interactions between the mesenchyme and the overlying epithelium, which are critical for cellular differentiation and normal tooth development (Kratowil et al., 1996; Satokata and Maas, 1994; van Genderen et al., 1994; Peters et al., 1998). Based on these findings and their expression patterns, we suggest that *Lhx6* and *Lhx7*, in addition to their initial role in patterning the mandible and maxilla, are also involved in signalling between the epithelial and mesenchymal cell layers that is necessary for normal progression of odontogenesis.

### Expression of *Lhx6* and *Lhx7* in the basal forebrain suggests a role in the development of the medial ganglionic eminence

Work over the last several years has identified the basic cellular and molecular mechanisms responsible for patterning of the vertebrate CNS along the main anteroposterior and dorsoventral axes (Lumsden and Krumlauf, 1996; Tanabe and Jessell, 1996). However, further elaboration of this conserved ground pattern is required to generate the specific structures of the brain and spinal cord that perform specialised functions. It is therefore crucial to understand the genetic mechanisms which operate to bring about the regionalization of the vertebrate CNS and identify the molecules that confer specific developmental potential(s) and region-specific cell identity. The pattern of expression of *Lhx6* and *Lhx7* in the developing mammalian CNS suggests that these genes are part of the genetic programme which defines a basal telencephalon territory associated with formation of the MGE. Such a genetic programme might be responsible, for example through expression of differential adhesion systems, for the formation and maintenance of specific boundaries between the MGE and the rest of the developing forebrain. Our data also show that *Lhx6* and *Lhx7* are expressed in distinct subdomains of the MGE; *Lhx6* is expressed in the subventricular and submantle zones, while *Lhx7* is expressed in the submantle and mantle zones. The ventricular zone is negative for both genes. Given that cells located at different positions along the ventricular-pial axis of the embryonic MGE are characterised by distinct

proliferative or differentiation states, the sequential and overlapping expression of *Lhx6* and *Lhx7* along this axis, indicates a potential role of these genes in the progression of the neuroepithelial progenitor cells from the highly dividing and undifferentiated state to that of postmitotic neurons expressing a specific phenotype. For example, it is possible that *Lhx6* and *Lhx7*, uniquely or in combination, are involved in the production of particular neurotransmitters, the selection of specific axonal pathways or innervation of unique targets. Such potential functions would be consistent with similar roles ascribed to other members of the LIM homeodomain family of transcription factors, in both invertebrates and vertebrates (Lundgren et al., 1995; Tsuchida et al., 1994).

The ability of members of the Fgf family of growth factors to induce expression of *Lhx6* and *Lhx7* in de-epithelialised mandibular processes in vitro, raises the possibility that a member of this family of growth factors also controls expression of these genes in the basal forebrain. Consistent with this hypothesis, Fgf8 mRNA is localised in a group of midline cells which borders the *Lhx6*- and *Lhx7*-positive domains of the basal telencephalon. Furthermore, Fgf8 has already been shown to have a crucial role in pattern formation and differentiation of the forebrain in vertebrates (Shimamura and Rubenstein, 1997). However, the positive identification of the signal(s) which induces expression of *Lhx6* and *Lhx7* in the CNS requires further experimentation.

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