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Combinatorial Expression of Three Zebrafish Genes Related to *Distal-Less*: Part of a Homeobox Gene Code for the Head

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We describe analysis of zebrafish *distal-less*–related homeobox genes that may serve as specifiers of positional information in anterior regions of the CNS and in peripheral structures. We isolated three zebrafish genes, *dlx2*, *dlx3*, and *dlx4*, by screening embryonic cDNA libraries. Comparisons of the predicted sequences of the Dlx2, Dlx3, and Dlx4 proteins with *distal-less* proteins from other species suggest that vertebrate *distal-less* genes can be divided into four orthologous groups. We observed similarities but also unique features of the expression patterns of the zebrafish *dlx* genes. Among the three genes, *dlx3* alone is expressed during gastrulation. Shortly after gastrulation, cells in the ventral forebrain rudiment express *dlx2* and *dlx4*, but not *dlx3*, and hindbrain neural crest cells express only *dlx2*. Presumptive precursor cells of the olfactory placodes express *dlx3* and *dlx4* but not *dlx2*. Transcripts of *dlx3* and *dlx4* are present in overlapping subsets of cells in the auditory vesicle and in cells of the median fin fold, whereas *dlx2* is never expressed in the auditory vesicle and only at low levels in localized regions of the median fin fold. Cells of the visceral arches and their primordia express all three *dlx* genes, but with different developmental time courses. We suggest that combinatorial expression of the *dlx* genes is part of a homeobox gene code specifying pattern formation or cell fate determination in the forebrain, in peripheral structures of the head, and in the fins.

[Key words: branchial arches, cranial neural crest, ear, fin, forebrain, homeobox gene]

In vertebrates, homeodomain proteins may participate in the coding of positional information during the development of segmented structures (McGinnis and Krumlauf, 1992). The homeodomain, a highly conserved DNA-binding domain, has been found in the product of a large number of genes that encode putative or known transcription factors. The most widely studied vertebrate homeobox genes are members of the four mammalian *Hox* gene complexes that belong to the *Antennapedia* class, as deduced from sequence comparisons (Boncinelli et al., 1988; Gaunt et al., 1988; Duboule and Dollé, 1989; Graham et al., 1989).

Combinations of functionally active *Hox* genes, or *Hox* codes, are thought to play a determinant role in the development of the vertebrae (Kessel and Gruss, 1991), the hindbrain, the branchial arches (Hunt et al., 1991a,b), and the limbs (Morgan et al., 1992). Combinatorial expression of *Hox* genes may specify the identity of vertebral segments, as suggested by the homeotic transformations produced by alterations in the expression patterns of the *Hox* genes by either ectopic expression (Kessel et al., 1990; Kessel and Gruss, 1991; Jegalian and DeRobertis, 1992) or loss of function (Wright et al., 1989; Le Mouellic et al., 1992). The mouse *HoxA7* gene placed under the control of the chicken β-actin promoter was ectopically expressed in regions anterior to its normal boundary of expression, resulting in the formation of an additional vertebra, the proatlas (Kessel et al., 1990). A targeted null mutation in the *HoxC8* gene resulted in anterior transformation of several skeletal segments (Le Mouellic et al., 1992). In the avian limb bud, alteration of *HoxD11* expression and, consequently, of the combinatorial expression of the *HoxD* complex genes produced a posterior homeotic transformation (Morgan et al., 1992). In these cases, although not in others (Pollock et al., 1992), the homeotic transformations obeyed a rule analogous to the posterior transformation rule first suggested in *Drosophila* (Lewis, 1978; McGinnis and Krumlauf, 1992). The molecular mechanisms by which combinations of active *Hox* genes determine segment or digit identities are presently unknown, and genetic coding of positional information in other regions of vertebrate embryos, particularly the head, has yet to be elucidated.

Here we describe the cloning of three zebrafish homeobox genes, *dlx2*, *dlx3*, and *dlx4*, that belong to the *distal-less* family of genes and that may specify positional information in the head. Vertebrate members of the *distal-less* family include the mouse *Dlx1* and *Dlx2* (*Tes-1*) genes (Porteus et al., 1991; Price et al., 1991; Robinson et al., 1991), the newt *NvHBox-4* and *NvHBox-5* genes (Beauchemin and Savard, 1992), and the *Xenopus Xdll*, *X-dll1*, *X-dll2*, *X-dll3* and *X-dll4* genes (Asano et al., 1992; Dirksen et al., 1993; Papalopulu and Kintner, 1993). Comparisons of the expression patterns of *dlx2*, *dlx3*, and *dlx4* in zebrafish embryos indicate that distinct regions of the embryo express specific combinations of the three genes. We suggest that the *distal-less* genes could participate in a new type of homeobox gene code during development.

Materials and Methods

Animals. Embryos from the Oregon AB line were maintained using standard methods (Westerfield, 1993) and were staged at 28.5°C according to hours (h) and days (d) postfertilization.

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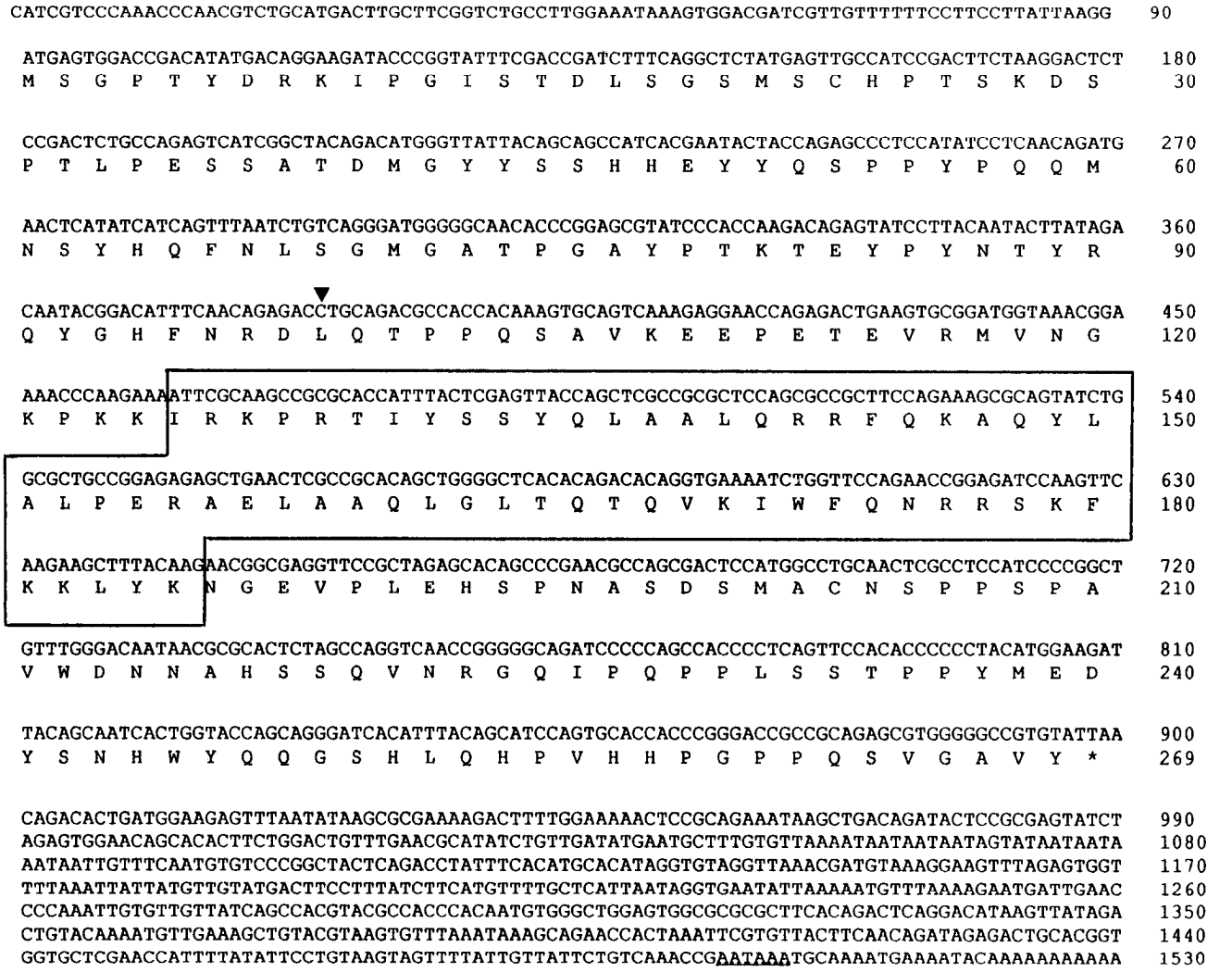

Figure 1. The nucleotide and the predicted amino acid sequences of the zebrafish *dlx3* gene. The homeodomain is *boxed.* The probe (see Materials and Methods) used for *in situ* hybridization was made from the sequences between nucleotide position 1 and the *Pst*I site at position 385 (*arrowhead*). A potential polyadenylation site is *underlined.*

Isolation and sequencing of zebrafish dlx *genes. Dlx3* was isolated during the screening of a lambda gt-11 cDNA library, prepared from 2 d zebrafish mRNA, with a probe that includes the entire homeobox sequence of the mouse *Msx1*(*Hox-7*) gene (Robert et al., 1989; Ekker et al., 1992a). *Dlx2*, *dlx4*, and additional *dlx3* cDNA clones were obtained by screening two Lambda ZAP II (Stratagene, San Diego, CA) cDNA libraries prepared from 9–16 h and 20–28 h zebrafish mRNA with a 150 bp *Xho*I- *Hind*III restriction fragment of the *dlx3* cDNA corresponding to the 5′ portion of the *dlx3* homeobox. Restriction fragments of the cDNAs were isolated and subcloned into Bluescript phagemids (Stratagene, San Diego, CA). Single-strand templates were prepared from these phagemids according to the manufacturer's instructions. DNA sequencing was performed by the dideoxy-termination method using Sequenase (U.S. Biochemicals, Inc.), according to the manufacturer's directions. Sequences were analyzed with the GCG package and the evolutionary tree was constructed using the Phylip package kindly provided by J. Felsenstein (U. of Washington).

In situ *hybridization.* Zebrafish embryos were fixed with paraformaldehyde and *in situ* hybridization was performed on whole-mount or sectioned embryos using antisense riboprobes as previously described (Ekker et al., 1992a; Püschel et al., 1992). The following DNA fragments were used as templates for the synthesis of antisense riboprobes: *dlx2*, either the entire 1667 bp cDNA or a 473 bp *Eco*RI fragment in which one *Eco*RI occurs at position 496, the cloning site of a shorter cDNA clone, and the other at position 968 (Fig. 2); *dlx3*, either the entire 1532 bp cDNA or a 390 bp *Eco*RI-*Pst*I fragment in which the *Eco*RI site was the 5′ cloning site and the *Pst*I site was located at position 385 (Fig. 1); *dlx4*, either the entire 1123 bp cDNA, a 744 bp *Pst*I-*Eco*RI fragment with the *Pst*I site at position 136 and the *Eco*RI site at position 879, or a 484 bp *Eco*RI-*Hinc*II fragment from the 5′ *Eco*RI clone site to the *Hinc*II site at position 484 (Fig. 3). In all cases, different probes from each gene produced identical expression patterns except that the intensity of the signal was strongest with the longest probe.

Despite the high degree of sequence similarity among the three *dlx* genes and our use of at least part of the coding region, cross-hybridization among probes was minimal, as indicated by the unique features of each *in situ* hybridization pattern.

Results

Molecular cloning of three zebrafish homeobox genes related to distal-less

We isolated cDNAs that correspond to the transcripts of genes with homeoboxes related in sequence to the *Drosophila distal-less* gene (Cohen et al., 1989; Vachon et al., 1992). We recovered the first of these genes, which we named *dlx3* during a screen for zebrafish genes belonging to the *msx* family of homeobox genes (Akimenko et al., 1991; Ekker et al., 1992a). Southern analysis of zebrafish genomic DNA with a probe corresponding to the homeobox of *dlx3* (not shown) suggested that the zebrafish

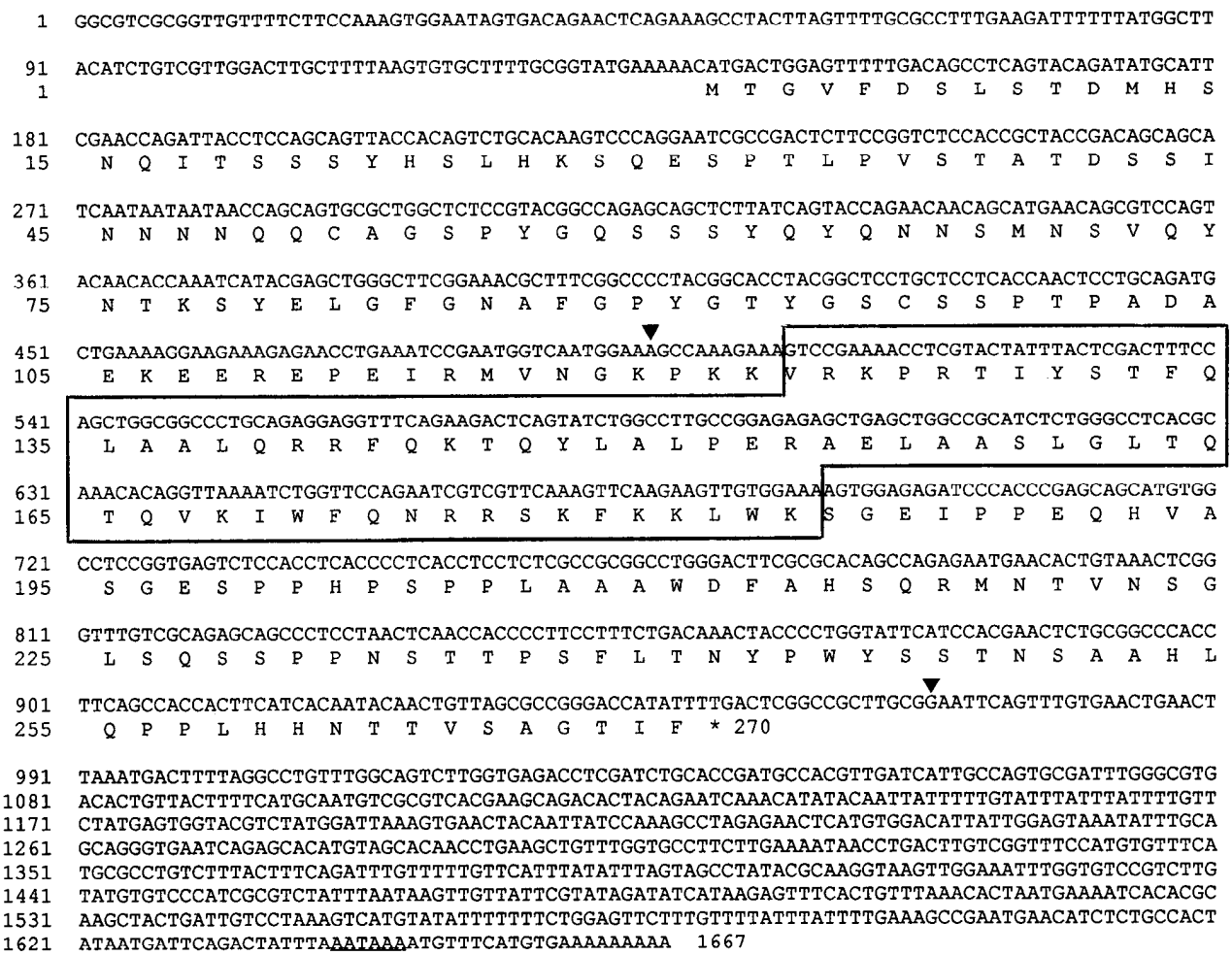

Figure 2. The nucleotide and the predicted amino acid sequences of the zebrafish *dlx2* gene. The homeodomain is *boxed.* The probe used for *in situ* hybridization was made from the sequences between nucleotide position 496 (5′ end of a shorter cDNA clone) and the *Eco*RI site at position 968 (*arrowheads*). A potential polyadenylation site is *underlined.* The sequence accession number for *dlx2* is U03875.

genome might contain additional genes with related homeoboxes. We therefore screened embryonic cDNA libraries and obtained clones corresponding to two additional *dlx* genes, *dlx2* and *dlx4*, and several more *dlx3* cDNA clones.

The longest *dlx3* cDNA clone, with 1532 bp, contains an open reading frame of 843 bp (Fig. 1). Several potential ATG initiation codons are located near the 5′ end of the open reading frame; the ATG at position 91 is the last ATG in the 5′ direction before an in-frame stop codon and the only ATG upstream from a region that is well conserved at the amino acid level between *dlx3* and a related newt gene (see below). If used as the starting codon, this ATG would initiate translation of a 269 amino acid protein. A polyadenylation site occurs at positions 1498–1503.

We isolated three cDNAs corresponding to the *dlx2* gene. The longest of these cDNAs (1667 bp; Fig. 2) contains an open reading frame of 834 bp. Two ATG initiation codons are present in the 5′ end of the open reading frame. Use of the more upstream ATG would produce a protein of 273 AA and use of the next ATG would encode a protein with a predicted size of 270 AA. Amino acid sequence comparisons with the product of the mouse *Dlx2* gene (not shown) suggest that the amino terminus of the protein is conserved between fish and mice and that the second ATG serves as the initiation codon. A polyadenylation site is found at positions 1641 to 1646.

We isolated three cDNAs corresponding to the *dlx4* gene. The longest is 1123 bp (Fig. 3) and the largest open reading frame within this cDNA is 906 bp. Using the ATG initiation codon nearest to the 5′ end of this long open reading frame predicts a protein of 283 AA.

The predicted homeodomains of *dlx2*, *dlx3*, and *dlx4* (Fig. 4*A*) closely resemble those of the mouse *Dlx1* and *Dlx2* (*Tes-1*) genes (Porteus et al., 1991; Robinson et al., 1991), the newt *NvHBox-4* and *NvHBox-5* genes (Beauchemin and Savard, 1992), and the *Xenopus X-dll1-4* gene (Asano et al., 1992; Dirksen et al., 1993; Papalopulu and Kintner, 1993).

The zebrafish Dlx proteins are related to each other and to Dlx proteins from other species

The homeodomains of the three zebrafish Dlx proteins are identical at 51 positions (84%) and the three proteins are 80% identical over a stretch of 85 AA that includes the 17 AA residues that precede the homeodomain, the homeodomain itself, and the 7 AA that follow it (Fig. 4*A*). One additional region of high similarity is found in a 19 AA stretch near the amino terminal of the predicted proteins (Fig. 4*B*), where the three proteins are 53% identical. Interestingly, a similar sequence is found in the mouse *Dlx2* (*Tes-1*) (Porteus et al., 1991; Robinson et al., 1991), the newt *NvHBox-4* (Beauchemin and Savard, 1992), and the

```
   1  GAGACGCTTGACGCTTGCACGCACAATGTTAGTGTAGACTCTTTGGACCCCTATAGAGTTAGACTGCCTTTTTTTACGTCGTCTTATCCG

  91  AACTATGACTGGAGTATTCGACAGAAGGATTCCGAGTATTAAACCTGCAGATTTTCAAAACCCTTTTCAGCTCTCCACGATGCATCATCC
   1      M  T  G  V  F  D  R  R  I  P  S  I  K  P  A  D  F  Q  N  P  F  Q  L  S  T  M  H  H  P

 181  GTCTCAGGAATCTCCAACCCTACCGGAGTCCACAGCCACGGATTCTGGCTATTACAGCCCTGCTGGAGGAGTTCATCATGGCTATTGTTC
  30   S  Q  E  S  P  T  L  P  E  S  T  A  T  D  S  G  Y  Y  S  P  A  G  G  V  H  H  G  Y  C  S

 271  ACCGAACTCGGGCACCTATGGGAAACCTCTTAATGCCTATCAGTACCAATACCACGGAGTCAATGGATCTTCTGGAAATTACTCTGCAAA
  60   P  N  S  G  T  Y  G  K  P  L  N  A  Y  Q  Y  Q  Y  H  G  V  N  G  S  S  G  N  Y  S  A  K

 361  ATCCTACCCTGATTACGGCTCATACTCCACAGCGTATCACCAATACGCAGGAACATATAACAGAGTGCAATCACAACCGAGCCCGCAAGA
  90   S  Y  P  D  Y  G  S  Y  S  T  A  Y  H  Q  Y  A  G  T  Y  N  R  V  Q  S  Q  P  S  P  Q  E

 451  AAAAGAAACAGCCGAGCCCGAAGTAAGGATGGTCAACGGAAAACCCAAAAAAGTCCGGAAGCCCCGAACCATTTACTCCAGTTTCCAGCT
 120   K  E  T  A  E  P  E  V  R  M  V  N  G  K  P  K  K  V  R  K  P  R  T  I  Y  S  S  F  Q  L

 541  CGCAGCTTTACAGAGAAGGTTTCAGAACACGCAATACCTCGCGCTTCCAGAAAGAGCCGAGCTCGCCGCATCGCTGGGACTCACACAGAC
 150   A  A  L  Q  R  R  F  Q  N  T  Q  Y  L  A  L  P  E  R  A  E  L  A  A  S  L  G  L  T  Q  T

 631  ACAGGTGAAAATCTGGTTCCAGAACAAAAGATCAAAACTAAAGAAGATTATGAAAAACGGCGAACTGCCCCCAGAACACAGCCCCAGCTC
 180   Q  V  K  I  W  F  Q  N  K  R  S  K  L  K  K  I  M  K  N  G  E  L  P  P  E  H  S  P  S  S

 721  CAGCGACCCAATGGCGTGTAACTCACCGCAGTCTCCCGCGGTCTGGGACTCACAGGGTCCTCAGAGACCTCACCATCAGCCGCAAAATAT
 210   S  D  P  M  A  C  N  S  P  Q  S  P  A  V  W  D  S  Q  G  P  Q  R  P  H  H  Q  P  Q  N  I

 811  TAACACAGCGGCATCCACGTTTCTGGAAATGCGCGAGCTCTTCGTGGTATTCCTCTACCGGGGCGATGAATTCTTCACCCTTCAGGCACC
 240   N  T  A  A  S  T  F  L  E  M  R  E  L  F  V  V  F  L  Y  R  G  D  E  F  F  T  L  Q  A  P

 901  CGGCACGTTACACTCGTTGGCACTCGGATCAGGAACGTTGTACTGAAAATTGTTTATTATTTTTGTTGTATATTGGACTGGTTGTTAACA
 270   G  T  L  H  S  L  A  L  G  S  G  T  L  Y  *  283

 991  ATTTTTTTGAGGAATATGCAATGTATCGATATGGCAGTCCTAGAAGAACGTGTATAATGTGTAAATTGTGTGCATGTAATTTATTGCATT
1081  TGGAAGAATTATTAAATGTTTTAAATGGACAATGGAAAAAAAA  1123
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Figure 3. The nucleotide and the predicted amino acid sequences of the zebrafish *dlx4* gene. The homeodomain is *boxed.* The probe used for *in situ* hybridization was made from the sequences between the *Pst*I site at position 136 and the *Eco*RI site at position 879 (*arrowheads*). The sequence accession number for *dlx4* is U03876.

Xenopus X-dll1, X-dll2, X-dll3, and *X-dll4* (Dirksen et al., 1993; Papalopulu and Kintner, 1993) genes, but not in the newt *NvHBox-5* (Beauchemin and Savard, 1992), the mouse *Dlx1* (personal communication from J. L. R. Rubenstein), or the *Xenopus X-dll* (Asano et al., 1992) genes. Several interspersed amino acid residues are also well conserved among vertebrate *distal-less* proteins for which this information is available. Examples include the Q residue at AA position 65 in Figure 1, the Y at position 92, a WD sequence at positions 222–223, the LQXP sequence at positions 252–255, and the G located four residues before the stop codon (Fig. 1).

The vertebrate dlx *genes can be subdivided into four orthologous groups*

We performed pairwise and multiple alignments of the predicted protein sequences of the zebrafish and other vertebrate *dlx* genes. From the results of pairwise alignments we built an evolutionary tree that divides the vertebrate *dlx* genes into four orthologous groups (Fig. 5), with each of the zebrafish *dlx2*, *dlx3*, and *dlx4* genes belonging to a different group.

The product of the zebrafish *dlx2* gene shows extensive similarity to that of the mouse *Dlx2* (*Tes-1*) and the *Xenopus X-dll4* and *X-dll1* genes, both within and outside the homeodomain (97%, 95%, and 95% identity in the homeodomain, and 69%, 67%, and 67% overall identity, respectively). On the basis of this sequence analysis, we named this zebrafish gene *dlx2*. The homeodomain encoded by *dlx3* is identical to that of the newt *NvHBox-4* gene product and nearly identical to that of *X-dll2*, with only one substitution (Fig. 4*A*). The sequences outside the homeodomain are also very similar, 72% and 65% identity, respectively. Therefore, *dlx3*, *NvHBox-4*, and *X-dll2* could be the zebrafish, newt, and *Xenopus* versions of the same gene. The zebrafish Dlx4 protein shows particular sequence similarity only with the predicted product of *X-dll3* (72% identity).

Division of vertebrate *distal-less* genes into subfamilies was recently proposed on the basis of the sequence similarities of their homeodomains (Papalopulu and Kintner, 1993). According to this classification, the *Xdll-3* and *Xdll-4* genes belong to a single subfamily, while our analysis suggests that they belong to two different orthologous groups. Comparisons of complete protein sequences are more likely to reveal differences and similarities than comparisons of homeodomain sequences alone because of the high degree of sequence conservation in the homeodomain, even though there is somewhat greater conservation of the predicted homeodomains among members of a given orthologous group (Fig. 4*A*). Conservation of the 19 AA sequence found in the amino terminal regions of the proteins is also consistent with this division of the genes into four orthologous groups (Fig. 4*B*). Our analysis further suggests that the fourth group, which includes the mouse *Dlx1*, the newt *NvHBox-5*, and the *Xenopus Xdll* genes, may also contain a possible fourth and as yet unidentified zebrafish *distal-less*–related gene.

Mouse homeobox sequences named *Dlx3* and *Dlx4* were pre-

A

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                                        1
                                        VRKPRTIYST FQLAALQRRF QKTQYLALPE
    z-dlx2  KEEREPE IRMVNGKPKK
mouse Dlx2  ||DL||| ||I|||||| |||||||||S |||||||||| ||||||||||
    X-dll4  ||DC||| V|||||||| |||||||||S |||||||||| ||||||||||
    X-dll1  |||C||| V|||||||| |||||||||S ||M|||||| ||||||||||

    z-dlx3  |||P|T| V|||||||| I|||||||S Y|||||||| ||A|||||||
  NvHBox-4  |||P||| V|||||||| I|||||||S Y|||||||| ||A|||||||
    X-dll2  |||||T| V|||||||| I|||||||S Y|||||||| ||A|||||||

    z-dlx4  ||TA||| V|||||||| |||||||S |||||||||| |N|||||||
    X-dll3  ||VS||| V|||||||| I|||||||S |||||||||| ||||||||||

mouse Dlx1  STVV|GG EVRF|||G|| I|||||||S L||Q||N||| |Q|||||||
    X-dll   TTVI|NG EIRF|||G|| I|||||||S L||Q||NH|| |Q|||||||
  NvHBox-5  NTVI|NG EIRI|||G|| I|||||||S V||Q||NQ|| |Q|||||||

                                                    61
                                        RAELAASLGL TQTQVKIWFQ NRRSKFKKLWK SGEIPPEQH
    z-dlx2
mouse Dlx2  |||||||||| |||||||||| |||||||M|| |||||T|||
    X-dll4  |||||||||V |||||||||| |||||||M|| |||||SD|L
    X-dll1  |||||||||| |||||||||| |||||||M|| N||M|SD|L

    z-dlx3  ||||||Q||| |||||||||| |||||||||Y| N||V|.LE|
  NvHBox-4  ||||||Q||| |||||||||| |||||||||Y| N||V|GME|
    X-dll2  ||||||Q||| |||||||||| |||||||||Y| N||G|GLE|

    z-dlx4  |||||||||| |||||||||| |K|||L||I|| N||L|||.|
    X-dll3  |||||||||| |||||||||| |K|||I||M|| N||L|||.|

mouse Dlx1  |||||||||| |||||||||| |||||||||M| Q|GAAL|GS
    X-dll   |||||||||V |||||||||| |K|||Y|||I| Q|NPLEIDQ
  NvHBox-5  ||||||H||| |||||||||| |K|||Y||IM| Q|SSIQ|GE
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B

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    z-dlx4  SQESPTLPES TATDSGYYS
    X-dll3  ||D|||||||| |||||||||

    z-dlx2  |||||||||V| |||||SINN
mouse Dlx2  P|||||||V| |||||S||T
    X-dll4  |||||||||V| |||||S||T
    X-dll1  |||||||||V| |||||S||T

    z-dlx3  |KD||||||| S|||M||||
  NvHBox-4  |KD||||||| S|||L||||
    X-dll2  TKD||||||| S|||M||||
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Figure 4. Sequences of conserved regions in vertebrate Dlx proteins. The amino acid sequences of the homeodomain and surrounding amino acids (*A*) and of a region near the N-terminus (*B*) of the three zebrafish Dlx predicted proteins are aligned with the products of related vertebrate genes. Identities are indicated by *vertical bars*. Gaps are indicated by *dots*. The homeodomain in *A* is indicated with a *horizontal bar*. The sources for the sequences are, for mouse *dlx2*, Porteus et al. (1991), Robinson et al. (1991); *Xenopus X-dll1*, Dirksen et al. (1993); *X-dll*, Asano et al. (1992); *X-dll2*, *X-dll3*, and *X-dll4*, Papalopulu and Kintner, (1993); and newt *NvHBox-4* and *NvHBox-5*, Beauchemin and Savard (1992). The mouse *Dlx-1* sequence is a personal communication from J. L. R. Rubenstein.

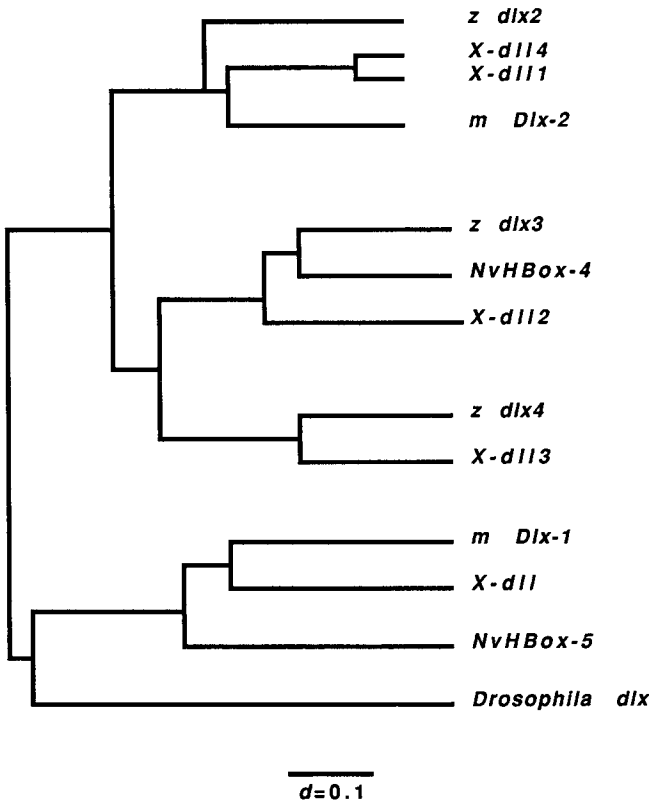

Figure 5. *Dlx* genes form four orthologous groups: evolutionary tree for 13 *distal-less*–related homeobox genes. The same nomenclature was used as for Figure 4. The tree was made from a distance matrix using the neighbor joining method (Saitou and Nei, 1987). The distance matrix was calculated from amino acid sequence identity values. A tree predicting the same relationships among the genes was obtained using amino acid sequence similarity values. The scale bar indicates the estimated evolutionary distance.

viously reported (Robinson et al., 1991) and the presence of a mouse *Dlx3* gene was also suggested by Dollé et al. (1992). However, in the former report, only the homeobox sequences are given, and in the latter report, no sequence information is available. Therefore, considering the high degree of sequence similarity among *distal-less* homeoboxes, it is not yet possible, only on the basis of homeobox sequence comparisons, to determine in which orthologous group these two mouse genes belong.

Expression of the dlx *genes in zebrafish embryos*

Gastrulation and the olfactory and auditory placodes. Expression of the *dlx* genes begins with *dlx3* during late gastrula stages. By 8 h (hours postfertilization) scattered cells on the ventral side of the embryo express detectable levels of *dlx3* transcripts. Within an hour, expressing cells coalesce into a stripe that extends around the lateral edge of the embryo, midway between dorsal and ventral, and which is shaped like the stitches on a baseball (Fig. 6*A*). Along its anterior half, the stripe is approximately six cells wide and includes presumptive ectodermal cells (Fig. 6*D*). Posteriorly, the stripe broadens and the scattered expressing cells are separated from one another by nonexpressing cells (Fig. 6*A*). During gastrulation, the lateral parts of the stripe converge toward the dorsal midline (Fig. 6*B*), probably due to convergence of the *dlx3*-expressing cells.

By the end of gastrulation, the lateral regions of the *dlx3* stripe have reached the edge of the developing neural keel and expression is progressively restricted to two patches on each side that later form the olfactory and auditory (Ekker et al., 1992a) placodes and a patch at the posterior end of the embryo that may contribute to the tail bud (Fig. 6*C*). The formation of these patches is probably due to downregulation of *dlx3* expression by cells in intervening regions of the stripe, rather than to migration of expressing cells into the patches, because there is no obvious increase in the numbers of expressing cells in the regions of the patches and because convergence of cells during gastrulation is accompanied by extension rather than contraction along the dorsal midline (Warga and Kimmel, 1990). Beginning around 24 h and continuing until about 48 h, cells of the olfactory placodes also weakly express *dlx4*.

Cranial neural crest. Presumptive neural crest cells in the head express *dlx2* beginning around 12 h. Initially, cells scattered over the dorsal surface of the hindbrain express *dlx2* in a pattern that shows no obvious segmental organization (Fig. 7*A*). By 13 h, the positive cells, which can then be recognized as neural

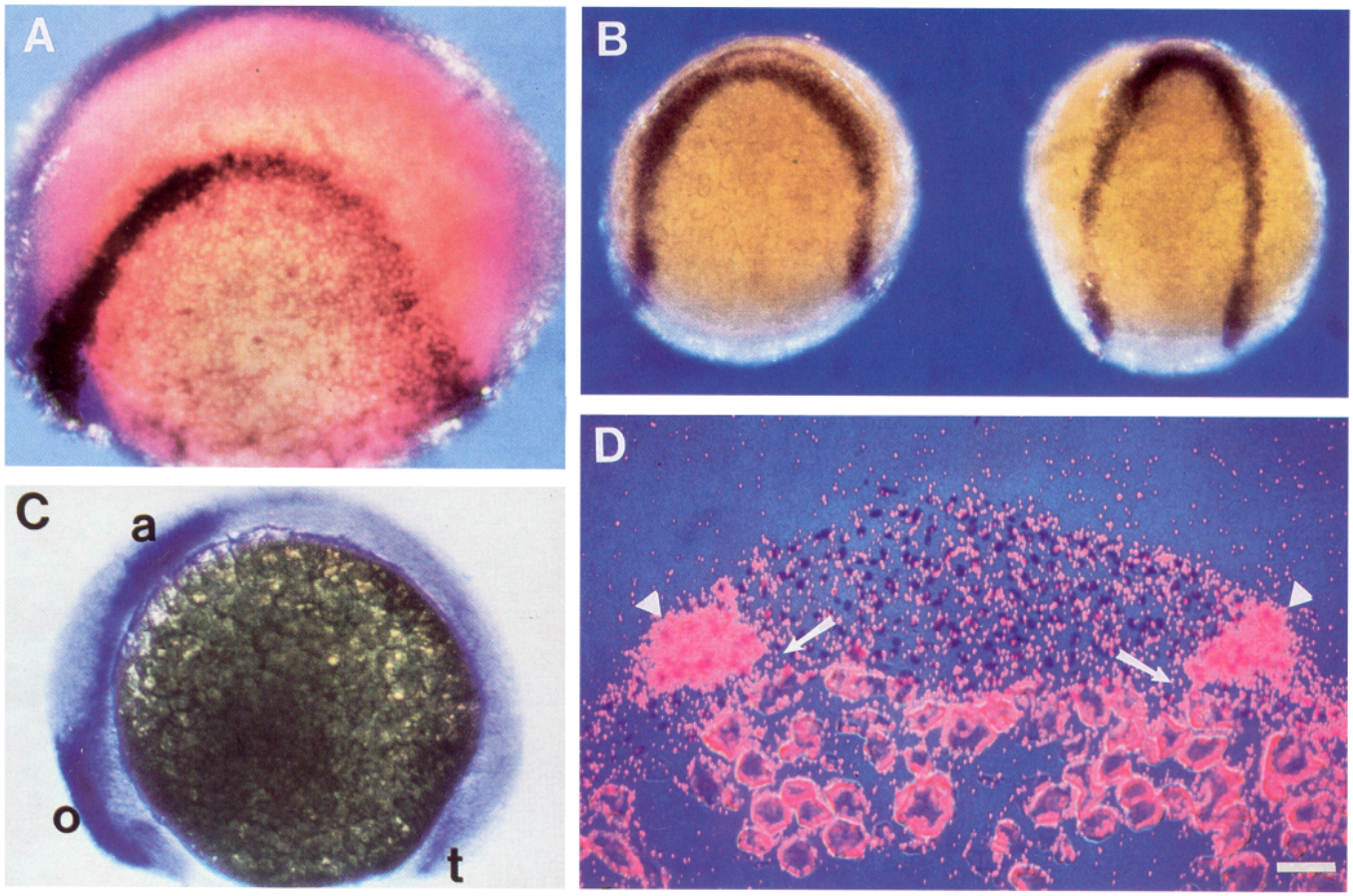

Figure 6. Ectodermal cells of the gastrula express *dlx3* transcripts: *in situ* hybridization of whole-mount embryos with a *dlx3* probe. *A*, At 9 h, scattered cells on the ventral surface of the embryo and a denser stripe of cells around the lateral edge of the presumptive neural plate express *dlx3*. *B*, The lateral regions of the stripe of *dlx3*-expressing cells rapidly converge toward the dorsal axis shown in 9.5 h (*left*) and 10.25 h (*right*) embryos. *C*, Expression becomes progressively restricted and includes precursors of the olfactory (*o*) and auditory (*a*) placodes and the tailbud (*t*) shown here at 12 h. *D*, Superficial cells express *dlx3*, shown here in a cross section through the anterior region of a 10 h embryo. The *dlx3* signal appears as *red grains* in this autoradiograph. The *red at the bottom* is produced by the yolk granules and is not due to specific hybridization as revealed by examination of nonhybridized sections. The *arrows* indicate unlabeled, presumptive mesodermal cells. Anterior to the left and dorsal to the top in *A* and *C*; dorsal midline view looking down on animal pole in *B* with anterior to the top; dorsal is up *D*. Scale bar: 60 μm for *A*, 100 μm for *B*, 85 μm for *C*, 40 μm for *D*.

crest cells by their morphologies and positions (Schilling, 1993), appear in a distinct segmental pattern as they seem to migrate into the periphery along pathways adjacent to rhombomeres 1, 2, 4, and 6 (Fig. 7*B*). Expression of *dlx2* persists in the migrating crest cells as they reach and enter the primordia of the branchial arches (Fig. 7*C*).

Forebrain. By 13 h, cells of the presumptive forebrain express *dlx2* and *dlx4*. During the first day of development (Fig. 8*A*), the expression patterns of the two genes are similar, with positive cells in the presumptive telencephalic and diencephalic bands (Wilson et al., 1990). These bands probably correspond to the two domains of *distal-less* expression in the forebrain of mouse (*Dlx1* and *Dlx2*; Bulfone et al., 1993b) and *Xenopus* (*X-dll3* and *X-dll4*; Papalopulu and Kintner, 1993). During subsequent development, divergence of the zebrafish *dlx2* and *dlx4* patterns becomes more apparent as overlapping but distinct sets of forebrain cells express the two genes (Figs. 8*B,C*; 9*A,C*). In the telencephalon, cells expressing *dlx2* are located medially (Figs. 8*B*, 9*A*) close to the ventricular surface, whereas *dlx4*-expressing cells appear as bilateral bands located more superficially (Figs.

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Figure 7. Cells of the cranial neural crest and precursors of the branchial arches express the *dlx2* gene. *A*, Presumptive neural crest cells along the dorsal and dorsal lateral sides of the hindbrain express *dlx2* at 12 h. *B*, Neural crest cells migrating from the hindbrain rhombomeres of a 13 h embryo express *dlx2* while hindbrain cells in rhombomeres 3 and 5 (*arrowheads*) express *krx20* (Oxtoby and Jowett, 1993). *C*, Cells in the mandibular (*m*), hyoid (*h*), and first through third gill arches (*1–3*) express *dlx2*, shown here at 24 h. *D*, Mesenchymal cells in the arches express *dlx2*, whereas cells of the pharyngeal endoderm lining the arches do not (indicated here at 48 h and in *C* by *arrows*). Anterior is to the left and dorsal to the top; lateral views in *A*, *C*, and *D*; dorsal view in *B*. Scale bar, 60 μm for *A* and *B*, 25 μm for *C*, 16 μm for *D*.

Figure 8. Overlapping but distinct sets of forebrain cells express *dlx2* and *dlx4*. *A*, Side view of head of whole-mount embryo at 24 h hybridized with *dlx2* probe. *B* and *C*, Approximately horizontal sections through heads of 27 h embryos hybridized with *dlx2* (*B*) or *dlx4* (*C*) probes and counterstained with basic fuchsin. The *arrows* in *A* and *C* indicate expression in the line of cells that extends through the presumptive hypothalamus. *t*, telencephalic band; *d*, diencephalic band; *e*, eye. Scale bar, 25 μm.

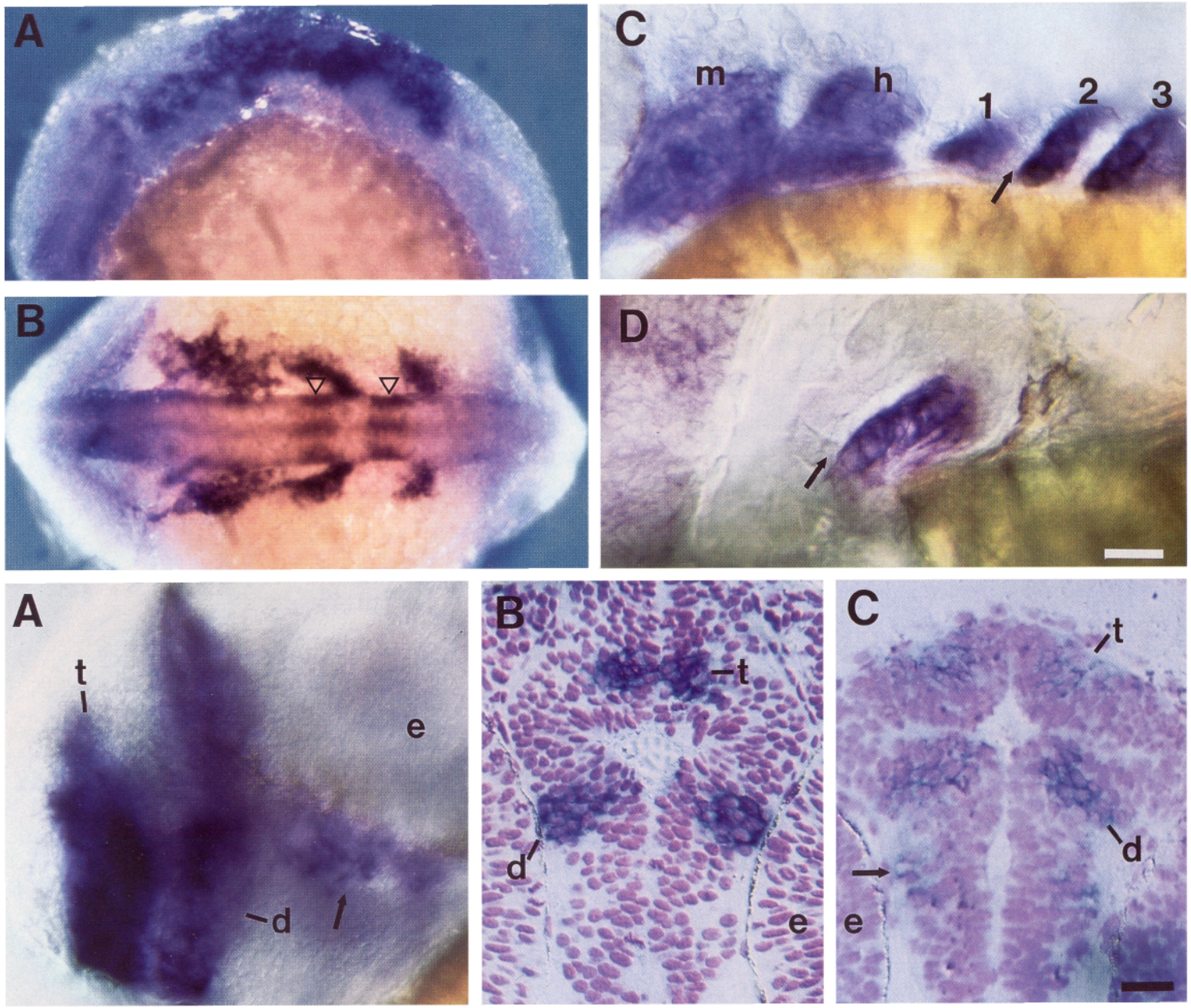
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Table 1. Summary of expression patterns of three zebrafish *dlx* genes

	16 h	24 h	48 h	55–60 h
Telencephalon	2 – 4	2 – 4	2 – 4	2 – 4
Diencephalon	2 – 4	2 – 4	2 – 4	2 – 4
Olfactory placodes and precursor cells	– 3 –	– 3 4	– 3 4	– 3 –
Migrating neural crest	2 – –	– – –	– – –	– – –
Pectoral girdle	– – –	– 3 –	– 3 –	– 3 –
Otic placode and vesicle	– 3 –	– 3 4	– 3 4	– – 4
Sensory maculae	– – –	– – –	2 – 4	2 – 4
Pectoral fin bud	2 – –	2 – –	2 3 4	2 3 4
Median fin fold	– 3 4	2 3 4	– – –	– – –
Visceral arches				
Mandibular	2 – –	2 3 4	2 3 –	2 3 –
Hyoid	2 – –	2 3 4	2 3 4	2 3 4
Gill arch 1	2 – –	2 – 4	– 3 4	– 3 4
Gill arch 2	2 – –	2 3 4	2 3 4	– 3 4
Gill arch 3	2 – –	2 – –	2 3 4	2 3 4
Gill arch 4	2 – –	2 – –	2 3 4	2 3 4

Expression of *dlx2* (2), *dlx3* (3), or *dlx4* (4) was detected by *in situ* hybridization in structures, as listed at the left, at the times listed at the top. The – symbol indicates undetectable levels of expression.

8*C*, 9*C*), corresponding very well to the expression patterns of the *Xenopus X-dll4* and *X-dll3* genes, respectively (Papalopulu and Kintner, 1993). The expression patterns of the two zebrafish genes are similar in the diencephalon (Fig. 8*B,C*), and as in *Xenopus*, include bilateral punctate lines of expressing cells that extend from the diencephalic bands through the presumptive hypothalamus (Fig. 8*A,C*, arrows).

Branchial arches and pectoral girdle. Cells of the branchial arches express the three *dlx* genes with different time courses. Of the three genes, *dlx2* is expressed first, appearing in all the arch primordia by 16 h, a time consistent with the arrival of the *dlx2*-expressing hindbrain neural crest cells (Schilling, 1993). By 24 h, cells in the mandibular, hyoid, and second gill arch primordia express all three genes, whereas the primordium of the first gill arch expresses only *dlx2* and *dlx4*, and the primordia of the posterior arches express only *dlx2* (Fig. 7*C*). By 36 h, cells in all the arches express all three genes (Table 1), but by 48 h, *dlx4* expression disappears from the mandibular arch (Fig. 9*C*) followed by a rostral-to-caudal loss of *dlx2* expression from the gill arches (Table 1). Cells in all the gill arches still express the *dlx3* and *dlx4* genes at 55 h. In each gill arch, expression of all three *dlx* genes appears in the lateral, mesenchymal cells but is absent from the medial, endodermal cells (Fig. 7*C,D*).

Beginning at 24 h, an accumulation of cells posterior to the last gill arch primordium expresses *dlx3*, but not *dlx2* or *dlx4* transcripts. During subsequent development, this expression domain extends posteriorly and includes precursor cells of the presumptive pectoral girdle (not shown).

Auditory vesicle. Cells oriented along the future axes of the auditory vesicle express *dlx3* and *dlx4*, but not *dlx2* (Fig. 9*D–F*). By 24 h, cells on the medial and caudal surface of the vesicle express *dlx3* (Fig. 9*E*). The ventral edge of this expression domain is aligned with one of the future axes of the semicircular canals, as we have described previously (Ekker et al., 1992a). At this stage, cells on the medial and anterior surface of the vesicle express *dlx4*. The ventral edge of this *dlx4* expression domain is oriented obliquely to the ventral edge of the *dlx3* domain (Fig. 9*F*) and may also be related to the future axes of the inner ear.

As the sensory maculae differentiate from the epithelium of the auditory vesicle, cells in the region of the maculae begin to express the *dlx2* and *dlx4* genes, but not *dlx3* (not shown). Expression is apparent by 48 h, a time when the sensory hair cells express the *msxC* and *msxD* genes (Ekker et al., 1992a).

Fins. Subsets of cells in the pectoral fin buds (Fig. 9*G–I*) and in the median fin fold (Fig. 10*B*) express the three *dlx* genes in distinct patterns. By 16 h, bilateral crescents of cells express the *dlx2* gene in the presumptive pectoral fin buds over the yolk lateral to the anterior somites. During the following several hours of development, these cells rise up off the surface of the yolk as other cells coalesce beneath them to form the pectoral fin buds by about 28 h. The crescent is located at the distal edge of the fin bud and is equivalent to the apical ectodermal ridge (AER) of other vertebrates (Wood, 1982). After the first day of development, expression of the *dlx3* and *dlx4* genes begins in the AER. Cells in the most anterior region of the AER express *dlx3* (Fig. 9*H*) and *dlx4* (Fig. 9*I*), whereas cells in the most posterior region express *dlx2* (Fig. 9*G*) and *dlx3* (Fig. 9*H*). By 55 h, expression of all three *dlx* genes is confined to mesenchymal cells in the tip of the extending pectoral fin (Fig. 10*A*).

In the tailbud, a stripe of midline cells expresses the *dlx* genes at 16 h (not shown). These cells include superficial epithelial cells along the length of the tail and deeper mesenchymal cells in the tail bud. The superficial cells probably contribute to the median fin fold.

At this stage, the expression of *dlx3* and *dlx4* in superficial cells is uniform around the tail bud, whereas *dlx2* expression is restricted to a subset of cells on the ventral surface. At 24 h (Fig. 10*B*), cells of the median fin fold express all three genes although *dlx2* expression appears to be weaker than that of *dlx3* and *dlx4*. At later stages, expression of the *dlx* genes becomes restricted to the caudal fin primordium.

Discussion

Four orthologous groups of dlx *genes*

We identified three zebrafish homeobox-containing genes, *dlx2*, *dlx3*, and *dlx4*. On the basis of the predicted amino acid sequences of their homeodomains, the three genes belong to the *distal-less* family. In addition to the homeodomain and the amino acid residues that surround it, the sequences of the DLX2, DLX3, and DLX4 proteins are highly similar in a region of 19 amino acids near the amino-terminal end (Fig. 4*B*). Other regions of the protein sequence show less similarity, although several conserved amino acid residues are interspersed throughout the length of the predicted proteins. Comparisons with the sequences of other vertebrate homeobox genes of the *distal-less* family (Fig. 5) indicate that *dlx2* is probably the ortholog of the mouse *Dlx2* (*Tes-1*; Porteus et al., 1991; Robinson et al., 1991) and the *Xenopus X-dll4* and *X-dll1* genes (Dirksen et al., 1993; Papalopulu and Kintner, 1993). The *Xenopus X-dll1* and *X-dll4* are very similar (92% identity), and it is not yet clear whether they are distinct genes or two alleles of the same gene. The zebrafish *dlx3* gene is probably the ortholog of the newt *NvHBox-4* (Beauchemin and Savard, 1992) and *Xenopus X-dll2* (Papalopulu and Kintner, 1993) genes, and *dlx4* is most likely the ortholog of the *Xenopus X-dll3* (Papalopulu and Kintner, 1993) gene.

The products of the *distal-less* genes differ from the *Antennapedia* class of homeodomain proteins that, in vertebrates, are organized into multigene complexes such as the mouse *HoxA* through *HoxD* complexes. However, *Dlx1* and *Dlx2* are linked and located near the *HoxD* complex on mouse chromosome 2 (McGuinness et al., 1992; Ozcelik et al., 1992). It will now be interesting to determine if two or more of the zebrafish *dlx* genes are linked, thus suggesting that like the *Hox* genes, *dlx* genes evolved as linked multigene complexes. If a fourth zebrafish *dlx* gene belonging to the *dlx1* orthologous group exists and if linkage has been conserved, this fourth *dlx* gene would probably be linked to *dlx2*. Linkage could provide an explanation for the large number of conserved amino acid residues among the three *dlx* genes, because linkage would facilitate gene conversion events that result in sequence conservation among genes, provided the conversions affect amino acid residues that do not influence the functional specificity of the protein.

Combinatorial expression of dlx *genes*

The three zebrafish *dlx* genes resemble each other not only in the sequences of their predicted protein products but also in some but not all aspects of their embryonic patterns of expression. These patterns, summarized in Table 1, suggest that distinct combinations of functionally active *dlx* genes participate in the development of specific regions of the embryo. The forebrain expresses a combination of *dlx2* and *dlx4*. The olfactory placodes, the auditory vesicle, and the median fin fold or their precursors express, at some stages of their development, a combination of *dlx3* and *dlx4*. Cells in the primordia of the visceral arches express all three *dlx* genes, but the developmental time course of expression is unique for each gene.

Comparisons of gene expression patterns among the three zebrafish *dlx* genes and related genes from other vertebrates suggest a conservation of function among orthologs. The combinatorial expression that we observe for the zebrafish genes may also be found for the *distal-less* genes of other vertebrates. For example, the regions of the forebrain that express *dlx2* and *dlx4* in zebrafish appear to correspond to the forebrain regions that express the orthologs *X-dll4* and *X-dll3*, respectively, in *Xenopus* (Papalopulu and Kintner, 1993), and *dlx2* in the mouse (Bulfone et al., 1993b). In the telencephalon, differences between the zebrafish *dlx2* and *dlx4* expression patterns are reflected in the patterns of their *Xenopus* orthologs, *X-dll4* and *X-dll3*, respectively. No differences between the expression patterns of the *Dlx1* and *Dlx2* genes in the forebrain of the mouse have yet been reported. In contrast, and consistent with this conservation of the expression patterns of orthologous genes, cells of the forebrain fail to express two members of the *dlx3* orthologous group, the zebrafish *dlx3*, and the *Xenopus X-dll2* genes (Fig. 9*B*; Papalopulu and Kintner, 1993). The corresponding information is presently unavailable for the third member of this group, the newt *NvHBox-4* gene (Beauchemin and Savard, 1992). Combinatorial expression among species seems to apply also to the auditory vesicle. Cells of the auditory vesicle express two members of the *dlx4* group (Fig. 9*F*; Papalopulu and Kintner, 1993), but not members of the *dlx2* group. The auditory vesicle also expresses the zebrafish *dlx3* gene (Ekker et al., 1992a). Unfortunately, because *dlx3* is the only member of this group for which embryonic patterns of expression have been described, it is not yet possible to determine whether this aspect of *dlx3* expression is conserved among species. Finally, expression of members of the *dlx3* and *dlx4* orthologous groups in the olfactory placodes is well conserved between zebrafish and *Xenopus* (there is no information on the expression of *X-dll2* in the olfactory placodes). Similarly, the zebrafish and *Xenopus* olfactory placodes fail to express members of the *dlx2* group. We suggest that many aspects of the combinatorial expression of *dlx* genes have been well conserved during evolution. Characterization of additional *dlx* genes in the mouse and in other species is required to learn how general this finding may be.

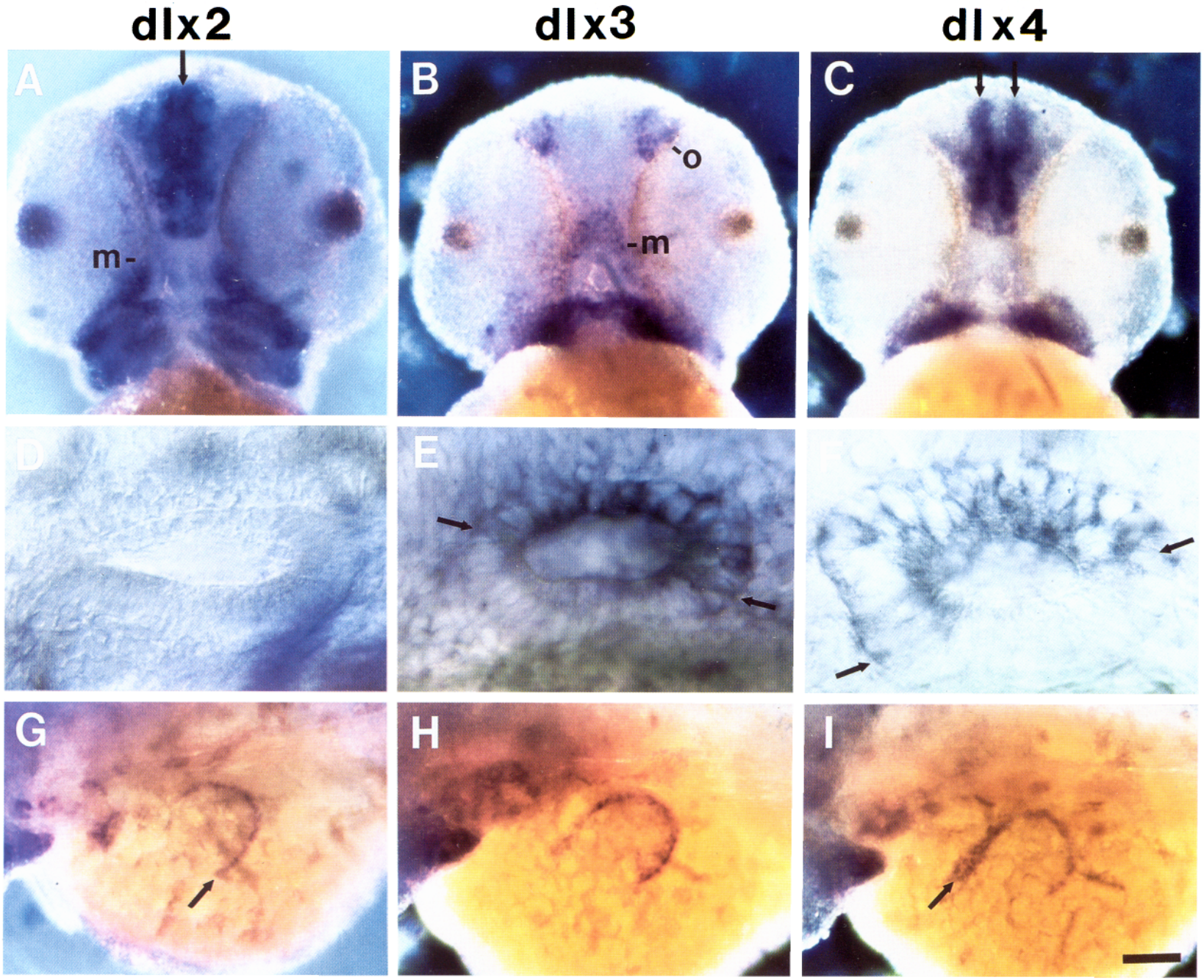
The three regions that express both *dlx3* and *dlx4* after 16 h, the olfactory placode and epithelium, the auditory vesicle, and the median fin fold, each appear to derive from cells that, at the end of gastrulation, express *dlx3* (Table 1). It is thus possible that *dlx3* expression in a subset of ectodermal cells persists in descendants of these cells and is related to the later expression of the *dlx4* gene (but not *dlx2*) and that the combined expression of the *dlx3* and *dlx4* proteins in a given cell participates in the regulatory mechanisms that influence the fate or the state of differentiation of this cell.

Similarly, expression of *dlx2* in the precursors of the branchial arches may be related to the later expression of *dlx3* and *dlx4*. Development of the branchial arches requires interactions between the neural crest cells that have migrated to this region and mesodermally derived cells (Noden, 1980, 1988). The expression of *dlx2* by neural crest cells in the hindbrain region that contributes crest to the branchial arches suggests that *dlx2*-expressing neural crest cells may migrate and contribute to the branchial arch region. Expression of *dlx3* and *dlx4* would be activated, following migration, in the same crest cells that express *dlx2* and/or in mesodermally derived cells as a result of interactions with these crest cells. The labeling of premigratory neural crest cells and the analysis of labeled cells after migration for the presence of *dlx* transcripts will help clarify this issue.

The segmental patterning of hindbrain neural crest cell migratory pathways, as marked by *dlx2*-expressing cells (Fig. 7*B*), matches the migratory patterns of hindbrain crest cells in chicks (Lumsden et al., 1991). In zebrafish, this patterning probably arises from the directed migration of crest cells as they leave the hindbrain, rather than from the absence of crest contributions from rhombomeres 3 and 5, as suggested for chick (Lumsden et al., 1991), because we see *dlx2*-positive cells in rhombomeres 3 and 5 prior to migration (Fig. 7*A*) and because previous fate mapping (Schilling, 1993) suggests that these rhombomeres, in addition to the others, can contribute crest to the branchial arches. Recent fate mapping in the chick agrees with this view that all rhombomeres can contribute neural crest cells to the branchial arches (Sechrist et al., 1993).

Migrating neural crest cells express *dlx2* in the mouse (Bulfone et al., 1993a) and *dlx2* in zebrafish (Fig. 7*B*). The most closely related *Xenopus* gene, *X-dll4*, does not seem to be expressed in premigratory neural crest cells (Papalopulu and Kintner, 1993), and its expression in migrating neural crest cells has not been reported.

Our results suggest that homeobox genes, in addition to those of the *Antennapedia* class, may provide combinatorial homeobox gene codes. We have previously observed the coordinate expression of zebrafish engrailed genes at the midbrain–hindbrain junction, in the jaw, and in myotomal muscle pioneers (Ekker et al., 1992b) and the coordinate expression of zebrafish members of the *msx* homeobox gene family in macular cells of the inner ear (Ekker et al., 1992a). The results of the present study add the *dlx* genes to this growing list of potential tran-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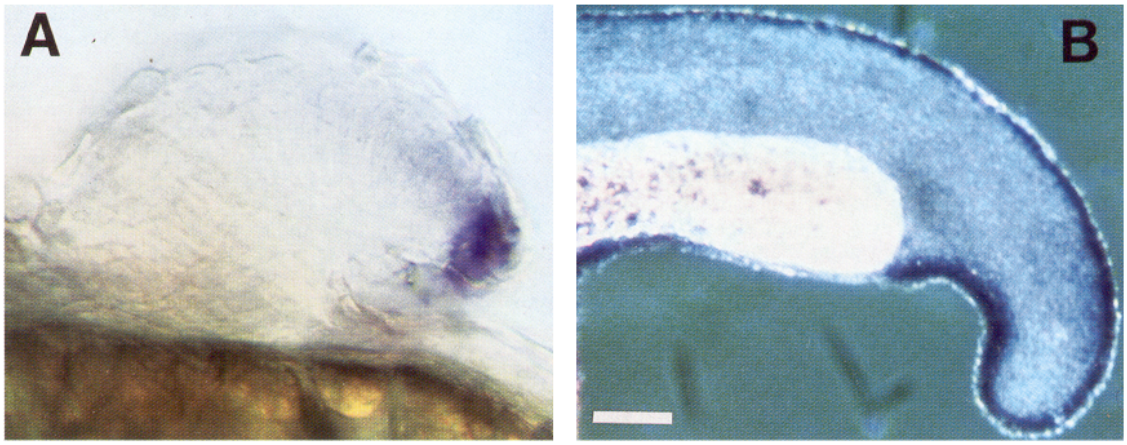

Figure 10. Cells of the fins express the *dlx* genes. *A*, Cells in the tip of the extending pectoral fin express the *dlx2* gene, shown here at 55 h. *B*, Cells of the caudal fin fold express *dlx3*, shown here at 24 h. Scale bar: 25 μm for *A*, 100 μm for *B*.

scriptional regulators that appear in discrete regions of the head when cells are differentiating into various types.

Regulatory mechanisms involving combinatorial expression of structurally related proteins could be based upon the formation of multiprotein complexes, competition at similar DNA binding sites for accessory transcription factors, or both, resulting in the activation or repression of specific target genes. Synergistic activation of transcription, mediated by related homeodomain proteins, has been described *in vitro* (Han et al., 1989) and may require the DNA binding activity of only one of the homeodomain protein partners (Ananthan et al., 1993). The elucidation of such mechanisms *in vivo* will enhance our understanding of the genetic control of development and will provide valuable information about how developmental strategies involving multigene families evolved.

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Figure 9. Combinatorial expression of the three *dlx* genes in the head. *A–C*, Ventral views (with anterior to the top) of 55 h embryos hybridized with *dlx2* (*A*), *dlx3* (*B*), or *dlx4* (*C*) probes. The *arrows* indicate the medially (*A*) and bilaterally (*C*) located cells in the telencephalon expressing *dlx2* (*A*) and *dlx4* (*C*), respectively. *D–F*, Cells of the auditory vesicle express *dlx3* (*E*) and *dlx4* (*F*), but not *dlx2* (*D*) at 24 h. Side view; *arrows* indicate the ventral limits of expression within the vesicle. *G–I*, Cells in the AER of the pectoral fin bud express *dlx2* (*G*), *dlx3* (*H*), and *dlx4* (*I*) in overlapping but distinct patterns at 28 h. *Arrows* indicate the posterior (*G*) or anterior (*I*) extent of expressing cells. The blue signal in the lens (*A–C*) is probably due to background labeling because it appears variably with all probes. *m*, mandibular arch; *o*, olfactory pit; dorsal is to the top and anterior to the left in *D–I*. Scale bar: 85 μm for *A–C* and *G–I*, 16 μm for *E* and *F*, 25 μm for *D*.

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