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# Role of the Dlx Homeobox Genes in Proximodistal Patterning of the Branchial Arches: Mutations of Dlx-1, Dlx-2, and Dlx-1 and -2 Alter Morphogenesis of Proximal Skeletal and Soft Tissue Structures Derived from the First and Second Arches

**Mengsheng Qiu,[1] Alessandro Bulfone,[1,2] Ingrid Ghattas,[1] Juanito J. Meneses,* Lars Christensen,† Paul T. Sharpe,† Robert Presley,‡ Roger A. Pedersen,* and John L. R. Rubenstein[3]**

*Nina Ireland Laboratory of Developmental Neurobiology, Center for Neurobiology and Psychiatry, Department of Psychiatry and Programs in Neuroscience and Developmental Biology, 401 Parnassus Avenue, University of California at San Francisco, San Francisco, California 94143-0984; ‡Anatomy Unit, University of Wales, Cardiff, United Kingdom; †Department of Craniofacial Development, Guy's Hospital, University of London, United Kingdom; and *Reproductive Genetics Division, Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California at San Francisco, San Francisco, California 94143-0984*

**The Dlx homeobox gene family is expressed in a complex pattern within the embryonic craniofacial ectoderm and ectomesenchyme. A previous study established that Dlx-2 is essential for development of proximal regions of the murine first and second branchial arches. Here we describe the craniofacial phenotype of mice with mutations in Dlx-1 and Dlx-1 and -2. The skeletal and soft tissue analyses of mice with Dlx-1 and Dlx-1 and -2 mutations provide additional evidence that the Dlx genes regulate proximodistal patterning of the branchial arches. This analysis also elucidates distinct and overlapping roles for Dlx-1 and Dlx-2 in craniofacial development. Furthermore, mice lacking both Dlx-1 and -2 have unique abnormalities, including the absence of maxillary molars. Dlx-1 and -2 are expressed in the proximal and distal first and second arches, yet only the proximal regions are abnormal. The nested expression patterns of Dlx-1, -2, -3, -5, and -6 provide evidence for a model that predicts the region-specific requirements for each gene. Finally, the Dlx-2 and Dlx-1 and -2 mutants have ectopic skull components that resemble bones and cartilages found in phylogenetically more primitive vertebrates.** 

## INTRODUCTION

Development of craniofacial structures requires complex interactions involving multiple embryonic tissues (Hanken and Hall, 1993). The embryonic central nervous system (CNS) produces the cranial neural crest (CNC), which forms the visceral skeleton (dermatocranium and splanchnocranium), and the connective tissues that encase mesodermally derived muscles (Noden, 1988; Couly *et al.*, 1993; Serbedzija *et al.*, 1992; Osumi-Yamashita *et al.*, 1994, 1996; Schilling and Kimmel, 1994; Trainor and Tam, 1995; Kontges and Lumsden, 1996). The mesoderm also forms the neurocranial skeleton that encases the base of the brain, eye, cochlea, and vestibule. Specialized regions of the nonneural ectoderm, such as placodes, participate in the formation of the eye, inner ear, and several other structures (Fujiwara *et al.*, 1994; Northcutt, 1993).

The genetic control of these processes is beginning to be elucidated, as a large number of candidate regulatory genes

[1] Co-first authors.

[2] Present address: San Raffaele Institute, Milan, Italy.

[3] To whom correspondence should be addressed at 401 Parnassus Ave., UCSF, San Francisco, CA 94143-0984. Fax: 415-476-7884. E-mail: jlrr@cgl.ucsf.edu.

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have been identified and mutations in these genes are rapidly being made. Among these, homeobox transcription factors of the Hox family are the best characterized. The Hox genes are expressed in the surface ectoderm, the embryonic hindbrain, and the premigratory and migratory CNC that populate the branchial arches, except for the first arch (Krumlauf, 1993). Mutation of Hoxa-2, which is expressed in the pre- and postmigratory CNC that contribute to the second and more posterior arches, leads to an anterior transformation of the second arch into some of the bones found in the first arch (Rijii *et al.*, 1993; Gendron-Maguire *et al.*, 1993). This and other studies support the idea that the Hox genes regulate anteroposterior (A-P) identity of the CNC. This result is consistent with transplantation studies, suggesting that some aspects of the CNC developmental program are encoded in the premigratory CNC cells (Noden, 1983, 1988). While the Hox genes apparently can specify A-P properties of the CNC for the second and perhaps more posterior arches, other genes that are expressed in the central nervous system anterior of rhombomere 3, such as Otx-2, must be essential for regulating development of the first arch. Otx-2 is expressed in the mesencephalic neural plate (Simeone *et al.*, 1993), which contributes CNC to the first arch (Osumi-Yamashita *et al.*, 1994; Kontges and Lumsden, 1996). Mice that are heterozygotes for a mutation in Otx-2 have abnormal bones derived from the mandibular and maxillary components of the first arch (Matsuo *et al.*, 1995).

Other genes are likely to be responsible for programming development along the proximodistal (P-D) and mediolateral (M-L) dimensions of the arches. We have recently proposed that Dlx-2 participates in P-D patterning (Qiu *et al.*, 1995), based upon the phenotype of mice with a mutation of this gene. In these animals, bones that we interpreted to correspond to derivatives of the proximal first and second arches where either deleted or altered in their morphologies. There are at least six Dlx genes (Dlx-1, -2, -3, -5, -6, and -7) that are expressed in spatially restricted patterns in craniofacial mesenchyme and ectoderm of vertebrates (Porteus *et al.*, 1991; Price *et al.*, 1991; Robinson *et al.*, 1991; Beauchemin and Savard, 1992; Asano *et al.*, 1992; Dirkson *et al.*, 1993; Papalopulu and Kintner, 1993; Selski *et al.*, 1993; Akimenko *et al.*, 1994; Simeone *et al.*, 1994; Zhao *et al.*, 1994; Ferrari *et al.*, 1995; Nakamura *et al.*, 1996; Stock *et al.*, 1996). Dlx-3 is expressed in distal regions of the first and second arches, whereas Dlx-1 and -2 are expressed more extensively in these arches (Robinson and Mahon, 1994; Bulfone *et al.*, 1993), suggesting that different Dlx genes regulate development in different regions of the branchial arches. To address this point we have made mutations in Dlx-1 and in both Dlx-1 and -2. Here we have demonstrated unique and overlapping functions for these genes in patterning morphogenesis of structures derived from the proximal first and second arches.

## MATERIALS AND METHODS

***Isolation of Dlx-1 and -2 genomic clones and generation of targeting vectors, mutant ES cells, and mutant mice.*** Dlx-1 and -2 genomic DNA was isolated from 129-strain mice and was mapped as described in McGuinness *et al.* (1996). The organization of the targeting vector is shown in Fig. 1 (see Qiu *et al.*, 1995, for a description of the components).

The targeting vector was electroporated into JM-1 ES cells and mutant ES clones were selected and characterized using the methods described in Qiu *et al.* (1995). Genotyping of ES cells and mouse tails was performed using either Southern (Fig. 1) or PCR assays.

The PCR assay for the Dlx-1 mutation used oligonucleotide primers (Primer 1, 5′-AAG GCG GGG CAG CTC TGG AG-3′; Primer 2, 5′-AGG GAG ACG GGC AGG AAG CG-3′) that amplify a 215-bp fragment of Dlx-1 (nucleotides 1114–1339 in the third exon). The PCR conditions are 1 cycle, 94°C; 35 cycles, 94°C, 1 min, 65°C, 1 min, 72°C, 1 min; 1 cycle 72°C, 1 min. PCR for genotyping Dlx-1 and -2 double mutants uses the same conditions as those for genotyping the Dlx-1 or the Dlx-2 single mutants (see Qiu *et al.*, 1995).

Genotyping by the Southern blot method was performed as follows. For Dlx-1 mutants, the genomic DNA was digested with *Xba*I and analyzed with probe 1 (Fig. 1A). The wild-type allele is 8.5 kb, and the mutant allele is 12.5 kb. For Dlx-1 and -2 mutants, the genomic DNA was digested with *Eco*RI and analyzed with probe 3 (Fig. 1A). The wild-type allele is 12 kb, and the mutant allele is 4 kb.

***RNA analysis.*** Northern analysis was performed using standard methods (Sambrook *et al.*, 1989). Radioactive and nonradioactive *in situ* RNA hybridization was performed as described in Qiu *et al.* (1995) and Shimamura *et al.* (1995), respectively. The Dlx-1 probe was generated from a full-length (2.8-kb) Dlx-1 cDNA clone (McGuinness *et al.*, 1996). The Dlx-5 probe was produced from a 1-kb cDNA containing its homeobox and 3′ sequences (Liu and Rubenstein, unpublished). The Dlx-6 probe was produced from a 250-bp cDNA clone encoding its C-terminal region (Ghattas and Rubenstein, unpublished). The Dlx-3 probe was obtained from K. Mahon (Robinson and Mahon, 1994).

***Anatomical analyses.*** Bone and cartilage preparations were made as described by McLeod (1980). Soft tissue analysis was performed at the day of birth (P0) on two homozygous mutants and on one heterozygous and one wild-type control. These samples were fixed in 4% paraformaldehyde, processed, cut into serial paraffin sections, and stained with Masson's trichrome. Their histology and soft tissue topographical anatomy were studied by light microscopy, with graphic reconstruction (Peter, 1922).

## RESULTS

### *Generation of Mice with Mutations in Dlx-1 and Dlx-1 and -2*

The Dlx-1 and -2 genes are linked on mouse chromosome 2 (Ocelick *et al.*, 1992; McGuinness *et al.*, 1996). Previously, we used homologous recombination in embryonic stem (ES) cells (Thomas and Capecchi, 1987; Joyner, 1994) to generate a strain of mice that has a deletion in *Dlx-2* (Qiu *et al.*, 1995). To determine the effect of mutations in Dlx-1 and Dlx-1 and -2, we have now used targeted mutagenesis to introduce additional deletions into this locus. Figure 1A is a schematic of the genomic organization of this locus which also shows the locations of the deletions (and insertions of PGKneo). The Dlx-1 targeting vector has a 2.8-kb deletion that removes all of exons 2 and 3. The Dlx-1 and -2 targeting

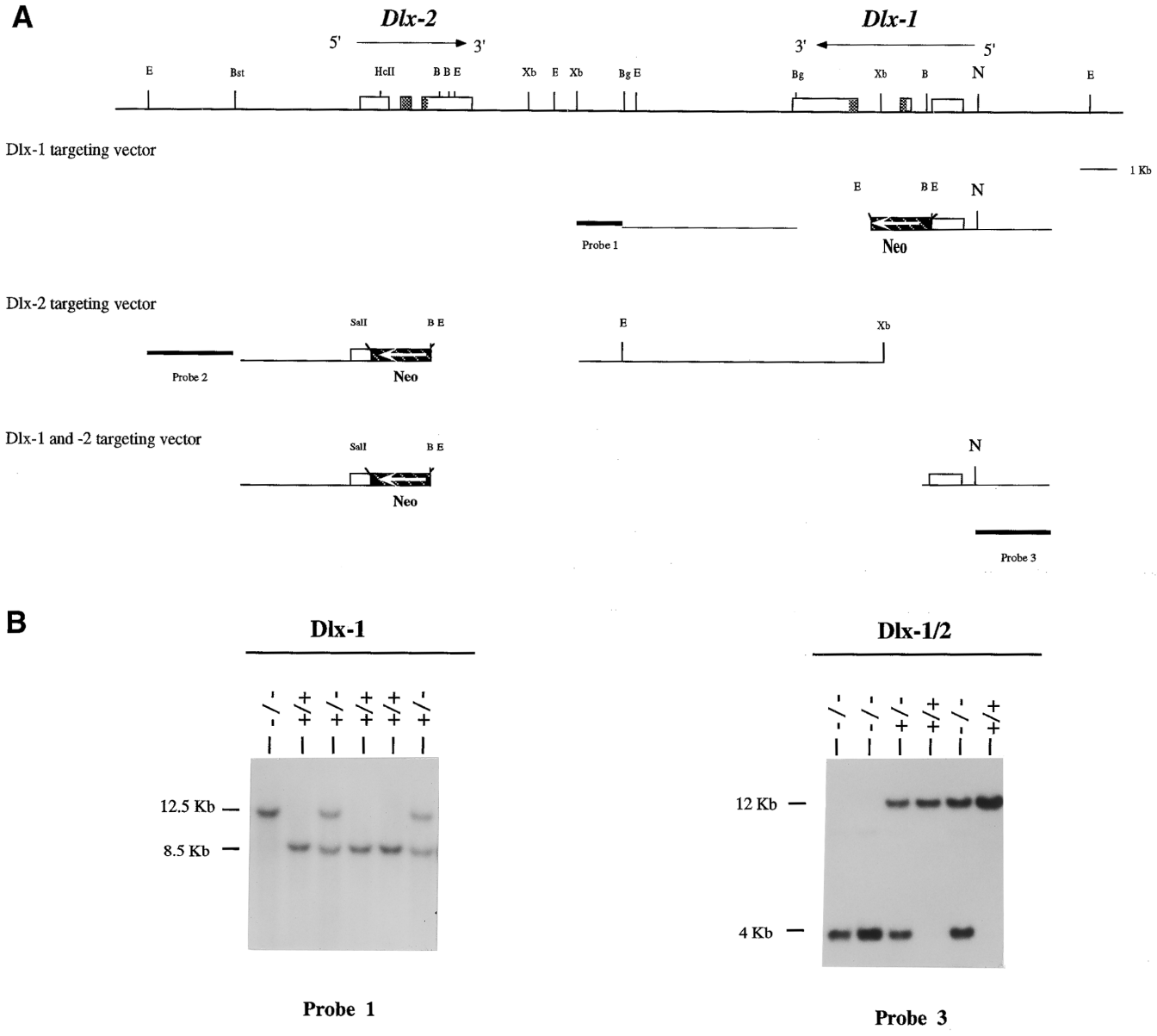
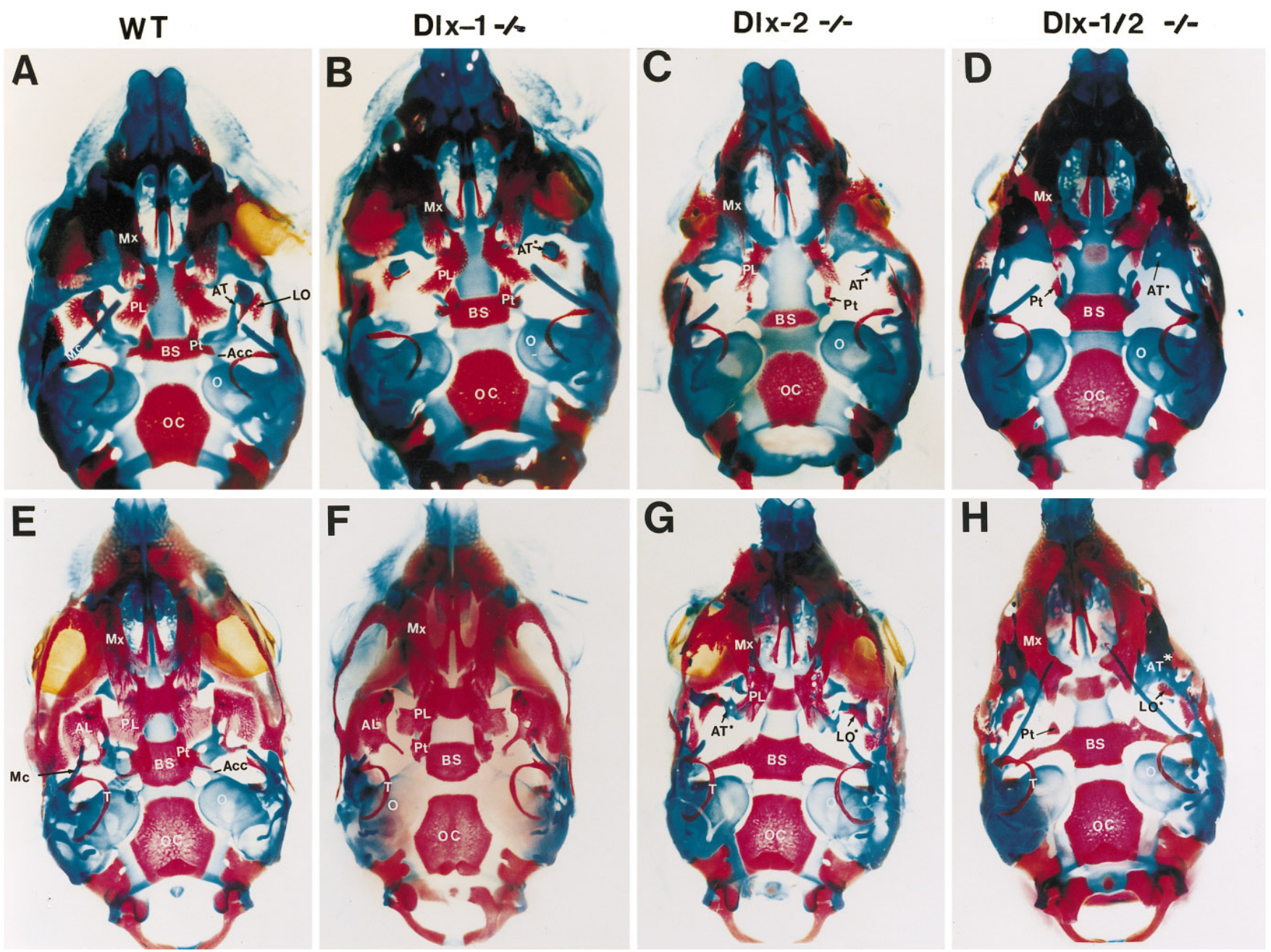



**FIG. 1.** (A) Targeting vectors used to mutate Dlx-1 and both Dlx-1 and -2. The Dlx-1 and -2 loci are schematized showing the exons (boxes) and homeodomains (stippled boxes) and the direction of transcription (for details see McGuinness *et al.*, 1996). Below this are shown the fragments of the Dlx locus and the inserted PGKneo gene that were used to construct the Dlx-1, Dlx-2, and Dlx-1 and -2 targeting vectors. The probes (1–3) that were used for genotyping are indicated. (B) Genotype analysis using the Southern method of mice harboring mutations in Dlx-1 and both Dlx-1 and -2. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; Bst, *Bst*I; E, *Eco*RI; HcII, *Hin*cII; N, *Not*I; Xb, *Xba*I.

vector has an 15.6-kb deletion that removes all of exons 2 and 3 of Dlx-1 and Dlx-2, the entire intergenic region, and part of exon 1 of Dlx-2 [the deletion begins at the *Hin*cII site (as in the Dlx-2 deletion described in Qiu *et al.*, 1995)]. Present evidence, based on sequencing and Northern analysis, suggests that the ~10-kb intergenic region does not encode an additional gene (McGuinness *et al.*, 1996).

To introduce deletions into Dlx-1 and Dlx-1 and -2 *in vivo*, the gene replacement vectors were electroporated into the JM-1 line of ES cells. To identify ES clones with a mutation in Dlx-1, we studied its genomic structure in three independent ES clones by Southern blot analysis (data not shown) using probes that flank the 3′ and 5′ regions of the Dlx-1 sequences in the replacement vector (probes 1 and 3, respectively, in Fig. 1A). We identified 10 ES clones that had the predicted deletion in one *Dlx-1* allele. We will refer to the mutant allele as Dlx-1-D2.8/neo (Dlx-1 2.8-kb deletion/neo insertion).


WT
Dlx-1 -/-
Dlx-2 -/-
Dlx-1/2 -/-
A
B
C
D
E
F
G
H
Mx
PL
AT
LO
Pt
BS
Acc
O
OC
Mc
AL
T

To identify ES clones with a mutation in both Dlx-1 and -2, we studied the structure of this locus in 750 independent ES clones by Southern blot analysis (data not shown) with flanking probes (probes 2 and 3, respectively, in Fig. 1A). We identified 2 ES clones that had the predicted deletion in Dlx-1 and Dlx-2. We will refer to the mutant allele as *Dlx-1&2-D15.6/neo* (*Dlx-1 and -2* 15.6-kb deletion/neo insertion).

The ES clones were karyotyped and then injected into blastocysts to produce chimeric mice. Each of these produced highly chimeric offspring that passed the mutant allele through their germline [mice were genotyped using Southern analysis (Fig. 1B) and/or a PCR assay; see Materials and Methods]. We generated two mouse lines derived from distinct ES clones with the Dlx-1 mutation and one mouse line generated from an ES clone with the Dlx-1 and -2 mutation (one of the mutant ES cell lines died).

Mice heterozygous for the mutant alleles are indistinguishable from wild-type littermates. Heterozygotes were mated to generate mice that were homozygous for the Dlx-1 and Dlx-1 and -2 mutations. Genotype analysis of the offspring from heterozygote crosses showed Mendelian segregation ratios in prenatal and newborn mice (data not shown).

Mice homozygous for the Dlx-1 mutation are viable at birth, but are small and all die within 1 month. The cause of death is uncertain, but may relate to dysfunction of the enteric nervous system, which appears to be abnormal (Gershon and Rubenstein, unpublished). Mice homozygous for the Dlx-1 and -2 mutation behave like Dlx-2 mutants (Qiu *et al.*, 1995) and all die within a few hours after birth. The external anatomy of the Dlx-1 mutant mice does not reveal obvious abnormalities, whereas the Dlx-1 and -2 mutants develop massive abdominal distention prior to their death. The limbs in all of these mice show no clear abnormality, despite the expression of these genes in the ventral ectoderm and apical ectodermal ridge of the limb bud (Fig. 6) and the role of the *Drosophila* Dlx gene, Distal-less, in regulating limb development (Cohen and Jürgens, 1989). The forebrain of the Dlx-1 mutants appeared normal, whereas the Dlx-1 and -2 mutants had a clear defect in basal ganglia development (Anderson *et al.*, manuscript submitted). As the Dlx-2 mutants have distinctive craniofacial skeletal defects, and both of these genes are expressed in the CNC, we investigated the skeletal morphology of the Dlx-1 and Dlx-1 and -2 mutant mice.

### *Dlx-1 and Dlx-1 and -2 Mutants Have Defects in Cranial Neural Crest-Derived Skeletal Components*

To examine the effect of the *Dlx-2* mutation on the development of the skull, we used the Alizarin red S and Alcian blue method (McLeod, 1980) to differentially stain bone (red) and cartilage (blue) in the skeletons of prenatal (E16.5) and newborn (P0) animals. Comparison of about 80 skeletons revealed no morphological differences between wild-type and heterozygous specimens. However, all the mutant homozygotes consistently had defects in a subset of the craniofacial bones and cartilages (Figs. 2 and 3; Tables 1 and 2).

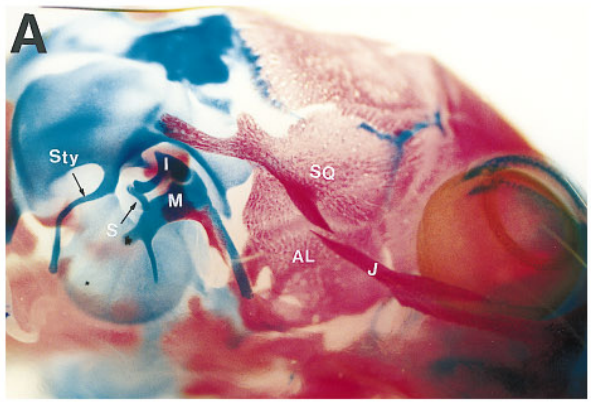
Components of the skull are generally categorized into three groups that have distinct embryological and/or evolutionary relationships: the neurocranium, the splanchnocranium, and the dermatocranium. We have subdivided our analysis of the *Dlx* mutants into alterations of these classes of bones and cartilage.

### *Alterations in the Base of the Skull: Defects Lateral to the Basisphenoid*

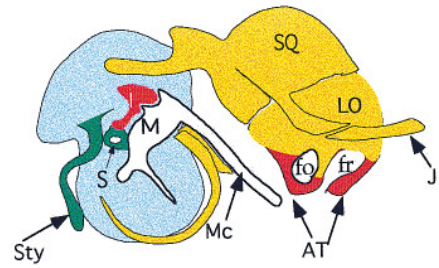
The neurocranium forms the base of the skull and the capsules that surround the sense organs (Barghusen and Hopson, 1979); fate-mapping studies in birds show that some neurocranial structures are derived from mesoderm (e.g., paraxial bones of the skull base that are adjacent to the notochord, such as the basisphenoid, BS), whereas more rostral bones are derived from CNC (Couly *et al.*, 1993).

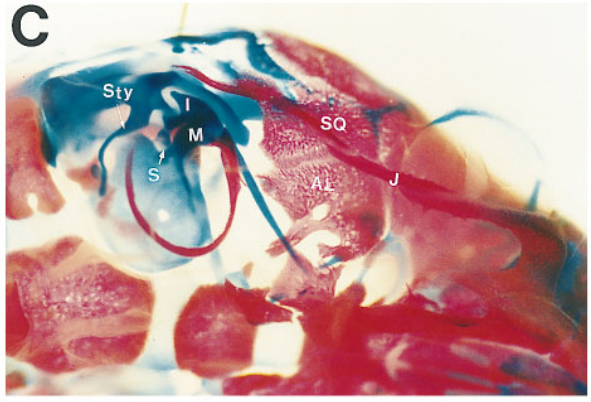
The skeletal elements lateral to the basisphenoid are abnormal in all of the *Dlx* mutants (Table 1); it is unclear whether the defective elements are of neurocranial or splanchnocranial origin, as the boundary between these tissues is unknown. In E16.5 and P0 wild-type and heterozygote animals, the basisphenoid is connected with other components of the neurocranium by cartilaginous bridges (Figs. 2A and 2E). At its lateral aspects are the cartilaginous

**FIG. 2.** Ventral views of E16.5 wild-type (A) and mutant (B–D) and P0 wild-type (E) and mutant (F–H) skulls. Dlx-1 mutants (B, F); Dlx-2 mutants (C, G); Dlx-1 and -2 mutants (D, H). This figure highlights the abnormal morphology of the regions of the alisphenoid and palate. Note that the alisphenoid (AL) bone is derived from two components: the splanchnocranial ala temporalis (AT) and a membrane bone called the lamina obturans (LO) (see A and E) (Presley and Steel, 1976). Normally, the AT connects with the basisphenoid by a basal process and with the otic capsule by the alicochlear commissure (Acc), which form respectively the anterior and lateral boundaries of the carotid foramen. A short (palatine) process of the AT extends rostrally and slightly ventrally to contact palatine and pterygoid in the lateral wall of the nasopharynx. Laterally, dorsally, and rostrally the AT forms the lamina ascendens, a free-ending sheet of cartilage between the mandibular and maxillary nerves and which is pierced by the alisphenoid canal for a branch of the pterygopalatine (stapedial) artery. The lamina obturans ossifies directly in a fibrous sheet which extends dorsally and laterally from the cartilaginous AT to join the neurocranium rostrally and dorsally and the roof of the tympanic cavity (tegmen tympani) caudally. This surrounds the emerging branches of the mandibular nerve. Abbreviations: Acc, alicochlear commissure; AL, alisphenoid; AT, ala temporalis; AT*, cartilage resembling the ala temporalis; BS, basisphenoid; LO, lamina obturans; LO*, bone resembling the lamina obturans; Mc, Meckel's cartilage; Mx, maxillary; O, otic capsule; OC, basisoccipital; PL, palatine; Pt, pterygoid; T, tympanic.

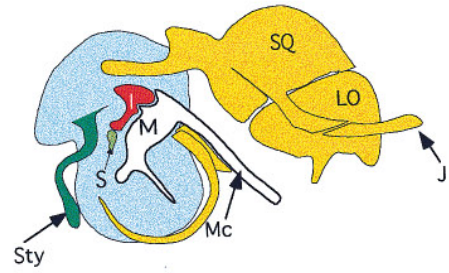


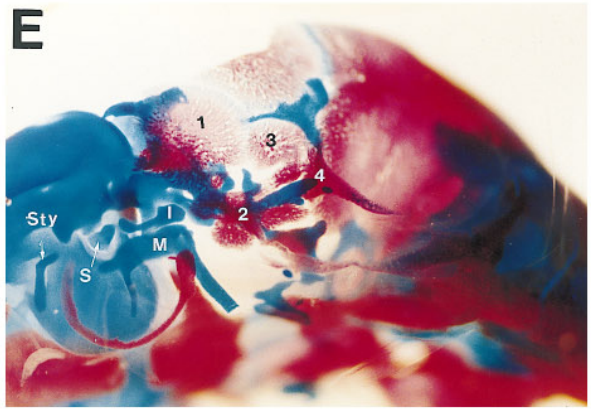

B Wildtype



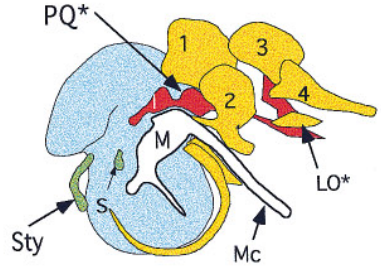



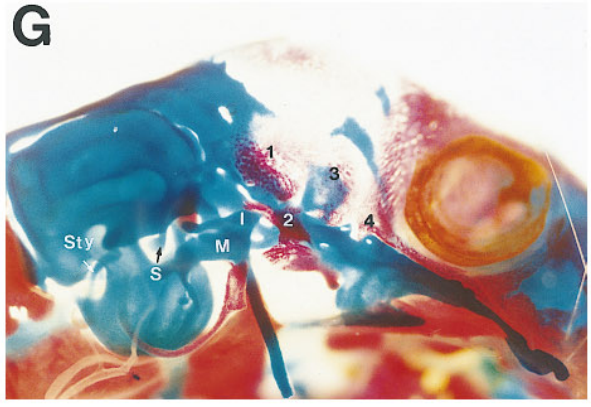

D Dlx-1 -/-




F Dlx-2 -/-




H Dlx-1/2 -/-

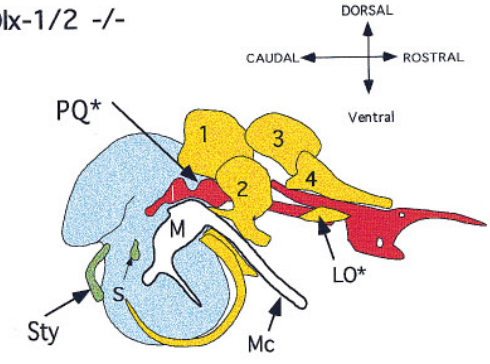


**TABLE 1**

A Summary of Abnormalities of Craniofacial Bones in the Dlx-1, Dlx-2, and Dlx-1 and -2 Mutants

| | | Dlx-1−/− | Dlx-2−/− | Dlx-1 and -2−/− |
|---|---|---|---|---|
| First arch | | | | |
| Splanchnocranium | | | | |
| Proximal | Ala temporalis[a] | − | − | − |
| | Incus | + | − | − |
| Distal | Malleus | + | + | + |
| | Meckel's cartilage | + | + | + |
| Dermatocranium | | | | |
| Proximal | Palatine | +/− | − | −− |
| | Pterygoid | +/− | − | − |
| | Lamina obturans[a] | + | − | − |
| | Maxillary | + | − | −− |
| | Jugal | + | − | − |
| | Squamosal | + | − | − |
| Distal | Mandible | + | + | + |
| | Tympanic | + | + | + |
| | Gonial | + | + | + |
| Second arch | | | | |
| Splanchnocranium | | | | |
| Proximal | Stapes | +/−[b] | − | − |
| | Styloid | +/−[b] | − | − |
| Distal | Hyoid (less horns and upper body) | + | + | + |
| Dermatocranium | | | | |
| No dermal bones are related to the second arch | | | | |

*Note.* Phenotypic effects of the Dlx mutations on the morphologies of splanchnocranial and dermatocranial bones in the first and second branchial arches. +, wild-type phenotype; −, abnormal phenotype; +/−, partial penetrance of an abnormal phenotype. At least 30 homozygous mutant skulls were analyzed for the Dlx-2 and Dlx-1 and -2 mutants, and over 15 Dlx-1 mutant skulls were analyzed.

[a] Two components of the alisphenoid: ala temporalis and lamina obturans.

[b] Affected in about 50% of cases.

components of the alisphenoid, which are called the ala temporalis (AT) (see legend to Fig. 2 for anatomical details of this region). From the caudal–lateral part of these processes arises the alicochlear commissure (ACC) that contacts the otic capsule (O).

In all of the Dlx mutants, the overall shape and the rostral and caudal connections of the basisphenoid are normal. However, the proximal part of the AT (the part adjacent to the basisphenoid) is largely absent in all of the E16.5 and P0 mutant animals (Fig. 2). The distal component of the AT may be present (AT*, Figs. 2B–2D).

In addition to deleted structures, the Dlx-2 and Dlx-1 and -2 mutants have structures not found in wild-type mice. Slightly caudal to the region of the deleted AT, there is a

**FIG. 3.** Realistic and schematic lateral views of wild-type (A, B) and mutant P0 skulls. Dlx-1 mutant (C, D); Dlx-2 mutant (E, F); Dlx-1 and -2 mutant (G, H). In the schemas, blue indicates chondrocranial otic cartilage, yellow indicates dermal bones, red indicates proximal first arch splanchnocranial cartilages, white indicates distal first arch splanchnocranial cartilages, and green indicates second arch splanchnocranial cartilages. Blue–green indicates second arch-derived cartilages with the normal morphology, whereas olive green indicates second arch-derived cartilages with abnormal morphologies. The temporal walls of newborn Dlx-2 and Dlx-1 and -2 mutants can have three kinds of morphology (Types I, II, and III) that differ in the patterns of articulations between five bones: maxillary and bones 1–4. Each of these skull types is present in about one-third of the cases. In Type I skulls (e.g., the Dlx-2 mutant in E and H), an inferior temporal arch is present (made up of the maxilla and bone 2); bones 3 and 4 form a postorbital bar that is not connected to the maxilla. In Type II skulls, an inferior temporal arch does not form; instead, a postorbital bar, made up of bones 3 and 4, articulates with the maxilla (e.g., the Dlx-1 and -2 mutant in G and H). The rostral process of bone 2 remains unattached. In Type III skulls, neither an inferior temporal arch nor a postorbital bar form (not shown). Abbreviations: AL, alisphenoid; fo, foramen ovale for the mandibular nerve; fr, foramen rotundum for the maxillary nerve; I, incus; J, jugal; LO, lamina obturans; LO*, bone resembling the lamina obturans; Mc, Meckel's cartilage; M, malleus; PQ*, cartilage resembling the palatopterygoquadrate; S, stapes; SQ, squamosal; Sty, styloid; 1, 2, 3, and 4, dermal bones found in the Dlx-2 and Dlx-1 and -2 mutants in the position of the squamosal (SQ) and jugal (J) bones.

**TABLE 2**
Regional Alterations of Craniofacial Bones in the Dlx-1, Dlx-2, and Dlx-1 and -2 Mutants

| | Dlx-1−/− | Dlx-2−/− | Dlx-1 and -2−/− |
|---|---|---|---|
| Lateral skull wall | | | |
| Wing of alisphenoid[a] | + | − | − |
| Squamosal | + | − | − |
| Jugal | + | − | − |
| Ectopic lateral cartilage[b] | − | + | + |
| Ectopic dermal bones | − | + | + |
| Ectopic dermal bones (1–4) | − | + | + |
| Palate | | | |
| Palatine | +/− | − | −/− |
| Pterygoid | +/− | − | − |
| Maxillary | + | − | −/− |
| Base of skull | | | |
| Alisphenoid[c] | − | − | − |
| Alicochlear commissure | + | − | − |
| Ectopic strut[d] | − | + | + |
| Teeth | | | |
| Maxillary molars missing | − | − | + |

*Note.* Regional phenotypes of the Dlx mutations. Phenotypic effects of the Dlx mutations on the morphologies of skull elements in three regions: the lateral skull wall, the palate, and the base of the skull. +, wild-type phenotype or indicates the presence of ectopic skeletal elements (lateral cartilage resembling the PQ and the dermal bones 1–4); −, abnormal phenotype or indicates the absence of ectopic skeletal elements; +/−, partial penetrance of an abnormal phenotype.

[a] Lamina obturans component of alisphenoid.

[b] Hypothesized to be homologous to reptilian palatopterygoquadrate (PQ) bone.

[c] Ala temporalis component of alisphenoid.

[d] Occurs in about 30% of cases.

new osseous process (the ''strut'') that extends laterocaudally from the basisphenoid in ~30% of the Dlx-2 and Dlx-1 and -2 mutant animals (Fig. 2). In serially sectioned Dlx-2 and Dlx-1 and -2 homozygous mutants that lack a cartilaginous strut, a sheet of fibrous tissue was found in its place (data not shown). Laterally the strut makes contact with an extensively modified tegmen tympani and neurocranium behind the jaw joint and above the tympanic cavity. In addition to the deletion of the proximal AT, the size of the ACC is greatly reduced in the Dlx-2 and Dlx-1 and -2 mutants (Fig. 2).

## *Splanchnocranial Alterations*

Splanchnocranial elements are endochondral bones derived from the CNC mesenchyme of the branchial arches (Couly *et al.*, 1993). The mutant E16.5 and P0 animals have abnormalities in bones/cartilages derived from parts of the first and second arches lying proximal with respect to the source of the CNC. The first arch has two major subdivisions: the maxillary (which can be considered as dorsal or proximal) and the mandibular (which can be considered as ventral or distal). It is generally believed that maxillary mesenchyme gives rise to the palatopterygoquadrate (PQ) cartilage of lower vertebrates (Barghusen and Hopson, 1979), which is subdivided into separate bones in mammals (proximocaudally the incus, distalorostrally the ala temporalis component of the alisphenoid) (Barghusen and Hopson, 1979; deBeer, 1985). The mandibular mesenchyme gives rise to Meckel's cartilage, whose proximocaudal region forms the malleus in mammals.

In the first arch, only maxillary derivatives are grossly abnormal in all three types of Dlx mutant mice (Table 1). Thus, the ventromedial part of the alisphenoid (AL) is absent (derived from the AT) (Figs. 2 and 3). The lateral wing of the alisphenoid (which is probably a dermatocranial bone that develops from lamina obturans, LO) is malformed in the Dlx-2 and Dlx-1 and -2 mutants (Figs. 3E–3H). In addition, the incus (I) is misshapen and often does not articulate with the stapes (S) in the Dlx-2 and Dlx-1 and-2 mutants (Figs. 3E–3H). These two mutants also have an extensive new area of cartilage that is lateral to the basisphenoid, which has features similar to the palatopterygoquadrate (PQ) (see Qiu *et al.*, 1995, and Discussion for details) (Figs. 3G and 3H).

Proximal (dorsal) second arch bones/cartilages are also abnormal in all three mutants, whereas again the distal (ventral) structures are unaffected (Fig. 3 and Qiu *et al.*, 1995). The second arch forms Reichert's cartilage, which proximally gives rise to the stapes and the styloid process (Sty), and distally forms part of the hyoid bone (Barghusen and Hopson, 1979; deBeer, 1985). In the mutant animals, the stapes is smaller than normal and lacks a central hole (~50% of the Dlx-1 mutants and 100% of the Dlx-2 and

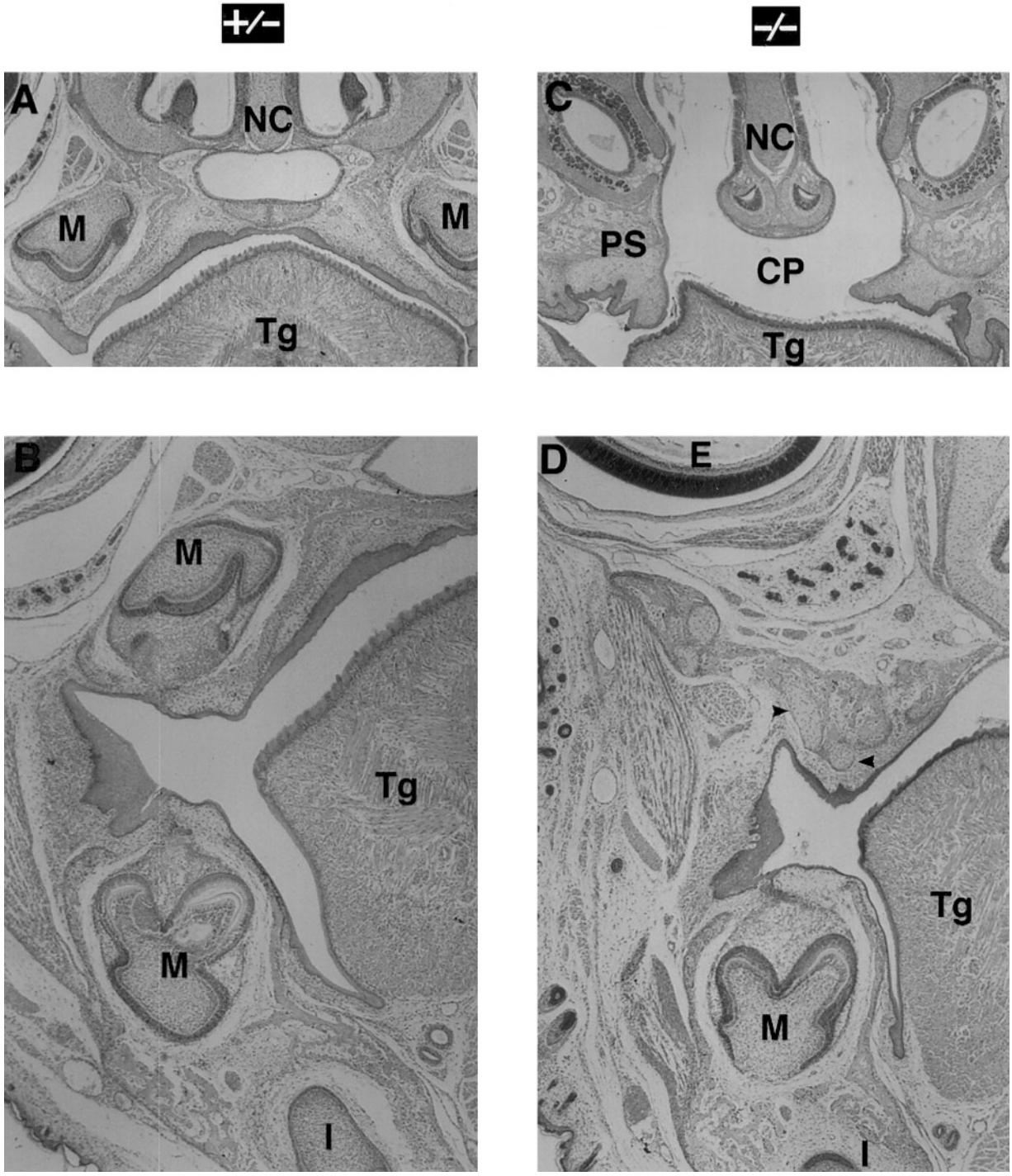



**FIG. 4.** Frontal sections through the oral cavity of a newborn homozygous Dlx-1 and -2 mutant (C, D) and a heterozygous littermate (A, B). A and C show the oral cavity including the palate; B and D focus on the region of the maxillary and mandibular molars. Arrowheads in D show ectopic cartilage formed in the position of maxillary molar teeth. Abbreviations: CP, cleft palate; E, eye; I, incisor; M, molar; NC, nasal cartilage; PS, palatal shelf; Tg, tongue.

Dlx-1 and -2 mutants) (Figs. 3C–3H); this is associated with the absence of the stapedial artery (see below). The styloids lack their connection with the crista parotica of the otic capsule (O) in all of the Dlx-2 and Dlx-1 and -2 mutants (Figs. 3E–3H) and in ~50% of the Dlx-1 mutants (not shown).

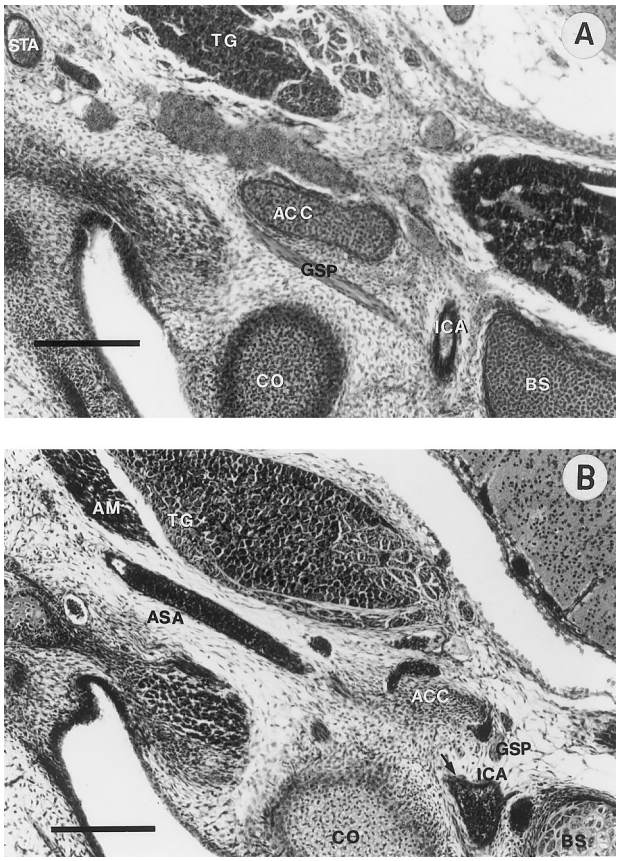



**FIG. 5.** Coronal sections through heads of P0 specimens passing through the carotid canal, cutting the tip of the cochlear promontory (CO), the alicochlear commissure (ACC), and the basisphenoid (BS). (A) Wild type showing the internal carotid artery (ICA) passing through the carotid canal without branching. The greater superficial petrosal nerve (GSP) passes extracranially, outside the ACC, to reach the external aperture of the carotid canal from a lateral position. (B) Dlx-2−/− showing the anomalous equivalent of the stapedial artery (ASA) running laterally, having just branched from the ICA (branching point, arrow). The GSP nerve is descending through the carotid canal having reached the internal opening by following an intracranial course. One of the intracranial fascicles of an adductor mandibulae muscle (AM) is seen in contact with the TG. Scale marker: 100 $\mu$m.

### *Dermatocranial Alterations Are Found Primarily in the Dlx-2 and Dlx-1 and -2 Mutants*

The dermatocranium comprises the CNC-derived dermal bones that encase chondrocranial and splanchnocranial structures and that also form the roof of the skull (calvaria) (Couly *et al.,* 1993). Abnormalities in the morphology of the maxillary (Mx), palatine (Pl), pterygoid (Pt), squamosal (Sq), jugal (J), and alisphenoid (lamina obturans-derived part) dermal bones are observed in the Dlx-2 and Dlx-1 and -2 mutants (Figs. 2 and 3; Qiu *et al.,* 1995). A subset of these dermal bones are abnormal in the Dlx-1 mutants (Table 1). The changes in the squamosal and jugal bones alter the temporal wall of the skull, whereas the changes in the maxillary, pterygoid, and palatine bones alter its ventral surface (Tables 1 and 2). The calvaria appears to be normal in the mutants.

In the Dlx-2 and Dlx-1 and -2 mutants, the temporal skull wall is altered. Normally, the squamosal, jugal, and maxillary bones contribute to form the zygomatic arch, which is a characteristic feature of the mammalian skull (Fig. 3). In the Dlx-2 and Dlx-1 and -2 mutants, the squamosal and jugal bones are replaced with four bones which we will name bones 1–4 (Figs. 3E–3H; Table 2); these dermal bones are derived from four independent ossification centers (Qiu *et al.,* 1995, and data not shown). In the Dlx-1 and -2 mutants the musculoskeletal relationships in this region are disrupted (data not shown). The caudal end of bone 2 ends in the sheath of a belly of the superficial masseter muscle. More caudally, the tip of bone 4 turns inward close to the front of the jaw joint to end among the sheaths of deeper bellies of the masseter (data not shown).

Alterations of three other dermatocranial bones (maxillary, pterygoid, and palatine) transform the morphology of the ventral surface of the skull (Fig. 2; Table 2). The palatine bones in the Dlx-2 and Dlx-1 and -2 mutants lack palatal processes and are flattened and displaced rostrolaterally (Figs. 2C, 2D, 2G, and 2H). In the Dlx-1 and -2 mutants, the caudal part of the maxillary bone is larger than normal. The palatal process of the maxillary bone is absent in the Dlx-2 and Dlx-1 and -2 mutants. In these animals, the pterygoids are rostrally displaced, smaller than normal. The maxillary, palatine, and pterygoid alterations lead to cleft secondary palate in ~80% of the mutant Dlx-2 and 100% of the mutant Dlx-1 and -2 animals (Figs. 2G and 2H). In about 10% of the Dlx-1 mutants, a small cleft palate is observed, even though there is no clear change in the sizes of the palatine, maxillary, or pterygoid bones (there may be a rostral shift in the position of the pterygoids which laterally displaces the palatine bones) (Fig. 2F).

### *Absence of the Maxillary Molars in the Dlx-1 and -2 Mutants*

Dlx-1 and -2 mutants lack all maxillary molar teeth (M) (Fig. 4). Maxillary incisor (I), mandibular incisor, and molars are all present in their correct positions and appear normal with respect to morphology and cytodifferentiation of ameloblasts and odontoblasts. The Dlx-1 and Dlx-2 single mutants have normal teeth. No evidence of aborted/regressed maxillary molar tooth development was observed in the Dlx-1 and -2 mutants, except in one embryo, which had an epithelial downgrowth, lacking tooth elements, in the upper left molar region. The tissue replacing the absent teeth consists of ectopic cartilage nodules with some bone and connective tissue (Fig. 4D, arrowheads).

### *Alterations of the Craniofacial Soft Tissues*

Abnormalities in the vasculature, peripheral nervous system, and muscles adjacent to the dysmorphic bones and

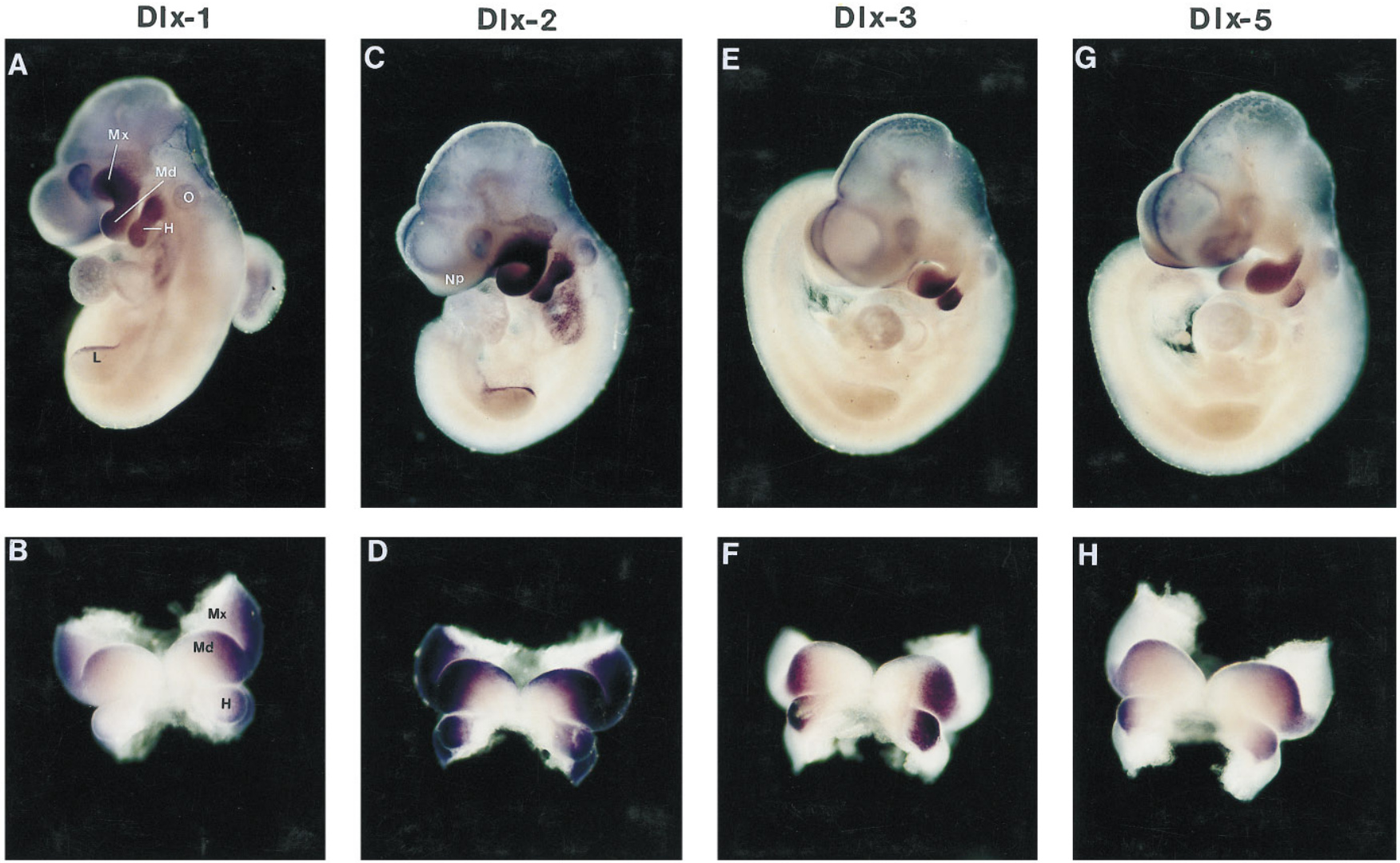
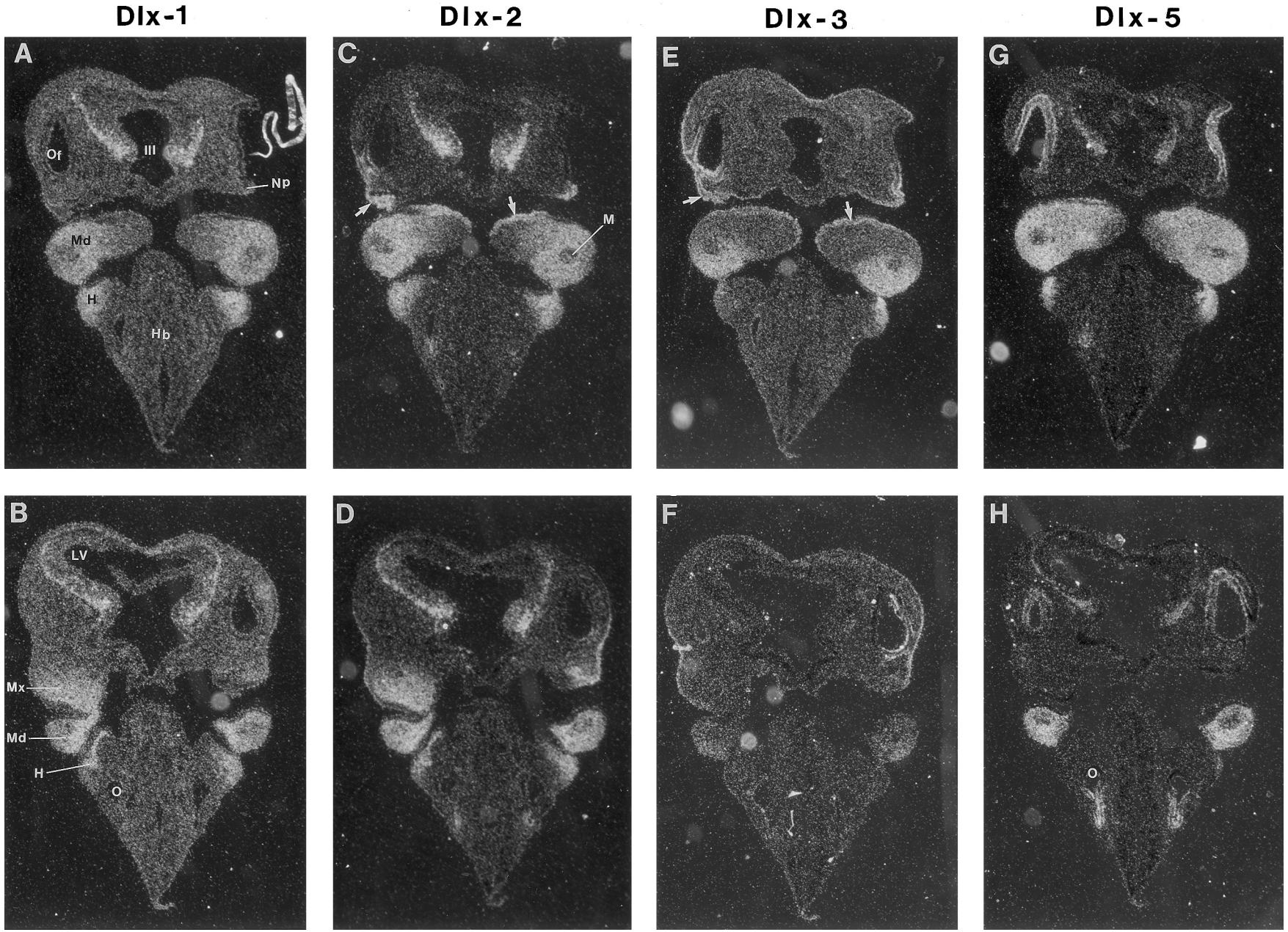



**FIG. 6.** Expression patterns of Dlx-1 (A, B), -2 (C, D), -3 (E, F), and -5 (G, H) at E9.5 revealed using whole-mount *in situ* hybridization. The upper tier shows a lateral view of the entire embryo; the lower tier shows a frontal view of dissected first and second arches. Abbreviations: H, hyoid (second) branchial arch; L, forelimb bud; Md, mandibular branch of first branchial arch; Mx, maxillary branch of first branchial arch; Np, nasal prominence; O, otic vesicle.


Dlx-1
Dlx-2
Dlx-3
Dlx-5
A
B
C
D
E
F
G
H
Of
III
Np
Md
H
Hb
LV
Mx
Md
H
O
M
O

cartilages of the first and second arches were identified in all three Dlx mutations. Because these defects are complex, we will only briefly describe three salient abnormalities here. The first two defects are found in all three mutants, whereas the third abnormality is not found in the Dlx-1 mutants.

The stapedial artery is absent. Normally, it is a branch from the internal carotid artery (ICA), running through the foramen in the stapes. It provides vascular supply to structures developing in the first arch, such as the jaws and their musculature. In the mutant, this territory is supplied by an artery (ASA) that branches, in the region derived from the maxillary arch, from the carotid artery at the carotid foramen (Fig. 5).

The trajectory of a branch of the facial nerve is also abnormal (Fig. 5). While the main branch and chorda tympani branch follow normal courses, the facial nerve's proximal parasympathetic branch to the maxillary region (greater superficial petrosal nerve, GSP) runs medially over the dorsum of the cochlear promontory (CO) to leave the cranial cavity through the carotid foramen. Normally, this nerve is entirely extracranial, passing laterally and ventrally over the cochlear promontory and not through the carotid foramen.

The cranial origins of the proximal jaw adductor musculature are abnormal; the distal musculature is generally normal. While the insertions of the proximal adductors on the mandible have only minor modifications, some of them have intracranial insertions (AM), something that is never found in mammals (Fig. 5). In addition, there are many more muscle bellies than normal. In particular, the medial pterygoideus has several bellies that originate intracranially. These reach the jaw through a fenestra bounded anterolaterally by the novel palatopterygoquadrate cartilage and caudally by the strut (or its fibrous replacement). Other intracranial muscle fascicles originate on one part of the palatopterygoquadrate and reinsert on another part, rather than reaching the mandible.

### *Expression of Dlx Genes in the Branchial Arches*

The loss of function mutations in Dlx-1 and Dlx-2 demonstrates that these genes are required for development of the proximal parts of the first and second arches. Previous studies show that Dlx-1 and Dlx-2 are expressed in the mesenchyme of both the proximal and distal domains of the arches (Dolle *et al.,* 1992; Bulfone *et al.*, 1993; Robinson and Mahon, 1994). Based on this result, it is unclear why the phenotypic effect of mutations in these genes is restricted to the proximal domains. Previous studies of Dlx-3, -5 and, -6 show that these genes have craniofacial expression patterns distinct from Dlx-1 and -2 (Akimenko *et al.*, 1994; Zhao *et al.*, 1994; Robinson and Mahon, 1994; Simeone *et al.*, 1994). This suggests that the restriction of the mutant phenotype to the proximal parts of the Dlx-1 and -2 expression domains may be due to compensation by other Dlx genes. To address this hypothesis, we systematically analyzed the craniofacial expression of the five known murine Dlx genes.

Whole-mount *in situ* RNA hybridization on E8.5–E9.0 embryos suggests that Dlx-1 and -2 are expressed in migratory and postmigratory CNC, whereas Dlx-3 and -5 appear to be expressed in postmigratory CNC beginning around E9.5 [data not shown and Robinson and Mahon, 1994; Dlx-6 expression was studied using *in situ* hybridization to sections (Fig. 8) because the signal was too low to reliably study using whole-mount *in situ* RNA hybridization]. By E9.5, Dlx-1 and -2 are expressed in the mesenchyme along much or all of the proximodistal axis of the first and second arches, whereas Dlx-3 and -5 are expressed in progressively more distal domains (Fig. 6) (note: Dlx-5 and -6 have very similar spatial expression; data not shown). For instance, only Dlx-1 and -2 are expressed in the maxillary process of the first arch (the tissue that is sensitive to the loss of Dlx-1 and -2 expression).

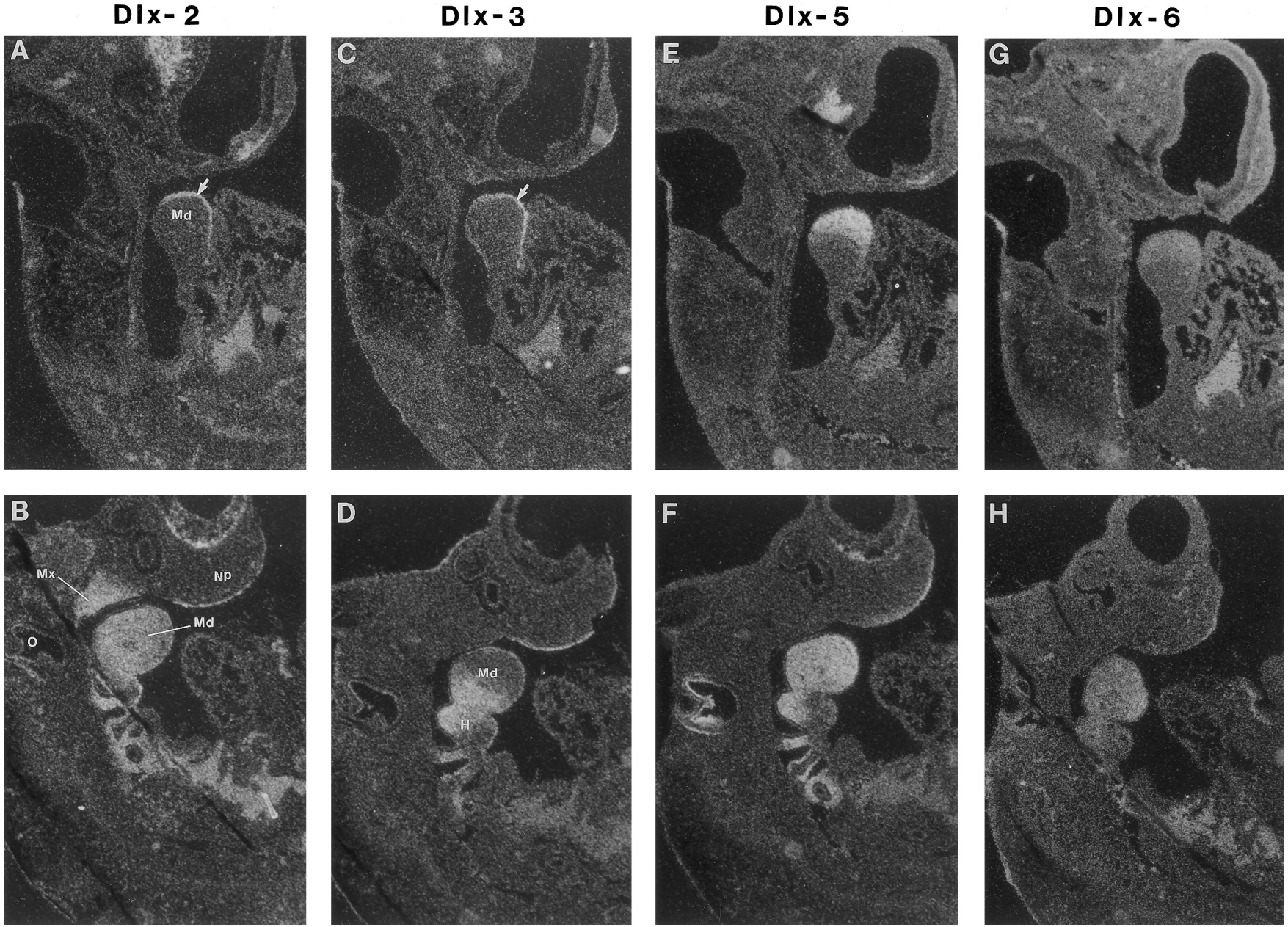
*In situ* hybridization to sections of E10.5 embryos also shows that Dlx-1 and -2 are expressed in both proximal and distal regions of the first and second arches, whereas Dlx-3, -5, and -6 expression is restricted to more distal domains (Figs. 6 and 7). The Dlx genes also have a graded expression along the mediolateral axis of the arches, with much lower levels of Dlx-2 expression in the extreme medial mesenchyme (Fig. 8; Bulfone *et al.,* 1993). Dlx-5 and -6 are expressed most medially, followed by Dlx-1 and -2; Dlx-3 expression is the most lateral (Robinson and Mahon, 1994).

Dlx-1 and -2 have very similar expression patterns in the branchial arches, yet mutation in these genes leads to distinct phenotypes (e.g., only the Dlx-2 and Dlx-1 and -2 mutants have abnormal dermatocranial lateral skull structures; Tables 1 and 2). The different phenotypes of these mutations may be due to subtle differences in Dlx-1 and -2 expression. For instance, Dlx-2 is expressed at higher levels than Dlx-1 in the ectoderm overlying the first arch (Figs. 7A–7D).

## DISCUSSION

Analysis of mice homozygous for mutations in Dlx-1, Dlx-2, and Dlx-1 and -2 demonstrates that these genes are essential for craniofacial development. These mutations also affect forebrain and enteric nervous system development (Qiu *et al.,* 1995; Anderson *et al.,* manuscript submitted and unpublished results). The mutations are deletions that eliminate most of the coding regions of these genes

**FIG. 7.** Expression patterns of Dlx-1 (A, B), -2 (C, D), -3 (E, F), and -5 (G, H) revealed using *in situ* hybridization to cross-sections of E10.5 wild-type embryos. Arrows point to expression of Dlx-2 and -3 in the surface ectoderm of the first arch. Abbreviations: H, hyoid (second) branchial arch; Hb, hindbrain; LV, lateral ventricle; M, presumed mesoderm of mandibular arch; Md, mandibular branch of first branchial arch; Mx, maxillary branch of first branchial arch; Np, nasal prominence; O, otic vesicle; Of, olfactory pit; III, third ventricle.


Dlx-2
Dlx-3
Dlx-5
Dlx-6
A
B
C
D
E
F
G
H
Md
Mx
NP
O
Md
Md
H

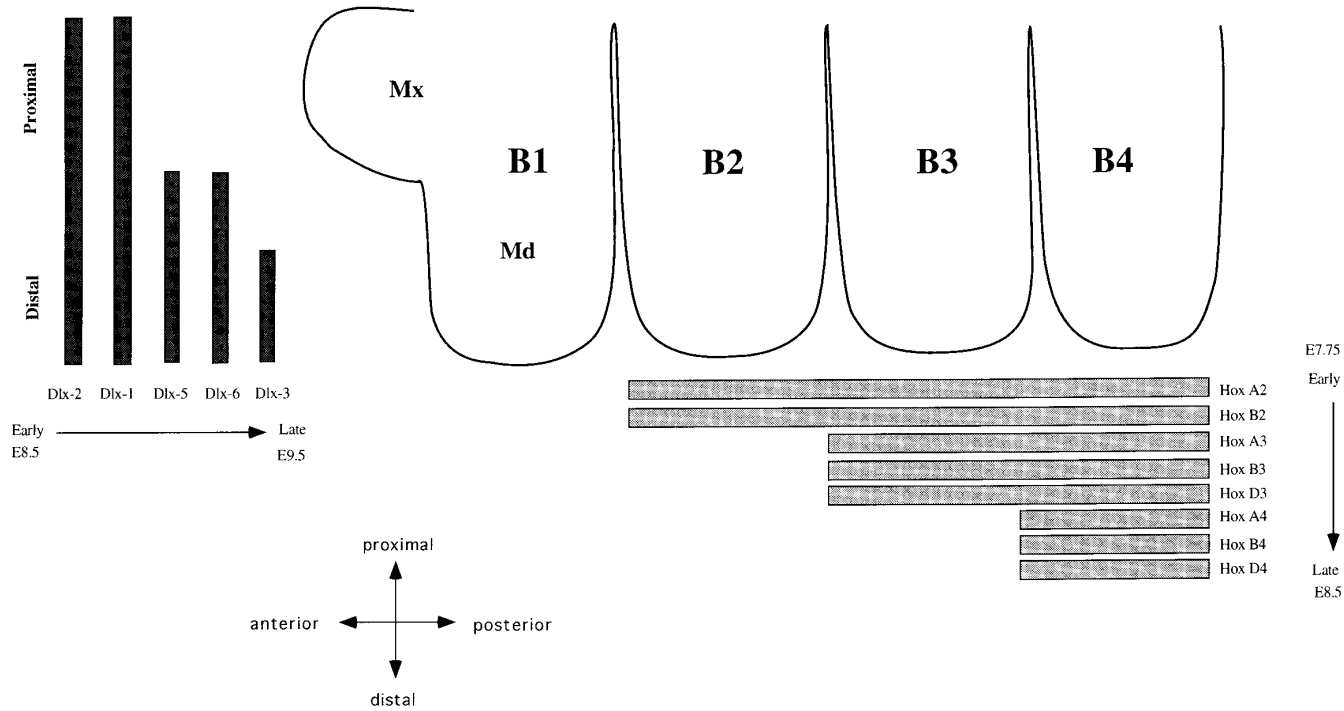



**FIG. 9.** Schema showing the postulated roles of the Dlx genes in proximodistal patterning and the Hox genes in anteroposterior patterning of the branchial arches. The branchial arches are labeled B1 (first arch), B2 (second arch), B3 (third arch), and B4 (fourth arch). B1 has two components: maxillary (Mx) and mandibular (Md). We interpret that the maxillary region is proximal to the mandibular region. Note that alternative topological relationships are also possible between the maxillary and mandibular processes, including that they are complementary subdivisions of the first arch, each with its own proximodistal axis. Anteroposterior patterning of B2–B4 would be regulated by the Hox genes, whereas proximodistal patterning would be regulated by the Dlx genes. The approximate time in development when these genes begin to be expressed is indicated. Note that within the first arch, the Dlx-1, Dlx-2, and Dlx-1 and -2 mutations have their greatest effects on morphogenesis of the proximal part of the maxillary process (the incus and alisphenoid are abnormal, whereas most of the maxillary and premaxillary bones are normal). In addition, the maxillary molars do not form, whereas the maxillary incisors are normal.

(including most of the homeodomains), suggesting that these are null alleles. Although the N-termini of the coding regions have not been deleted, which could lead to the production of biologically active truncated proteins with dominant effects, the lack of any abnormal phenotype in heterozygotes argues against this possibility.

Here we have demonstrated that both Dlx-1 and Dlx-2 are required for development of CNC-derived skeletal elements of the proximal first and second arches (Table 1). There are two recognized types of CNC-derived cartilage and bone: splanchnocranial and dermatocranial. The splanchnocranial structures have cartilaginous precursors, whereas the dermatocranial bones ossify without a cartilaginous intermediate. Both Dlx-1 and Dlx-2 are required for splanchnocranial development, whereas only Dlx-2 is clearly essential for the normal pattern of dermal bones in the lateral skull wall (Table 2). Below we discuss potential mechanisms that may explain why Dlx-1 and Dlx-2 regulate morphogenesis of only proximal arch structures and why mutations of Dlx-1 and Dlx-2 have different phenotypes.

Patterning of the skeletal elements within craniofacial structures probably involves interactions between multiple embryonic tissues including the CNC and the surface ectoderm. Available evidence suggests that the Hox genes have a role in specifying A-P information in the CNC (Fig. 9; Rijii *et al.*, 1993; Gendron-Maguire *et al.*, 1993). Previously, we provided evidence based upon the mutation of Dlx-2 that the Dlx genes contribute toward specifying P-D fate within the first and second arches (Qiu *et al.*, 1995). Here we have shown that mutation of Dlx-1 and both Dlx-1 and

**FIG. 8.** Expression patterns of Dlx-2 (A, B), -3 (C, D), -5 (E, F), and -6 (G, H) revealed using *in situ* hybridization to parasagittal sections of E10.5 wild-type embryos. Arrows indicate ectodermal expression of Dlx-2 and -3 in the medial mandibular arch. Abbreviations: H, hyoid (second) branchial arch; Md, mandibular branch of first branchial arch; Mx, maxillary branch of first branchial arch; Np, nasal prominence; O, otic vesicle.

-2 also affects morphogenesis of proximal splanchnocranial structures of the first two arches (Table 1).

Analysis of Dlx-1, -2, -3, -5, and -6 expression provides information that may explain why the phenotypic effects of the Dlx-1 and -2 mutations are focused on proximal structures. While Dlx-1 and -2 are expressed along most or all of the P-D axis, the expression of Dlx-3, -5, and -6 overlaps with Dlx-1 and -2 in more distal regions of arches 1 and 2 (Figs. 6–8). If Dlx-3, -5, and, -6 are functionally redundant for Dlx-1 and -2, this could explain why distal regions of these arches are not affected by these mutations.

It is intriguing that the Drosophila Dlx homolog Distal-less regulates P-D growth and/or patterning of appendages (Cohen and Jurgens, 1989), although in that case Distal-less functions in the absence of other known family members. Also, while the Distal-less gene is required for limb development, mutations of Dlx-1 and -2 apparently do not affect mouse limb formation (data not shown), despite their expression in the AER (Bulfone *et al.*, 1993; Fig. 7).

The Dlx expression patterns also suggest a reason why Dlx-1 and Dlx-2 mutations have different phenotypes, particularly in the lateral skull dermatocranium (Tables 1 and 2). Dlx-2 is expressed at much higher levels in the first arch ectoderm than Dlx-1 (Fig. 7; Bulfone *et al.*, 1993). The expression in the maxillary arch ectoderm implies that Dlx-2 may regulate gene expression in the surface ectoderm that in turn can modulate development of dermal bones (squamosal, jugal, and lamina obturans). This hypothesis is consistent with tissue recombination and ablation studies that have demonstrated a role of the ectoderm and its basal lamina in the differentiation of CNC-derived dermal bones (Hall *et al.*, 1983; Tyler and Hall, 1977) and CNC-derived odontoblasts (Lumsden, 1988). Thus, perhaps the restricted patterns of Dlx-2 and Dlx-3 ectodermal expression in the maxillary and mandibular primordia have important roles in patterning dermal bone morphogenesis. Accordingly, the head ectoderm may have a highly organized array of specialized tissues (such as the lens and olfactory placodes) that regulate development of underlying mesenchymal and neuroectodermal tissues.

Some cranial components have nearly identical phenotypes in the Dlx-1 and Dlx-2 mutants, particularly the deletion of the proximal ala temporalis (Figs. 2B–2D) (Tables 1 and 2). This suggests several models for the epistatic relationship of these genes. These genes encode homeodomain proteins that function as transcriptional regulators (Yu *et al.*, unpublished). Preliminary evidence suggests that Dlx-1 and -2 proteins are coexpressed in the same mesenchymal cells (Eisenstat and Rubenstein, unpublished); thus, Dlx-1 and -2 proteins could have cooperative interactions, like a1 and $\alpha$2 homeodomain proteins (Goutte and Johnson, 1988); however, to date no heteromeric interactions have been detected using the yeast two-hybrid method (Yu and Rubenstein, unpublished). Other possibilities include that Dlx-1 and -2 could regulate different sets of genes that are essential for the development of the ala temporalis. Because the homeodomains of Dlx-1 and -2 are similar (52 of 60 amino acids are identical; McGuinness *et al.*, 1996), it is likely that the other regions of these proteins would be responsible for regulating distinct sets of genes. On the other hand, Dlx-1 and -2 could regulate the same set of genes, but both proteins could be required. Finally, Dlx-1 and -2 could operate in series in the same genetic pathway (e.g., Dlx-2 could regulate Dlx-1). Additional studies are needed to determine the biochemical and cellular mechanisms that underlie the phenotypic effects of these mutations.

Loss of Dlx-2 and Dlx-1 and -2 results in the formation of novel structures (the PQ and strut) (Figs. 2 and 3; Tables 1 and 2; Qiu *et al.*, 1995). Previously, we discussed the possibility that the Dlx-2 mutation leads to the formation of a splanchnocranial structure similar to the palatopterygoquadrate (PQ), a skeletal element that is present in evolutionarily more primitive vertebrates (Qiu *et al.*, 1995). While the Dlx-1 mutant does not form such a PQ, the Dlx-1 and -2 double mutant has a larger PQ (Figs. 3G and 3H), indicating synergy between Dlx-1 and Dlx-2 in the formation of this structure. Dlx-2 mutants also form the strut (~50% of the time; Fig. 2G), whereas Dlx-1 mutants do not. The evolutionary and developmental significance of the strut is unclear. The strut in the double mutants appears as frequently, and with the same morphology, as in the Dlx-2 mutants, suggesting that Dlx-1 does not play a role in the formation of this structure.

The formation of structural patterns that resemble those found in evolutionarily older species is not unique to the Dlx-2 and Dlx-1 and -2 mutants. Loss of function mutations of Hoxa-2 (Rijii *et al.*, 1993; Gendron-Maguire *et al.*, 1993; Mark *et al.*, 1995), MHox (Martin *et al.*, 1995), and RAR double mutants (Lohnes *et al.*, 1994) also lead to the formation of structures resembling parts of the palatopterygoquadrate. These results suggest, not surprisingly, that homeobox genes and retinoid signaling pathways have had a central role in the evolution of craniofacial structures. This implies that these studies will begin to shed light on the genetic constraints that are the framework for phylogenetic morphological changes.

### *Dlx-1 and -2 Have a Role in Odontogenic Patterning*

Mammalian teeth develop from a series of interactions between oral epithelial cells and neural crest-derived ectomesenchymal cells (Theseleff *et al.*, 1995). The molecular mechanisms controlling dental patterning (e.g., tooth shape) are unknown but have been proposed to be controlled by homeobox gene expression in the ectomesenchyme (the ''odontogenic homeobox code'' model; Sharpe, 1995). This model, proposed on the basis of overlapping domains of expression of several homeobox genes in odontogenic ectomesenchyme, predicts that Dlx-1 and/or Dlx-2 would be required for molar development but not for incisor tooth development.

Dlx-1 and Dlx-2 have dynamic expression patterns during odontogenesis (Thomas *et al.*, 1995; Bulfone *et al.*, 1993). Prior to overt tooth development (E9), Dlx-2 is expressed

predominantly in the lateral ectomesenchyme of both the mandibular and maxillary areas where presumptive molar tooth germs will form. Dlx-2 transcripts are also distributed in the oral epithelium in a pattern that is a mirror image of the mesenchymal expression (Bulfone *et al.*, 1993; Thomas *et al.*, 1995). The position where the epithelial and mesenchymal expression domains of Dlx-2 coincide corresponds to the position where the oral epithelium will thicken to produce the primary epithelial band where tooth germs will develop. Dlx-1 expression in the ectomesenchyme of the first branchial arch is very similar to that of Dlx-2, but Dlx-1 is expressed in the oral epithelium of the developing maxilla at much lower levels (Fig. 7).

The failure of maxillary molar teeth development in the Dlx-1 and -2 mutants (Fig. 4) supports the odontogenic homeobox code model for patterning of maxilla tooth development and suggests that Dlx-1 and Dlx-2 are required for the specification of a subpopulation (maxillary molar) of odontogenic neural crest cells. The normal development of mandibular molars implies that tooth patterning in the upper jaw is controlled independently of that in the lower jaw.

Mutations in two other genes involved in tooth development, Msx-1 and Lef-1, result in defects that affect all teeth, indicating that these gene products have a role in processes common to development of all teeth (van Genderen *et al.*, 1994; Satokata and Maas, 1994; Kratochwil *et al.*, 1996). In contrast, Dlx-1 and Dlx-2 are required for development of only maxillary molars. The defects in Msx-1 and Lef-1 mutant mice are believed to affect the signaling pathways between condensing mesenchyme and the epithelial tooth bud (Satokata and Maas, 1994; Kratochwil *et al.*, 1996). The regional defects in the Dlx-1 and -2 mutants suggest a specific role for these genes in regional specification of a subpopulation of odontogenic neural crest cells. The presence of ectopic cartilage in the region of the maxillary molars implies either that the crest cells are respecified or that cartilage is a default state in the mutants. Since no tooth abnormalities are observed in the Dlx-1 or Dlx-2 mutants, there appears to be functional redundancy of these genes in dental patterning.

### *Defects in Vascular, Neuronal, and Muscular Tissue*

The changes in the soft tissue anatomy are restricted to the proximal regions of the first and second arches, consistent with the location of the skeletal defects, demonstrating that Dlx-1 and -2 are essential for patterning multiple tissues in these regions. At present, the mechanisms underlying these vascular, neuronal, and musculoskeletal abnormalities have defied a simple explanation, although they suggest some interesting hypotheses.

The stapedial artery is a branch from the dorsal part of the second aortic arch (Goodrich, 1930; MacPhee, 1981; Wible, 1987). The Dlx mutants lack this artery but have an anomalous artery (ASA) that appears to take over the territory of the stapedial artery (Fig. 5). The ASA arises near where the first aortic arch enters the dorsal aorta. Normally, an artery in this region, the first aortic arch artery, degenerates, suggesting that in the Dlx mutants the ASA represents persistence of the first arch artery.

The course of the greater superficial petrosal nerve is abnormal in its proximal part, where it passes from its origin from the facial nerve (nerve of the second arch) into the most proximal maxillary process (Fig. 5). Distal aspects of its trajectory to the sphenopalatine ganglion appear normal, allowing for distortion due to abnormal skeletal anatomy in this region. This suggests that guidance mechanisms acting on the nerve are anomalous in the proximal maxillary and hyoid arches. In addition to the facial nerve abnormality, we previously reported that proximal fascicles of the mandibular branch of the trigeminal nerve (nerve of the first arch) had abnormal trajectories at E10.5 (Qiu *et al.*, 1995); we suggest that these might relate to the markedly abnormal pattern of jaw adductor muscles discussed below.

The proximal jaw adductors in the Dlx-2 and Dlx-1 and -2 mutants are remarkable for two reasons: their intracranial origins (Fig. 5) (wild-type proximal jaw adductors do not have intracranial origins) and they have many more bellies. In wild-type rodents, compared to other mammals, the rostral jaw musculature has multiple bellies that migrate through extracranial openings (e.g., the infraorbital canal). Thus, in this regard, the proximal musculature in the Dlx mutants has features in common with the distal musculature in the wild-type animals. While this is a provocative finding, it must be regarded tentatively, primarily because of the complex anatomy of the jaw musculature of rodents (Wood, 1974; Woods and Hermanson 1985).

### *Genetic Hierarchy Regulating Craniofacial Development*

Understanding the genetic control of CNC development is best considered in the context of the embryology of these cells. The CNC are derived from neuroepithelial cells of the lateral rhombencephalic, mesencephalic, and prosencephalic neural plate (Noden, 1988; Couly *et al.*, 1993; Serbedzija *et al.*, 1992; Osumi-Yamashita *et al.*, 1994, 1996; Schilling and Kimmel, 1994). It is likely that the premigratory CNC precursors, which reside in the CNS, acquire positional information that results in at least partial A-P specification (Fig. 9). Once the CNC migrate out of the CNS to populate the branchial arches and other craniofacial structures, signals arising from tissues such as the nonneural ectoderm, axial mesendoderm, mesoderm, and endoderm may provide P-D and M-L positional information.

Hox genes appear to have a central role in specifying A-P positional information to the CNC up to rhombomere 3 and therefore may regulate the morphogenetic programs of the hyoid (B2) and more posterior branchial arches. Mice lacking Hoxa-2 form skeletal structures in B2 that resemble proximal first arch elements (Rijii *et al.*, 1993; Gendron-Maguire *et al.*, 1993). Thus, in B2, Hoxa-2 and perhaps Hoxb-2 may affect the expression or function of genes involved in P-D patterning (e.g., Dlx-1 and -2). This would be similar to the role of a *Drosophila* Hox gene (Deformed) in

regulating Distal-less during development of the maxillary segment (O'Hara *et al.*, 1993).

Anterior of B2, where Hox genes are not expressed, the Dlx genes must be regulated by other transcription factors. Since the CNC that contributes to the maxillary and mandibular components of the first arch (B1) is derived from the posterior midbrain and rhombomeres 1 and 2 (Osumi-Yamashita *et al.*, 1994; Kontges and Lumsden, 1996), candidate regulators of the Dlx genes must be expressed in this neuroepithelium. For instance, Otx-2 could regulate the Dlx genes, because it is expressed in the posterior midbrain, and loss of Otx-2 function alters morphogenesis of multiple first arch bones (Matsuo *et al.*, 1995).

While understanding the genetic control of first arch development is at an early stage, the identification of essential transcription factors (Cart-1, Dlx-1, Dlx-2, Gsc, Msx-1, MHox, Pax-7, and RAR; Zhao *et al.*, 1996; Qiu *et al.*, 1995; Yamada *et al.*, 1995; Rivera-Perez *et al.*, 1995; Satokata and Maas, 1994; Martin *et al.*, 1995; Mansouri *et al.*, 1996; Lohnes *et al.*, 1994) and secreted molecules (e.g., BMP-7 and activin; Luo *et al.*, 1995; Matzuk *et al.*, 1995) has begun the process of elucidating the molecular and cellular mechanisms that regulate craniofacial morphogenesis.

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