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Multiple functions of *Dlx* genes

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ABSTRACT ***Dlx*** **genes comprise a highly conserved family of homeobox genes homologous to the *distal-less* (*Dll*) gene of *Drosophila*. They are thought to act as transcription factors. All *Dlx* genes are expressed in spatially and temporally restricted patterns in craniofacial primordia, basal telencephalon and diencephalon, and in distal regions of extending appendages, including the limb and the genital bud. Most of them are expressed during morphogenesis of sensory organs and during migration of neural crest cells and interneurons. In addition, *Dlx5* and *Dlx6* are expressed in differentiating osteoblasts. Gene targeting of *Dlx1, Dlx2, Dlx3* and *Dlx5* in the mouse germ-line has revealed functions in craniofacial patterning, sensory organ morphogenesis, osteogenesis and placental formation. However, no effect on limb development has yet been revealed from gene inactivation studies. A role for these genes in limb development is however suggested by the linkage of the Split Foot/Hand Malformation human syndrome to a region containing *DLX5* and *DLX6*. As for most transcription factors, these genes seem to have multiple functions at different stages of development or in different tissues and cell types.**

KEY WORDS: *Dlx, craniofacial, limb, osteogenesis, brain development.*

Introduction

The *Distal-less* (*Dll*) gene of *Drosophila* encodes a homeodomain protein expressed in leg primordia of the thoracic segments and in anterior regions of the *Drosophila* embryo (Cohen *et al*., 1989; O'Hara *et al*., 1993). During insect limb development *Dll* is expressed in the center of the outgrowing leg primordium and in the distal segments of the leg, throughout the entire larval stage (Diaz-Benjumea *et al*., 1994; Lecuit and Cohen, 1997). *Distal-less* mutant *Drosophila* show various extents of size reduction and dysmorphogenesis of distal segments of the legs in the adult fly, indicating the *Dll* activity is required during early larval stages for the development of the entire limb and for correct proximo-distal (PD) organization (Cohen and Jurgens, 1989; Cohen *et al*., 1989). Homeotic genes of the *Bithorax* complex repress *Dll* transcription in the abdominal segments (Vachon *et al*., 1992). Presumably this mechanism is at the basis of the absence of legs in posterior *Drosophila* abdominal segments (Carrol, 1994; Panganiban *et al*., 1997).

In anterior regions of the *Drosophila* embryo, *Dll* is expressed in the antennal, maxillary and labial primordia. *Dll*-mutant flies show abnormalities of these appendages consistent with a PD growth and morphogenesis defect (Sunkel and Whittle, 1987; Cohen, 1990). It has been shown that *Dll* is activated by the HOM gene *deformed*, in the maxillary primordium. In the insect, therefore, there is a different regulation of *Dll* in gnathal and thoracic segments.

Dll-related genes have been identified and cloned in several species, from Hydra to man. In the mouse, there are six known *Dlx* genes arranged as pairs facing each other through the 3' end and located near *Hox* clusters (Simeone *et al.*, 1994; Nakamura *et al*., 1996; McGuinness *et al*., 1996; Liu *et al*., 1997). The spatial expression of these genes in vertebrates is somehow reminiscent of that observed in insects. Namely they are expressed during limb bud development and in head structures (pharingeal arches, olfactory epithelium, etc.). Although recent data obtained by gene targeting in the mouse begin to cast some light on the role of *distal-less*-related genes in mammals, their function and mode of action, as well as the regulatory cascades of which they are part, remain still to be defined.

Sequence, structure and organization of *Dlx* genes in vertebrates

Vertebrate *Dlx* genes share a highly conserved homeodomain (Fig. 1A) with the *Drosophila distal-less* gene. *Dlx* genes in mouse

Abbreviations used in this paper: AER, Apical Ectodermal Ridge; CNC, Cephalic Neural Crest; dpc, days post coitum; PD, Proximo-Distal; SHFM, Split Hand/Foot Malformation; SVZ, Sub-Ventricular Zone; TDO, Tricho-Dento-Osseous Syndrome.

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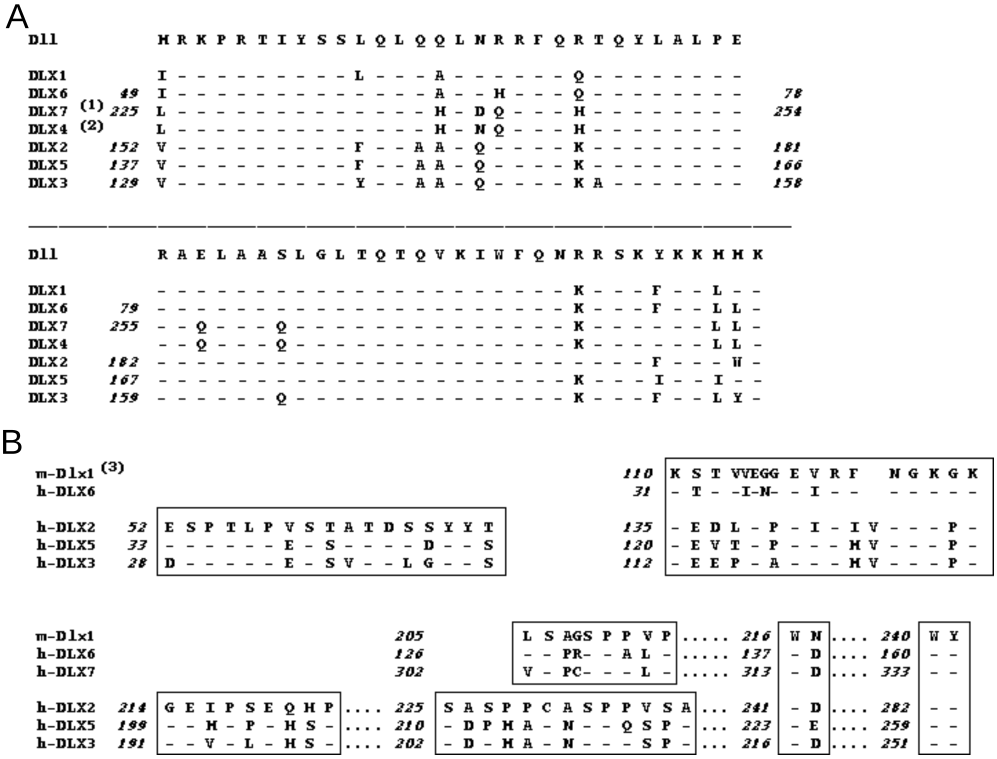

Fig. 1. Amino acid sequence alignment and homology between mammalian *Dlx* genes. (A) *Comparison of the homeodomain sequences of Dlx genes to the Dll homeodomain (human and murine homeodomains are identical).* **(B)** *Other regions of homology between Dlx genes, that support the subdivision of the Dlx family members in two evolutionary distinct subgroups. Dashes indicates amino acid identity. The numbers on the left indicate the codon position. For m-Dlx6 and m-Dlx7, the Start ATG codon has not been reported. m, murine; h, human.*
Notes: (1) h-DLX7 (gi3068596) is identical to h-DLX7 (gi4503342) and to h-DLX4 (gi1657866), so this gene is here reported simply as h-DLX7. (2) This is a partial cDNA sequence (gi1335787) that contains a DLX7-like homeodomain, but shows no similarity in the remaining sequence. This gene encodes a distinct mRNA and is differentially expressed in cell lines (N. Ferrari, personal communication). No corresponding murine sequence has been reported. (3) The human sequence of DLX1 is only partially available.

and man are linked in pairs, in a tandem convergent configuration, in the following order: *Dlx1* and *Dlx2*; *Dlx5* and *Dlx6*; *Dlx3* and *Dlx7*. The only exception appears to be the *DLX4* gene, that is located on the same chromosomal region as *DLX3* and *DLX7*, but for which no tandem partner has been reported. Within each pair, one member shows a higher degree of homology to one gene of another pair, rather that the other gene on the same pair. This has led to a subdivision of the *Dlx* gene family in two subfamily, one including *Dlx*1, -6, -7, and -4, the other including *Dlx*2, -5, -3 (Fig. 1B). These data can be interpreted as an indication of an initial *distal-less* duplication event that occurred in the early chordates, that yielded an ancestor tandem, and a series of subsequent duplications of the entire tandem to yield the mammalian configuration. The analysis of *distal-less* related genes in different vertebrate species substantially supports this hypothesis (Stock *et al.*, 1996).

Further support for this notion comes from the finding that the *Drosophila distal-less* gene is located near the *HOM-C* complex. In human, the *DLX3* and *DLX7* genes are located on chromosome 17q21, as is *DLX4* (Scherer *et al.*, 1995; Nakamura *et al.*, 1996; Quinn *et al.*, 1997; Morasso *et al.*, 1997), near the *HOX-B* homeobox gene cluster. The *DLX1* and *DLX2* genes are linked to the *HOX-D* gene cluster on chromosome 2 (McGuinness *et al.*, 1996), while the *DLX5* and *DLX6* genes are linked to the *HOX-A* cluster on chromosome 7 (Simeone *et al.*, 1994). The same linkage of *Dlx* genes to *Hox* clusters is respected in the mouse genome.

It has been observed that the expression of linked *Dlx* genes is in general very similar or indistinguishable (Simeone *et al.*, 1994; Chen *et al.*, 1996; Ellies *et al.*, 1997a). This suggests that linked *Dlx* genes may share cis-acting sequences that control their pattern of expression in the embryo and in the adult (Zerucha *et al.,* 2000).

Distal-less proteins are transcription factors

Dlx proteins share similar DNA-binding properties *in vitro* (Liu *et al.*, 1997; Zhang *et al.*, 1997; Feledy *et al.*, 1999) and are expected to act as homeodomain transcription factors. Transcriptional activation by Dlx3 protein on reporter construct *in vitro* depends on two subdomains located on either sides of the homeobox (Feledy *et al.*, 1999).

The transcriptional activity of Dlx proteins might be modulated by other proteins. In particular, Msx homeoproteins have been shown to bind to Dlx *in vitro*. The binding is mediated by their homeodomain, and results in a mutual functional antagonism. It is important to note that this effect can only take place *in vivo* in cells in which the two proteins are co-expressed. Indeed partial co-expression of *Msx* and *Dlx* genes occurs in the apical ectodermal ridge (AER) and underlying mesenchyme of the limb bud and in the pharingeal arches (Zhang *et al.*, 1997). However the resolution of *in situ* hybridization experiments is not sufficient to warrant for their cellular coexpression and hence their reciprocal inhibition *in vivo*. If the hypothesis of mutual inhibition is correct then some of the defects observed in *Msx* (Satokata and Maas, 1994; Houzelstein *et al.*, 1997) or *Dlx* mutants might be due to improper activation of partner genes. Analysis of *Msx / Dlx* double mutants will be particularly interesting in this respect.

Dlx genes during limb and appendages development

The expression of the *Dll* or *Dlx* homeoproteins seems to be a common features of appendage outgrowth from arthropods to man. Panganiban *et al.* (1997) have examined the expression of these regulatory genes in protostomes and deuterostomes finding that *Dll* is expressed along the PD axis of such diverse structures as the developing polychaete annelid parapodia, the onychophoran lobopodia, the ascidian ampullae, and even the echinoderm tube feet. In the mouse and chick embryo, the *Dlx* genes are coexpressed in the AER and in the underlying cells of the progress zone of the developing limb bud (Dollé *et al.*, 1992; Bulfone *et al.*, 1993a; Zhao *et al.*, 1994; Simeone *et al.*, 1994; Ferrari *et al.*, 1995; Zhang *et al.*, 1997; Acampora *et al.*, 1999; Ferrari *et al.*, 1999) (Fig. 2A-C). Although there is no evolutionary relation between insect appendages and mammalian limbs, the similarity in terms of territory of expression is striking. *Dll / Dlx* expression in such diverse appendages could be convergent, but this would have required the independent co-option of *Dll / Dlx* several times in evolution. Alternatively, appendicular *Dll / Dlx* expression might have been originated in a common ancestor and been used subsequently to pattern body wall outgrowths in a variety of organisms, including vertebrates. In this regard, it interesting to note that other non-limb appendages express *Dlx* genes. For example, the *Dlx5* gene is strongly expressed in the external ear lobes of newborn mice (Merlo and Levi, unpublished observations) and in the distal part of the genital bud of the mouse embryo, with a complex pattern reminiscent of that of *Hox* genes (Fig. 2 D,E). This later observation is interesting if we consider that *Dll* is expressed in the *Drosophila* genital disk, and that the overall pattern of expression of *Dll* and of the morphogens *wingless* and *decapentaplegic* is similar to that of the leg imaginal disk (Gorfinkiel *et al.*, 1999).

In spite of their strong expression during early and late phases of limb outgrowth, no limb or genital phenotype has been reported for any of the *Dlx* deficient mice obtained so far (Qiu *et al.*, 1995; Qiu *et al.*, 1997; Acampora *et al.*, 1999, Depew *et al.*, 1999). It is conceivable that the various *Dlx* genes coexpressed in the AER serve some redundant function. If this is the case, the disruption of more than one *Dlx* genes might be necessary to uncover their function in limb development.

A strong point in favor of a role of *Dlx* genes in limb development comes from the genetic analysis of families affected by split hand/foot malformations (SHFM). In man *DLX5* and *6* genes are considered as candidate genes for certain types of SHFM since they map to the critical interval of SHFM1 on chromosome 7q21.1 (Sherer *et al.*, 1994). Furthermore, in some families, SHFM with complete penetrance is correlated to deletions, inversions or translocations of the chromosomal region 7q21.3-q21.1 (Sherer *et al.*, 1994). We have recently found (Pfeffer *et al.*, submitted) that the first exon of human and mouse *DLX6* genes contain a CAG/CCG (polyglutamine/poly-proline) repeat region strikingly similar to the trinucleotide repeat present in the Huntington's disease gene. This CAG repeat is polymorphic in the normal human population suggesting that *DLX6* could have a role in the control of limb patterning. Mutation analysis of *Dlx6*–null mutant mice will contribute to answer this question.

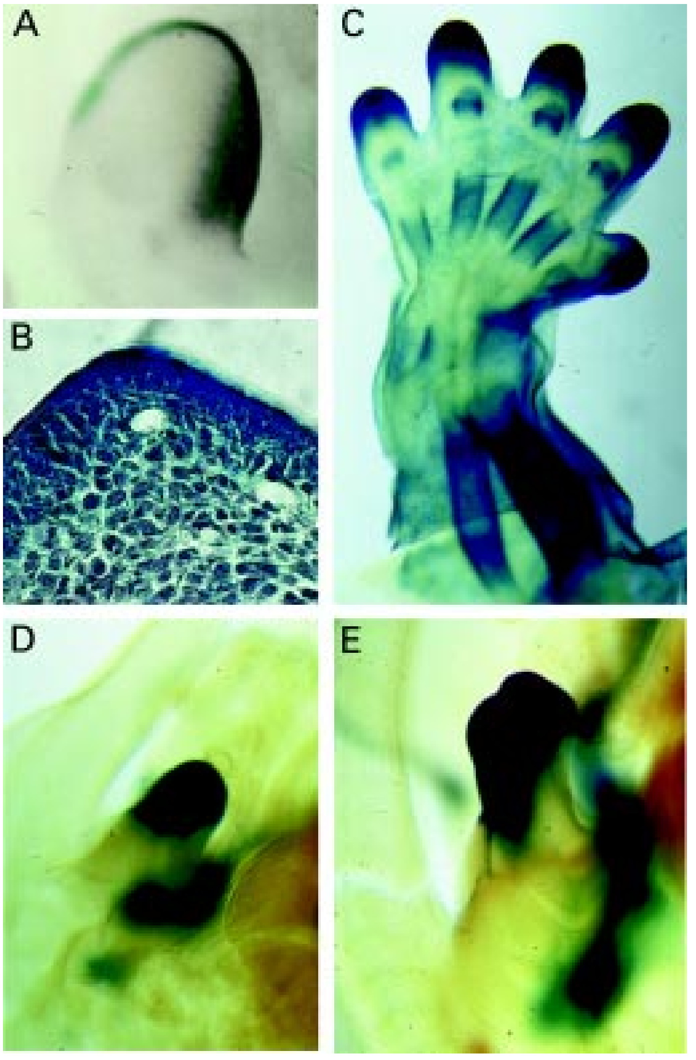

Fig. 2. *Dlx5* expression in extending appendages in the mouse embryo. *Images correspond to heterozygous lac-Z/Dlx5 mutant embryos stained in whole mount for the expression of lac-Z and clarified with benzyl benzoate/ benzyl alcohol to reveal staining of internal structures. We have shown (Acampora et al., 1999) that in these heterozygous mutants, development is normal and lac-Z expression faithfully reproduced the pattern of expression of Dlx5. Expression of Dlx5 in the limb bud at (A, B) 10.5 dpc is seen in the AER and in the underlying mesenchymal cells, by* **(A)** *whole-mount staining and* **(B)** *by histology. At later developmental stages* **(C**, *14.5 dpc***)** *Dlx5 expression is strong in the progress zone at the tip of each finger and in all skeletal elements. Expression of Dlx5 in the genital bud of* **(D)** *12.5 and* **(E)** *14.5 dpc embryos is confined to the distal part with a clear dorso-ventral asymmetry and PD gradient.*

Dlx genes in craniofacial development

The earliest skeletal elements to appear during mammalian skull development are cartilage structures, evolved from modification of ancient elements of more primitive vertebrates (reviewed in: Hanken and Thorogood, 1993), collectively known as chondrocranium. Part of the chondrocranium gives rise to the skeleton around the nose, eye, inner ear, and the base of the brain, and is known as neurocranium. The chondrocranium derived from the branchial arches gives rise to most of the facial skeleton and is known as splanchnocranium. Most of the chondrocranial elements undergo ossification, but some regress (i.e. Meckel's cartilage of the first arch). A third component of the skull appears later and originates by intramembranous ossification, as is known as dermatocranium. This type of bone formation is characteristic of the calvaria but is seen also around previously formed chondrocranial elements. In Fig. 3A we summarize the developmental origin and the relative position of skeletal elements of the splanchnocranium.

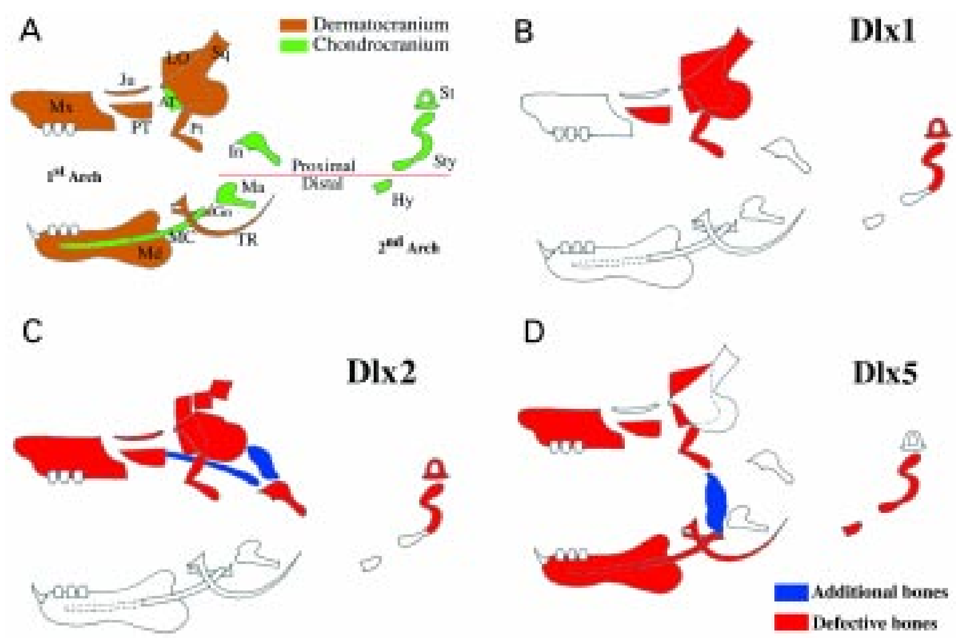

Fig. 3. Summary of craniofacial skeletal defects in *Dlx* mutant mice. (A) *Schematic drawing of splanchnocranial-derived skeletal elements, with indications for first and second pharingeal arch, for proximal / distal boundary (red line), and for chondro- or dermato-cranial developmental origin.* **(B-D)** *Morphogenetic defects observed at birth in the splanchnocranium of Dlx mutant mice, classified as alteration of shape and/ or size (red) and appearance of additional elements (blue). The data are derived from: Qiu et al., 1995, 1997; Acampora et al., 1999; Depew et al., 1999. The double Dlx1 / Dlx2 mutant mice are discussed in the text. AT, Ala Temporalis of the Alisphenoid; Hy, Hyoid (superior horn); Go, Gonial; In, Incus; Ju, Jugal; LO, Lamina Obturans of the Alisphenoid; Ma, Malleus; MC, Meckel's cartilage; Md, Mandible; Mx, Maxillary; PT, Palatine; Pt, Pterygoid; Sq, Squamosal; St, Stapes; Sty, Styloid process; TR, Timpanic Ring.*

Fate-mapping studies in the chicken embryo indicate that the dermato- and splanchnocranium originate from cephalic neural crest (CNC)-derived mesenchyme (Le Douarin, 1982; Noden, 1983; Couly *et al.*, 1993; Koentges and Lumsden, 1996). Craniofacial-forming mesenchyme appears to possess early-established positional information. The complex genetic control of CNC migration and differentiation is gradually being elucidated through the analysis of mutant mice.

Members of the *Dlx* gene family are expressed early in CNC cells and later in craniofacial mesenchyme (Dollé *et al.*, 1992; Bulfone *et al.*, 1993a; Akimenko *et al.*, 1994; Robinson and Mahon, 1994; Simeone *et al.,* 1994; Ellies *et al.,*1997b; Qiu *et al.,* 1997; Yang *et al.,* 1998; Acampora *et al.,* 1999; Depew *et al.*, 1999). At 9.5 days of mouse development, *Dlx1* and *Dlx2* genes are expressed in both the maxillary (proximal) and the mandibular (distal) component of the first phayingeal arch, whereas the *Dlx3*, *Dlx5* and *Dlx6* are expressed only in the mandibular portion of the first branchial arch.

In Fig. 3B–D, we summarize the details the skull defects observed in *Dlx1*, *Dlx2*, and *Dlx5* deficient mice. Craniofacial defects of *Dlx2* mutant mice can be divided in first arch-derived (incus, alisphenoid, maxillary, jugal, squamosal, palatine and pterygoid) and second arch-derived (stapes and styloid). Notably, no distal elements (first arch: Meckel's cartilage, malleus, dental, tympanic and gonial; second arch: upper hyoid horns) are affected by this mutation (Qiu *et al.*, 1995). *Dlx1* null mutant mice exhibit defects affecting the first arch-derived alisphenoid and, to a lesser extent, the palatine and pterygoid bones. The double mutant *Dlx1* / *Dlx2* mice exhibit not only the defects of the single knockouts, but also a novel defect, namely the absence of upper molar teeth (Qiu *et al.*, 1997). The lack of distal defects has been interpreted as the indication of a functional redundancy of the various *Dlx* genes in the mandibular portion of the first arch where all of them are coexpressed (Qiu *et al.*, 1997).

In the *Dlx5* null mutants mice morphological alterations are observed in skeletal elements derived from both the proximal and the distal domains of the first branchial arch (Acampora *et al.*, 1999, Depew *et al.*, 1999). The presence of defects in derivatives of the mandibular arch of *Dlx5-/-*animals, where also *Dlx1*, *2*, *3* and *6* are expressed at 9.5 dpc suggests that redundancy between *Dlx* genes is not generalized, but occurs only in specific cases. The results of *Dlx1* and *Dlx2* knock-outs have led to the proposition that a PD pattern of nested *Dlx* gene expression might be the basis of PD specification of splanchnocranial skeletal elements (Qiu *et al.*, 1997). Although attractive, this hypothesis appears overly simplified in the light of the *Dlx5* knock-out mouse as some craniofacial defects observed in *Dlx5-/-* mice cannot be simply explained by a PD patterning of arch organization. For example the maxilla and the palatine bones are defective both in *Dlx2*$^{-/-}$ and in *Dlx5-/-* mice; these bones derive from the proximal part of the maxillary arch where *Dlx5* expression is not present at 9.5 dpc (but where is expressed at later stages). In our view, the origin of the molecular patterning within the branchial arches should be seen in a more dynamic perspective. Our data show (Fig. 4) that the territory of expression of *Dlx5* in the first branchial arch changes during development. At 9.5 dpc is expressed in the distal part of the mandibular portion of the first arch with low incipient expression in the maxillary arch. In subsequent stages the territory of expression rapidly extends to most of the mandibular and maxillary arch. The expression of the "proximal" *Dlx* genes (*Dlx1*, *Dlx2*) precedes that of the "distal" ones (*Dlx3*, *Dlx5*, *Dlx6*) in the proximal domain of the branchial arches. This dynamic view of *Dlx* gene expression appears to better explain the morphological defects resulting from *Dlx* gene disruption in the mouse. A precise knowledge of the timing of expression and of the exact distribution within the arch of sets of genes interacting in a complex spatio-temporal manner will be needed to understand the molecular codes governing craniofacial development. These complex patterns of expression will most probably cause local changes in cell proliferation and/or apoptosis ultimately resulting in the specific morphology of each bone. For example we have found that inactivation of *Dlx5* alters the territory with the highest density of proliferating cells within the first branchial arch. In the absence of *Dlx5* this territory results expanded and a new boundary is defined (Acampora *et al*., 1999). Alteration in cell proliferation might also help in understanding the frequent appearance of additional skeletal elements (referred as "os paradoxicum", or "strutt") in *Dlx* and other mutants (Qiu *et al.*, 1995; Depew *et al.*, 1999; Acampora *et al.*, 1999).

Dlx genes in sensory organs morphogenesis

Several *Dlx* genes are expressed in the otic vesicle (Robinson and Mahon, 1994; Qiu *et al.*, 1997; Acampora *et al.*, 1999; Depew *et al.*, 1999), the transitory embryonic structure that gives rise to the epithelial and neurosensory component of the vestibular and acoustic organs of the inner ear (Webb and Noden, 1993; Torres and Giraldez, 1998). *Dlx5* and *-6* expression can be detected as early as the otic pit stage, but is later confined to the dorso-lateral region of the vesicle, which originates the vestibular portion of the inner ear (Fig. 4). Accordingly, mice deficient for *Dlx5* show severe dysmorphogenesis of the semicircular canals while little or no defects are observed in the cochlea (Acampora *et al.*, 1999; Depew *et al.*, 1999).

A number of transcription factors are expressed in specific territories of the otic vesicle (Torres and Giraldez, 1998) and may be part of a transcriptional cascades involving *Dlx* genes. In an effort to identify genes upstream and downstream in the *Dlx* regulatory cascades that control otic development, mice with targeted inactivation of different genes showing similar inner ear defects may indicate a possible functional relation. For instance, mice deficient for the *Nkx-5.1/Hmx3* or for the *Dlx5* transcription factors show strikingly similar vestibular defects (Hadrys *et al.*, 1998; Wang *et al.*, 1998; Acampora *et al.*, 1999; Depew *et al.*, 1999). However, *in situ* hybridization with *Dlx5* or with *Nkx-5.1* probes on, respectively, *Nkx-5.1* or *Dlx5* null embryos failed to show changes in their expression profile, making it unlikely that these two genes be part of the same regulatory cascade (Acampora *et al.*, 1999; Zerega and Levi, unpublished observation).

A peculiarity of the *Dlx5* and *-6* genes is their early expression in defined regions of the frontonasal ectoderm (Yang *et al.,* 1998; Acampora *et al.,* 1999; Depew *et al.*, 1999), in the olfactory placodes, and subsequently in the olfactory and respiratory epithelium that lines the nasal cavities and the vomeronasal organ of the mouse (Fig. 4). At present, defects affecting the olfactory epithelium in *Dlx5* deficient mice have not been unequivocally demonstrated. The olfactory placode, similarly to the otic placode, invaginates and delaminates in a complex series of morphogenetic events, induced in part by signals from the surrounding mesenchyme (Webb and Noden, 1993). Although the *Dlx5* gene is expressed very early in both the olfactory and otic placodes, its activity does not seem to be required for their initial specification, since invagination and early morphogenesis takes place normally.

Dlx genes in brain development

Several transcription factors are expressed in subpopulations of neurons in the developing forebrain and olfactory bulb. Their restricted domains of expression define distinct regions of the early forebrain. Among these are members of the *Dlx* gene family (Price, 1993; Bulfone *et al.*, 1993b; Porteus *et al.*, 1994; Tole and Patterson, 1995). *Dlx1* and *-2* are expressed in cells of the subcortical telencephalon that migrate across the pallial-subpallial limit and enter the mantle and subventricular zone (SVZ) of the cerebral cortex in 12.5 dpc mouse embryos. Mice with a disrupting mutation of the *Dlx1* and *-2* genes exhibit a reduction in number and a defective differentiation of both striatal projection neurons and neocortical interneurons (Anderson *et al.*, 1997a,b). This supports the current hypothesis that cortical projection neurons and interneurons are derived from distinct regions of the telencephalon (Anderson *et al.*, 1999).

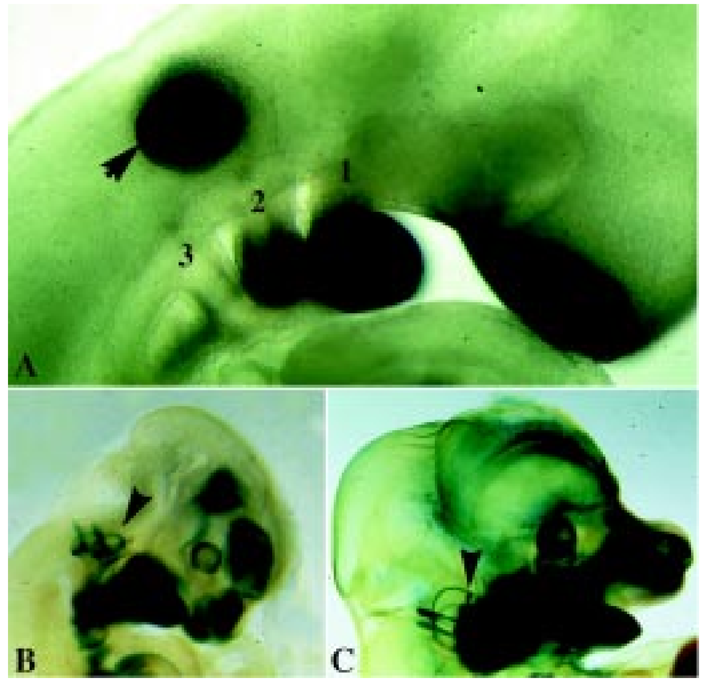

Fig. 4. Expression of *Dlx5* in the developing head of mouse embryo. *Head regions of lac-Z/Dlx5 embryos treated as in Fig. 1.* **(A)** *At 9.5 dpc, staining is seen in the distal part of the first two pharingeal arches (1, 2) and is incipient in the third arch (3); strong staining is also seen in the otic vesicle (arrowhead) and in the ventral cephalic epithelium.* **(B)** *At 11.5 dpc, the staining has extended to all pharingeal arches, is particularly strong in all the mandibular part of the first arch, and begins to extend to the maxillary region. Staining is also prominent in the vestibular system (arrowhead), in the olfactory epithelium and in restricted regions of the brain.* **(C)** *At 13.5 dpc, strong staining is observed in the mandibular and maxillary part of the first arch and persists in the vestibular organ and olfactory epithelium.*

Later in development *Dlx1* and *Dlx2* are also expressed in the interneurons of the olfactory bulb (Porteus *et al.,* 1994), cells derived from proliferation and migration from the SVZ. The *Dlx*1/*Dlx*2 knockout mice lack mature periglomerular and granule cells of the olfactory bulbs, represented by GABAergic interneurons. This defect results from a block in the migration and differentiation of SVZ-derived cells from the basal telencephalon (Qiu *et al.*, 1997). The interesting observation is that the development of the two major neuronal cell types in the olfactory bulb, the projection neurons and the inhibitory interneurons, are under distinct genetic control, and express different subset of transcription factors (Bulfone *et al.*, 1998). In addition there is evidence in favor of an anatomically distinct origin of these cells, as is the case for the cortical projection neurons and interneurons (Anderson *et al.*, 1999). Thus, a model has been suggested in which olfactory bulb projection neurons are generated from progenitors in the ventricular zone of the developing bulb and express transcription factors characteristic of the cerebral cortex, whereas most interneurons in the bulb are generated in the SVZ that express subcortical transcription factors.

The *Dlx5* and *-6* genes are expressed in the developing forebrain, with a very similar profile (Simeone *et al.*, 1994). Transcripts are detected early in the primordium of the ganglionic eminence, and in the ventral diencephalon. At 12.5 dpc these genes are expressed in the ventral thalamus, in both the medial and lateral ganglionic eminence, and in the basal telencephalic vesicle anterior to the preoptic area. At later stages *Dlx5* and *–6* are expressed in the SVZ of the olfactory area and in the developing olfactory bulbs. Finally, at birth expression is found also in the olfactory tuberculum and in the neocortex (Simeone *et al.*, 1994; Acampora *et al.*, 1999; Merlo and Levi, unpublished observation).

We have mentioned above that several *Dlx* genes are expressed in the primordia of the basal ganglia, in overlapping pattern according to the stage of cell differentiation (Liu *et al.*, 1997). *Dlx1* and *-2* are expressed in the least mature cells both in the ventricular and in the SVZ. In contrast *Dlx5* is expressed in cells of the SVZ and in post-mitotic cells of the mantle, but not in the ventricular zone, while *Dlx6* expression is higher in the mantle cells (Liu *et al.*, 1997). These data suggest that each *Dlx* gene may play a specific role in the differentiation of the cell types that compose the basal ganglia.

Dlx genes and osteogenesis

Unlike all other members of the mammalian *Dlx* family, the *Dlx5* and *Dlx6* genes are expressed in all skeletal elements from the time of initial cartilage formation onward (Simeone *et al.*, 1994; Zhao *et al.*, 1994). A further suggestion that *Dlx* genes might be important for the control of osteogenesis comes from the observation that Dlx5 protein represses osteocalcin gene transcription in cultured calvaria cells via a cis-acting homeodomain-binding site (Ryoo *et al.*, 1997). The mechanism of such regulation appears to involve antagonizing of the Msx-2 repression function on the same promoter (Newberry *et al.*, 1998). This is particularly relevant since Msx and Dlx proteins have been shown to heterodimerize and antagonize each other's activity (Zhang *et al.*, 1997).

As the *Dlx5* mutation that we have generated was characterized by an in-phase insertion of *lac-Z*, we could analyze in detail the expression of this gene during osteogenesis. We have shown that *Dlx5* expression is found in all bones during osteoblast differentiation (see Fig. 2C) and disappears in fully differentiated osteocytes. Its expression is prevalent in periosteal bone, but is also seen in a few cells of the endosteal compartment which might represent osteoblasts at a specific stage of differentiation. *Dlx5-/-* mice show a delayed ossification of dermatocranial bones which resemble that observed in mice in which one copy of *Cbfa1*, a key regulator of osteoblast differentiation, is inactivated (Otto *et al.*, 1997). However, in *Dlx5-/-* mice, we observe a mild defect of osteogenesis, which suggests that this gene plays a role in osteoblast differentiation and in bone formation (Acampora *et al.*, 1999). As *Dlx5* is coexpressed during osteogenesis with *Dlx6*, it is possible that a more severe effect will be observed when both genes will be inactivated.

A further possible involvement of *Dlx* genes in development of mineralized tissues comes from the identification of a 4 bp deletion in human *DLX3* which correlated with the tricho-dento-osseous (TDO; OMIM 190320) syndrome phenotype in 6 families. This mutation causes e a frameshift and a premature termination codon, resulting in a functionally altered DLX3 protein (Price *et al.,* 1998a,b). TDO is characterized by an autosomal dominant inheritance of enamel hypoplasia and hypocalcification with associated strikingly curly hair. Unfortunately it has not been possible so far to study the *in vivo* function of *Dlx3* in bone development, as the targeted deletion of the mouse *Dlx3* gene resulted in embryonic death between 9.5 and 10 dpc, due to placental defects that altered the development of the labyrinthine layer (Morasso *et al.*, 1999). *Dlx3* gene is initially expressed in ectoplacental cone cells and chorionic plate, and later in the labyrinthine trophoblast of the chorioallantoic placenta, where major defects are observed in the *Dlx3*$^{-/-}$ embryos.

Dlx genes and hematopoiesis

Shimamoto *et al.,* (1997) have shown that *DLX7* is expressed in normal bone marrow cells and at a particularly high levels in cell lines with the erythroid phenotype. Inhibition of *DLX7* gene expression by an antisense oligonucleotide directed against *DLX7* in erythroleukemia cell lines reduced the plating efficiency and induced apoptosis. The antisense treatment was accompanied by a reduction in GATA-1 and *c-myc* mRNA levels. These results suggested that the function of the *DLX7* gene may be linked to some aspect of erythropoiesis, possibly in the regulation of apoptosis that occurs during normal erythropoiesis. In our laboratory we have found a severe deregulation of several *DLX* genes in acute lymphoblastic leukemia patients which might also suggest a function of these genes in the control of apoptosis (Brigati *et al.*, in preparation).

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