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Cloning and characterization of two members of the vertebrate *Dlx* gene family

(brain/development/cartilage/gene expression/homeobox)

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ABSTRACT **A number of vertebrate genes of the *Dlx* gene family have been cloned in mouse, frog, and zebrafish. These genes contain a homeobox related to that of Distalless, a gene expressed in the developing head and limbs of *Drosophila* embryos. We cloned and studied the expression of two members of this family, which we named *Dlx5* and *Dlx6*, in human and mouse. The two human genes, *DLX5* and *DLX6*, are closely linked in an inverted convergent configuration in a region of chromosome 7, at 7q22. Similarly, the two human genes *DLX1* and *DLX2* are closely linked in a convergent configuration at 2q32, near the *HOXD* (previously *HOX4*) locus. *In situ* hybridization experiments in mouse embryos revealed expression of *Dlx5* and *Dlx6* mRNA in restricted regions of ventral diencephalon and basal telencephalon, with a distribution very similar to that reported for *Dlx1* and *Dlx2* mRNA. A surprising feature of *Dlx5* and *Dlx6* is that they are also expressed in all skeletal structures of midgestation embryos after the first cartilage formation. The expression pattern of these genes, together with their chromosome localization, may provide useful cues for the study of congenital disorders in which there is a combination of craniofacial and limb defects.**

Many vertebrate genes have been identified by virtue of their nucleotide sequence similarity with *Drosophila* developmental genes. Many homeobox-containing genes (1) have been identified on this basis. The *Dlx* gene family (2–7) has been identified because these genes contain a homeobox related to that of Distalless (*Dll*, also known as *Ba*) a gene expressed in the head and limbs of the developing fruit fly (8–9).

Cloned *Dlx* sequences in the mouse (2–4), frog (6, 7), and zebrafish (5) have been shown to correspond to at least four different genes, *Dlx1–Dlx4*. A detailed expression analysis has been carried out for murine *Dlx1* (2, 10, 11) and *Dlx2* (3, 4, 12) genes. They appear to be expressed within the central nervous system of midgestation mouse embryos in specific regions of the forebrain, but not in more posterior parts of the neural tube. In early embryos they are also expressed in branchial arches, in the otic vesicle, and in facial and limb primordia. Expression in the developing inner ear has been also reported (5) for the zebrafish cognate of *Dlx3*. With the notable exception of *Xdll2* (7), several frog genes (*Xenopus*) of the *Dlx* family have been identified (6, 7) that are similarly expressed in the anterior portion of the embryonic neural tube. In many instances, a correlation of their expression domain with forebrain regionalization (13, 14) has been suggested (2–7, 10–12).

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We previously reported (11) a detailed comparison of *Dlx1* expression in forebrain of midgestation mouse embryos with that of *Otx* genes. Here, we report the identification and characterization of two additional *Dlx* genes, termed *Dlx5* and *Dlx6*, in mouse and human. Their expression pattern in mouse embryogenesis includes forebrain regions already reported to be sites of *Dlx1* and *Dlx2* expression but also extends to developing skeletal structures. Thus, they are expressed in the developing head and limbs of the mouse with a remarkable parallelism with *Dll*, which is expressed in the developing head and limbs of *Drosophila* embryos.

MATERIALS AND METHODS

Expression Analysis. A cDNA library prepared from 8-week human embryos (15) was screened at low-stringency conditions with a short *Dll* genomic sequence including the homeobox (8). Four classes of homologous cDNA clones, corresponding to *DLX1*, *DLX2*, *DLX5*, and *DLX6*, were found. Using these cDNA clones as probes, we screened in turn a human genomic library constructed in cosmids (15) to study the transcriptional organization and genomic arrangement of these genes.

Human embryonal carcinoma cells of the NT2/D1 line were cultured and treated as reported (16). Northern blot experiments were performed by standard protocols (16), using as probes diverging portions of 3′ untranslated regions. The same regions were used for *in situ* hybridization experiments.

In situ hybridization was carried out as described (17) with minor modifications. Transcription reactions with T7 or SP6 phage polymerase (Riboprobe kit, Promega) were carried out in the presence of [α-[^{35}S]thio]CTP (Amersham). The template was then degraded with RNase-free DNase (Pharmacia), and the labeled RNA was purified through a Sephadex G-50 column and progressively degraded by random alkaline hydrolysis, to improve access to RNA *in situ*. The probes were dissolved at a working concentration of 10^5 cpm/μl in the hybridization mixture (17). Thirty microliters of the appropriate probe in hybridization mixture was added to each slide for hybridization overnight at 55°C. The slides were then washed under stringent conditions [65°C, 2× standard saline citrate (SSC; 1× is 0.15 M NaCl/0.015 M sodium citrate, pH 7) with 50% (vol/vol) formamide] and treated with RNase A (10 μg/ml). Autoradiography was performed with Kodak NT/B2 emulsion. Exposure times were 5–12 days.

Chromosomal Localization. Somatic hybrids used in this study have been described (18). Many of these hybrids were obtained from the Human Genetic Mutant Cell Repository at

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the Coriell Institute for Medical Research (Camden, NJ). Hybrid and control DNAs were digested with an appropriate restriction enzyme, electrophoresed in 0.8% agarose gels, transferred to nylon membranes (Duralon, Stratagene), and hybridized in 50% formamide/standard saline citrate/phosphate/EDTA (1× is 0.15 M NaCl/0.01 M sodium phosphate, pH 7.4/1 mM EDTA)/5× Denhardt's solution/0.1% SDS plus sonicated denatured salmon sperm DNA (100 μg/ml) for 16 hr at 42°C.

Fluorescence *in situ* hybridization was carried out (19) with probes prepared by nick-translation using biotin 11-dUTP (Bionick kit, BRL). Hybridization of biotin-labeled probes was detected with fluorescein isothiocyanate-conjugated avidin. Metaphase chromosomes were identified by Hoechst 33528 staining and UV irradiation (365 nm) followed by 4′,6-diamidino-2-phenylindole staining to produce the banding pattern. Fluorescent signals were observed with filter block 13 (BP450-490/LP515; Leitz Orthoplan) on the background of red chromosomes stained with propidium iodide. Q-banding was observed with filter block A (BP340-380/LP430).

RESULTS AND DISCUSSION

Structure and Arrangement of *DLX5* and *DLX6*. We cloned four human genes containing a homeobox encoding a homeodomain related to those present in gene products of the *Dlx* family. Two of these turned out to be *DLX1* and *DLX2*, cognates of murine *Dlx1* and *Dlx2* genes (2, 3), whereas two were new. Comparison of their homeodomains with those encoded by *Dlx* genes already reported in several vertebrate species (2–7) revealed (Fig. 1*a*) that they were different from the known *Dlx* genes *Dlx1–Dlx4*. For this reason we designated them *DLX5* and *DLX6*.

We first studied their genomic arrangement by using cosmid clones and genomic Southern blot experiments. We observed that *DLX5* and *DLX6* were closely linked in an inverted convergent arrangement within a 20-kb genomic region (Fig. 1*b*). Likewise, *DLX1* and *DLX2* were closely linked in an inverted convergent arrangement within a 25-kb genomic region (Fig. 1*b*). The same arrangement was displayed by the mouse cognates, *Dlx1–Dlx2* and *Dlx5–Dlx6* (data not shown). This type of genomic configuration of four *Dlx* genes in closely linked pairs is different from both the *Hox* genes, which are clustered in tandem (1), and the *Emx* (20) and *Otx* (21) genes, which do not appear to be linked. The genomic organization in clusters of homeotic genes of *Drosophila* HOM-C complexes and of *Hox* genes of vertebrates is believed to play a role in their biological action. In fact, genes belonging to any given *Hox*/HOM cluster are expressed along the embryonic anterior–posterior body axis in a colinear way according to a simple 5′-posterior/3′-anterior rule (1).

Northern blot analysis of RNA from differentiating human embryonal carcinoma NT2/D1 cells showed that *DLX5* and *DLX6* encode mRNAs of 1.8 and 2.7 kb, respectively (Fig. 1*c*). Both genes were transiently activated in these cells upon treatment with 10 μM retinoic acid for up to 2.5 days. Their expression subsequently declined. This temporal profile is in contrast with that observed in the same cells for other homeobox gene families (11, 20, 22).

Analysis of several rodent–human hybrids (data not shown) and fluorescence *in situ* hybridization experiments

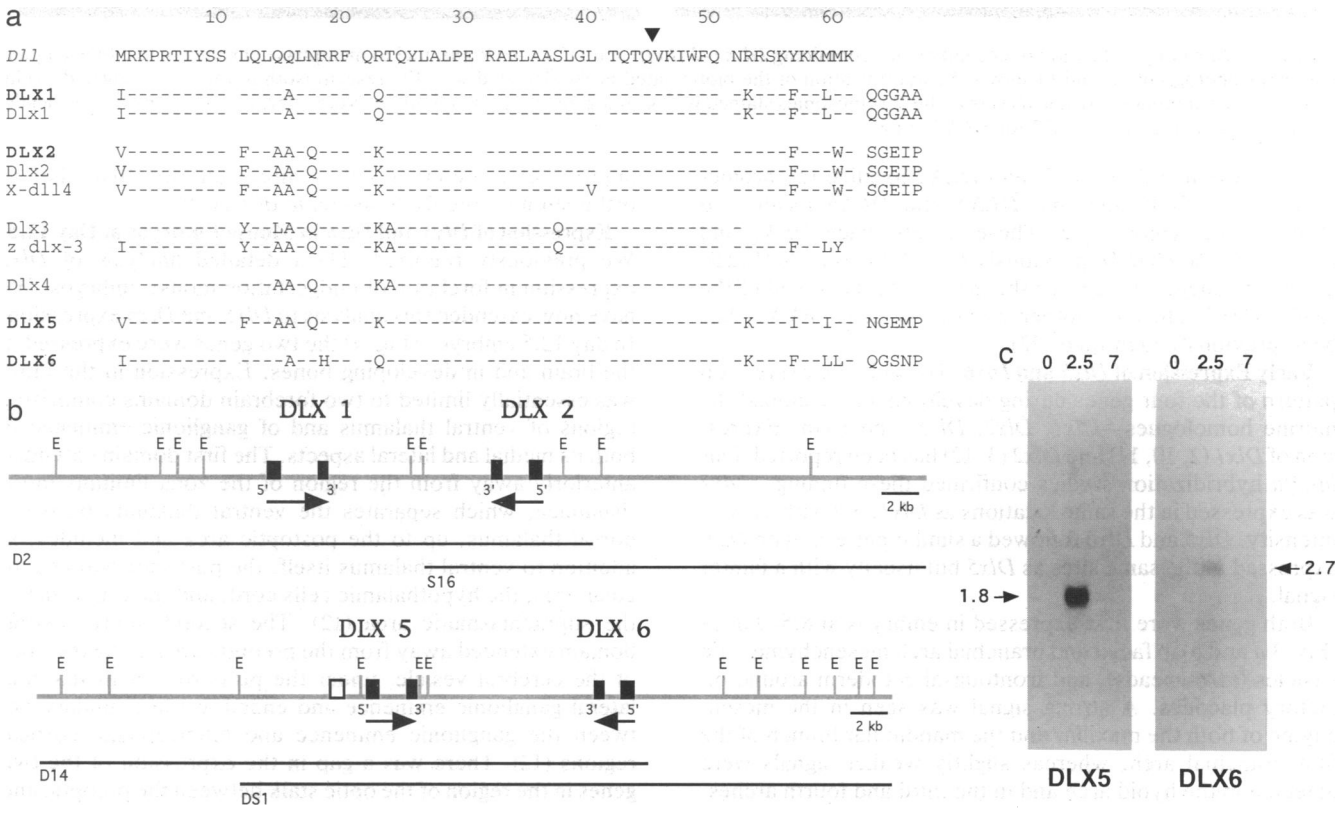

FIG. 1. Structure and organization of four human *DLX* genes. (*a*) Comparison of the four homeodomains with product of the *Drosophila* gene *Dll* (Ba) and other published *Dlx* homeodomains. The one-letter amino acid code is used. Dashes indicate amino acid identity with the *Dll* product and an arrowhead points to splice sites. (*b*) Genomic organization of human *DLX1*, *DLX2*, *DLX5*, and *DLX6* genes. Solid boxes indicate the two exons containing homeobox sequences; open box indicates an upstream exon. Corresponding cosmid clones are shown below genomic maps. *Eco*RI (E) restriction sites are shown. (*c*) Northern blot analysis [3 μg of poly(A)$^+$ RNA per lane] of *DLX5* and *DLX6* in differentiating human embryonal carcinoma NT2/D1 cells, untreated (lane 0) and after 2.5 and 7 days of 10 μM retinoic acid treatment. Approximate transcript sizes are shown in kilobases.

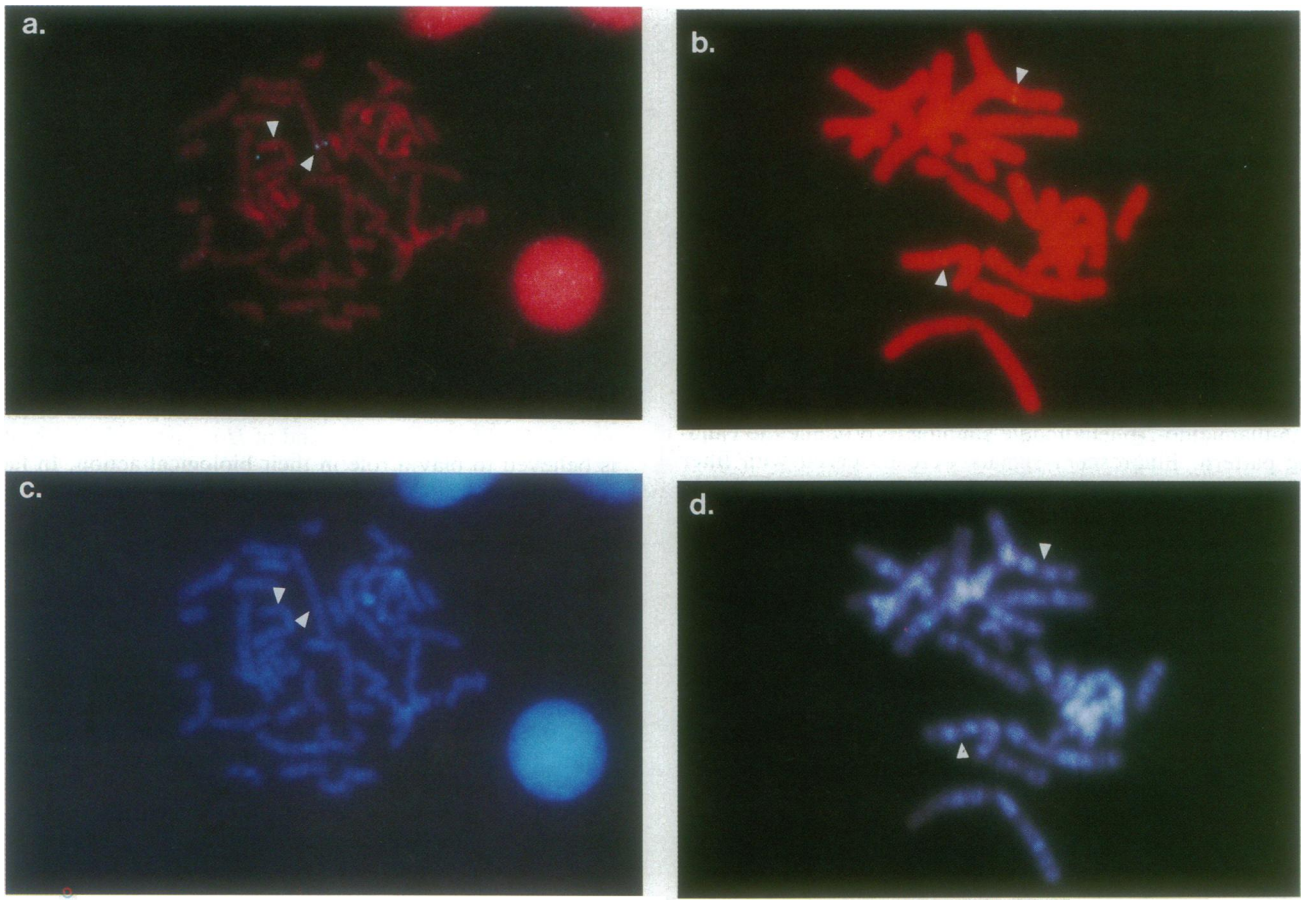

FIG. 2. *In situ* hybridization of cosmid clones containing *DLX1–2 (Left)* and *DLX5–6 (Right)* loci to metaphase chromosomes. In each pair, the upper photograph (*a* and *b*) shows the hybridization of the biotinylated probe detected with fluorescein isothiocyanate-conjugated avidin and the lower photograph (*c* and *d*) shows the chromosomes stained with 4′,6-diamidino-2-phenylindole. Note hybridization signal (arrowheads) at 2q32 for *DLX1–2* and at 7q22 for *DLX5–6* loci.

(Fig. 2) revealed that *DLX1* and *DLX2* localize to chromosome region 2q32 and that *DLX5* and *DLX6* localize to chromosome region 7q22. These results place *DLX1* and *DLX2* near the *HOXD* (previously *HOX4*) locus at 2q31 (22), as already suggested (23) for the mouse. At the 5′ end of the same *HOXD* cluster another homeobox gene, *EVX2*, has been previously identified (22).

Early Expression of *Dlx5* and *Dlx6*. To study the expression pattern of the four genes during development we cloned the murine homologues—*Dlx1*, *Dlx2*, *Dlx5*, and *Dlx6*. Expression of *Dlx1* (2, 10, 11) and *Dlx2* (3, 12) has been reported. Our *in situ* hybridization studies confirmed these findings. *Dlx2* was expressed in the same locations as *Dlx1*, but with a lower intensity. *Dlx5* and *Dlx6* followed a similar pattern, with *Dlx6* expressed in the same sites as *Dlx5* but usually with a fainter signal.

Both genes were first expressed in embryos at 8.5–9 days (Fig. 3 *a* and *b*) in facial and branchial arch mesenchyme, otic vesicles (arrowheads), and frontonasal ectoderm around olfactory placodes. A strong signal was seen in the mesenchyme of both the maxillar and the mandibular branch of the first branchial arch, whereas slightly weaker signals were detected in the hyoid arch and in the third and fourth arches. One day later both genes (Fig. 3 *c* and *d*) began to be expressed also in the developing forebrain in primordia of the ganglionic eminence and ventral diencephalic regions, whereas no expression was detectable in the region of the preoptic recess. Their expression domain in the most anterior region of the developing neural tube was more anterior than that of the *Otx* genes (11). Similarly, in fly embryos the anterior expression of *Dll* is more anterior than that of orthodenticle, the fly homologue of *Otx* (9).

Expression of *Dlx5* and *Dlx6* in Mouse Embryos at Day 12.5. We previously reported (11) a detailed analysis of *Dlx1* expression in forebrain of midgestation mouse embryos. We have now extended this analysis to *Dlx5* and *Dlx6* expression. In day 12.5 embryos (Fig. 4) the two genes were expressed in the brain and in developing bones. Expression in the brain was essentially limited to two forebrain domains comprising regions of ventral thalamus and of ganglionic eminence in both its medial and lateral aspects. The first domain extended anteriorly away from the region of the zona limitans intrathalamica, which separates the ventral thalamus from the dorsal thalamus, up to the postoptic area and includes, in addition to ventral thalamus itself, the posterior entopeduncolar area, the hypothalamic cells cord, and the primordia of the suprachiasmatic area (12). The second, more rostral, domain extended away from the preoptic area along the floor of the cerebral vesicle, under the primordia of medial and lateral ganglionic eminence and ended at the boundary between the ganglionic eminence and telencephalic cortical regions (12). There was a gap in the expression of the two genes in the region of the optic stalk between the preoptic and suprachiasmatic areas. This region gives rise to the eminentia thalami and several hypothalamic primordia.

As previously noticed for *Dlx1* (2, 10, 11) and *Dlx2* (3, 12), expression of these genes was absent in ventricular germinal cells and essentially confined to more internal regions. Their strongest expression was found in the subventricular zone. The exclusion of cells expressing *Dlx* transcripts from ven-

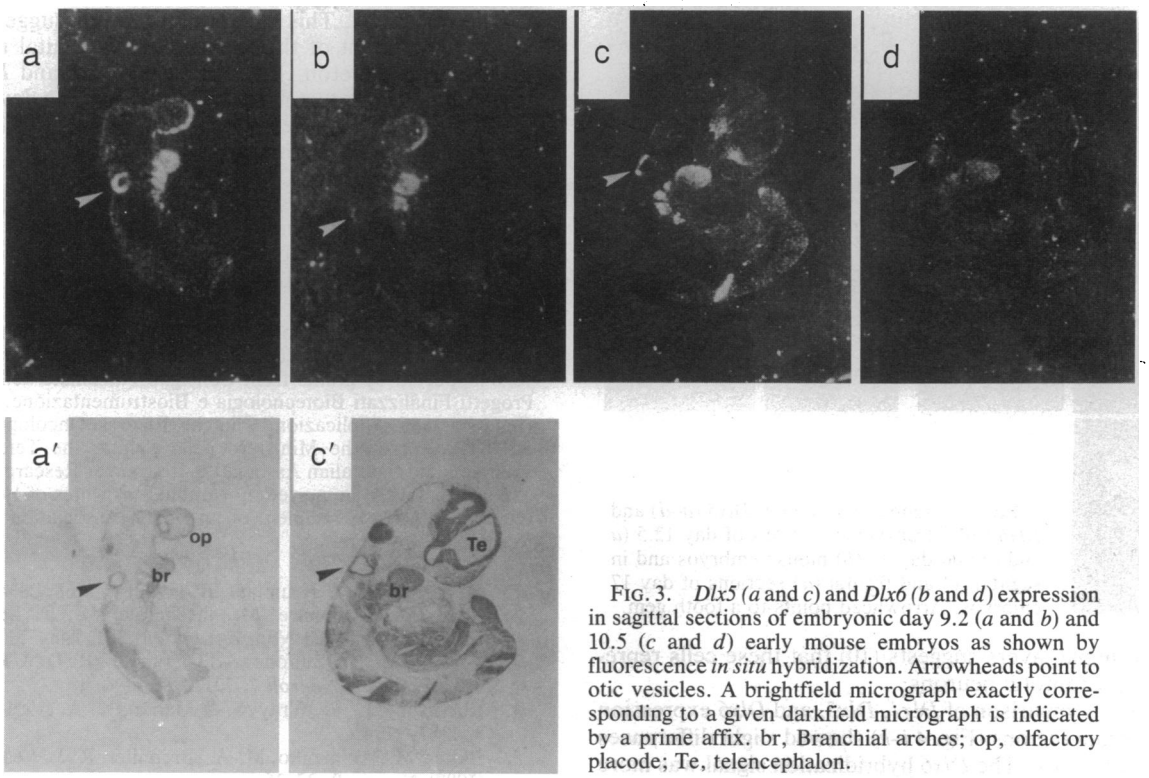

FIG. 3. *Dlx5* (*a* and *c*) and *Dlx6* (*b* and *d*) expression in sagittal sections of embryonic day 9.2 (*a* and *b*) and 10.5 (*c* and *d*) early mouse embryos as shown by fluorescence *in situ* hybridization. Arrowheads point to otic vesicles. A brightfield micrograph exactly corresponding to a given darkfield micrograph is indicated by a prime affix. br, Branchial arches; op, olfactory placode; Te, telencephalon.

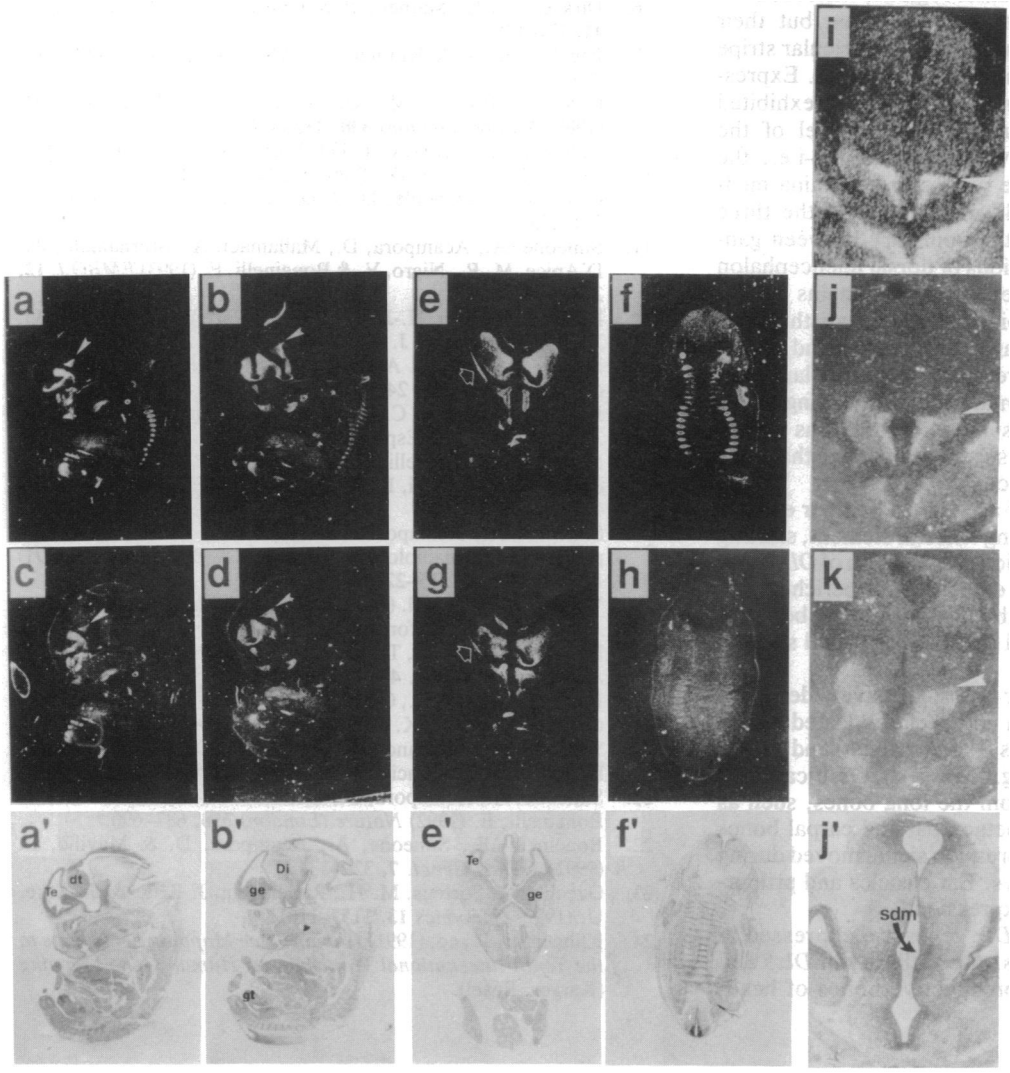

FIG. 4. *Dlx5* (*a*, *b*, *e*, and *f*) and *Dlx6* (*c*, *d*, *g*, and *h*) expression domains in sagittal (*a–d*) or frontal (*e–h*) sections of day 12.5 mouse embryos. Detailed comparison of *Dlx5* (*j*), *Dlx6* (*k*), and *Dlx1* (*i*) expression domains in frontal sections of diencephalon of day 12.5 mouse embryos is also shown. Arrowheads in *a–d* and *i–k* point to the dorsal boundary of ventral thalamus. An empty arrowhead in *e* and in *g* points to developing skull bones. ge, Ganglionic eminence; gt, genital tubercule; Di, diencephalon; dt, dorsal thalamus; sdm, sulcus diencephalicus medialis; Te, telencephalon.

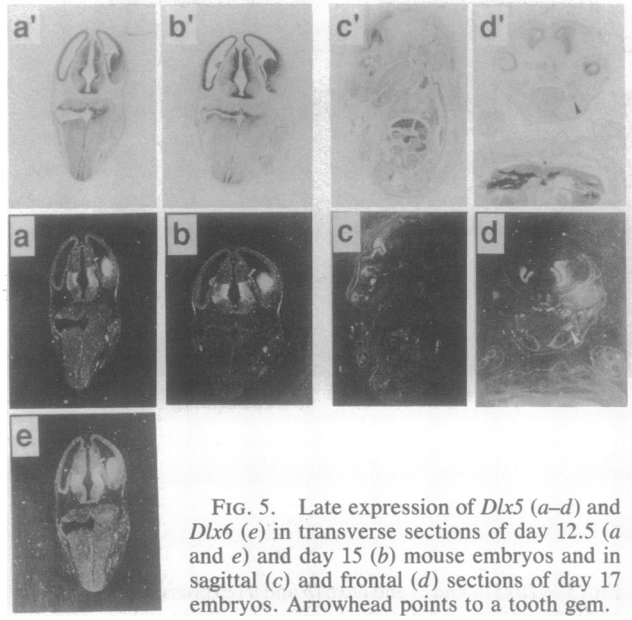

FIG. 5. Late expression of *Dlx5* (*a–d*) and *Dlx6* (*e*) in transverse sections of day 12.5 (*a* and *e*) and day 15 (*b*) mouse embryos and in sagittal (*c*) and frontal (*d*) sections of day 17 embryos. Arrowhead points to a tooth gem.

tricular germinal layers suggests (10) that these cells represent early postmitotic neurons.

A detailed comparison of *Dlx1*, *Dlx5*, and *Dlx6* expression in ventral brain regions (Fig. 4 *i–k*) showed slight differences in relative intensity. The *Dlx6* hybridization signal was more uniform in ventral thalamus and basal regions, whereas *Dlx1* and *Dlx5* were expressed in the same regions, but their expression appeared slightly higher in a subventricular stripe of cells, in both ventral thalamus and basal regions. Expression domains of the three genes in ventral thalamus exhibited a remarkably sharp dorsal boundary at the level of the boundary between dorsal and ventral thalamus—i.e., the zona limitans intrathalamica, the precursor of lamina medullaris externa (arrowheads) (14). Expression of the three genes also abruptly stopped at the boundary between ganglionic eminence and cortical regions of dorsal telencephalon (Fig. 4 *a–g*). Analysis of the expression domains of the various *Dlx* genes in the developing brain suggests that they may provide instructions for early subdivision and/or regional differentiation in the forebrain. In particular, they might be involved in developmental decisions leading to the specification of basal ganglia versus cortical areas, as well as of regions of ventral thalamus versus those of dorsal thalamus and of more posterior brain structures.

A peculiarity of *Dlx5* and *Dlx6* appears to be their expression in practically every developing skeletal element, starting from the first cartilage condensation. Expression of *Dlx5* and, to a minor extent, of *Dlx6* was detectable in perichondrial areas of developing vertebrae, ribs, long and short bones of trunk and limbs, and craniofacial cartilagineous and skeletal structures.

Whereas *Dlx6* expression later on progressively declined, expression of *Dlx5* in both brain and skeleton lasted essentially unaltered in day 15 embryos (Fig. 5 *a* and *b*) and slowly decreased in day 17 embryos (Fig. 5 *c* and *d*). Ossification in the limb progressively moves from the long bones, such as humerus, ulna, and radius, to metacarpal and carpal bones and phalanges. A strong *Dlx5* expression signal moved during this period along similar pathways. Ear ossicles and primordia of teeth also showed *Dlx5* expression.

In early embryos, all known *Dlx* genes are expressed in mesenchyme of branchial arches. A peculiarity of *Dlx5* and *Dlx6* is their expression in all prospective bones of head, trunk, and limbs. This expression pattern suggests a possible involvement of these genes in congenital malformations of the skeleton. In the mouse, *Dlx1* and *Dlx2* have been located near two mutations affecting the development of forelimbs and branchial arch derivatives—namely, Ulnaless and First branchial arch (12). In humans, a number of congenital disorders result in a combination of facial and limb defects. Only a few of them have been mapped. For example, the split hand/foot deformity type 1 (SHFD1) syndrome has been located at 7q21 (24), and we mapped *DLX5* and *DLX6* at 7q22.

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