The evolution of the vertebrate Dlx gene family
(homeobox/zebrafish/gene duplication/Hox cluster/transcription factor)

DAVID W. STOCK*,†, DEBRA L. ELLIES‡, ZHIYONG ZHAO*, MARC EKKER‡, FRANK H. RUDDLE§,
AND KENNETH M. WEISS*‡

*Department of Anthropology and †Graduate Program in Genetics, Pennsylvania State University, University Park, PA 16802; ‡Loeb Institute for Medical Research, Ottawa Civic Hospital and University of Ottawa, Ottawa, ON Canada K1Y 4E9; and §Department of Biology, Yale University, New Haven, CT 06520

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ABSTRACT

The vertebrate Dlx gene family consists of homeobox-containing transcription factors distributed in pairs on the same chromosomes as the Hox genes. To investigate the evolutionary history of Dlx genes, we have cloned five new zebrafish family members and have provided additional sequence information for two mouse genes. Phylogenetic analyses of Dlx gene sequences considered in the context of their chromosomal arrangements suggest that an initial tandem duplication produced a linked pair of Dlx genes after the divergence of chordates and arthropods but prior to the divergence of tunicates and vertebrates. This pair of Dlx genes was then duplicated in the chromosomal events that led to the four clusters of Hox genes characteristic of bony fish and tetrapods. It is possible that a pair of Dlx genes linked to the Hox cluster has been lost from mammals. We were unable to distinguish between independent duplication and retention of the ancestral state of bony vertebrates to explain the presence of a greater number of Dlx genes in zebrafish than mammals. Determination of the linkage relationship of these additional zebrafish Dlx genes to Hox clusters should help resolve this issue.

Ohno (1) proposed that multiple rounds of chromosomal duplication (polyploidization) early in vertebrate evolution were directly involved in the morphological diversification of the group. This hypothesis has received dramatic support in recent years with the cloning, mapping, and elucidation of developmental function of the Antennapedia-class Hox genes (2–8). Mammals have ~40 Hox genes arranged in four clusters on separate chromosomes. These genes are believed to be involved in pattern specification along the anterior–posterior axis, with the domain of function of a gene related to its position along the chromosome. It has been suggested that tandem duplications produced an array of Hox genes along a single chromosome in a common ancestor of insects and vertebrates, followed by at least two duplications of this array early in vertebrate evolution (5, 6, 8–12). A number of other gene families have members located on multiple Hox-containing chromosomes in vertebrates, suggesting that the Hox cluster duplications may have involved chromosomes or genomes (5, 6). One such gene family is a group of homeobox-containing transcription factors homologous to the Drosophila Distal-less (DII) gene (refs. 5, 6, 13–16 and 64; D.L.E., G. Giroux and M.E., unpublished data).

The DII gene of Drosophila is required for the formation of the distal portion of the legs, antennae, and mouthparts (17). Multiple homologues of DII (DIIx or distal-less-like genes) have been isolated from zebrafish (18), newt (19), Xenopus (20–23), mouse (24–28, 64), and human (14, 29, 64). These genes are expressed in a variety of sites, many of which are regions of epithelial-mesenchymal interaction, including the developing branchial arches, limb buds, forebrain, otic and olfactory vesicles, teeth, and hair follicles. Functional studies of DII genes are only beginning to appear (30), and their role in the development of these structures is not well understood. An understanding of the evolutionary relationships among vertebrate DII genes, both for the paralogues within a species as well as the orthologues between species, may allow the integration of developmental data from divergent species as well as suggest experiments for testing functional interactions among genes. To investigate these relationships, we have determined the sequence of five new DII genes from the zebrafish and have obtained additional sequence data from two mouse DII genes whose cloning but not sequencing has previously been reported. Phylogenetic analyses of these and other DII genes are consistent with an initial tandem duplication to give two linked genes, followed by at least two duplications of this cluster. It is most parsimonious to assume that these duplications were the same as those that gave rise to the multiple Hox clusters to which the DII genes are linked.

MATERIALS AND METHODS

RNA Extraction. RNA was extracted from the head of a single E14.5 mouse (Swiss Webster) embryo (E0.5 = folding of the vaginal plug). Zebrafish were obtained from a pet store in Monterey, CA, and embryos and larvae were raised at 26°C. Zebrafish RNA was extracted from a pool of ten 103-hr (postfertilization) larvae, as well as from the ovary of an adult female. All RNA extractions used the RNazol B reagent (Biotex Laboratories, Houston) according to the manufacturer’s instructions.

Reverse Transcription–PCR. A series of degenerate oligonucleotide primers was designed from an alignment of Drosophila DII and vertebrate DII sequences obtained from GenBank. The primers 5'-GCCGGAATCCGACACNGCNGCNGCNAGCCTGATCC-3' and 5'-TTTGGCAGACCCATATTGGNCT-3' (the underlined sequence is a restriction site added to the 5' end) bind to conserved amino acid sequences near the amino and carboxy ends, respectively, of the homeobox of all DII genes examined. Additional primers located outside of the homeobox and specific for particular DII orthologues or groups of orthologues were as follows: 5'-GCCGGAATCCGACACNGCNGCNGCNAGCCTGATCC-3', 5'-GCCGGAATCCGACACNGCNGCNGCNAGCCTGATCC-3', 5'-GCCGGAATCCGACACNGCNGCNGCNAGCCTGATCC-3', and 5'-GCCGGAATCCGACACNGCNGCNGCNAGCCTGATCC-3'.

Reverse transcription of total RNA was carried out at 42°C using Moloney murine leukemia virus reverse transcriptase and either random hexamers or an adaptor-dT primer (5'-GATGCTGACAGCTCAAGC(T)3'). PCR of the single-stranded cDNA from this reaction typically used the

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. U67840–U67846).

†To whom reprint requests should be addressed.

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following thermocycling protocol: denaturation at 94°C for 2 min, followed by 40 cycles of 1 min at 94°C, 1 min at 45–55°C, and 1 min at 72°C. A final extension was performed at 72°C for 5 min.

**PCR Amplification of 5' and 3' Ends of Dlx cDNAs.** The 5' and 3' ends of Dlx cDNAs were obtained by one of two different PCR-based methods. The 5' and 3' ends of mouse Dlx5 and the 3' ends of mouse Dlx6 and zebrafish Dlx5 and Dlx6 were obtained by the rapid amplification of cDNA ends (RACE) method essentially as described from Frohman (31).

The 5' RACE of mouse Dlx5 used a gene-specific primer for reverse transcription, followed by a single PCR reaction on the da-tailed first strand cDNA, with a nested gene-specific primer, and the dT-adaptor primer described above. PCR cycling conditions were identical to those described above except that 35 cycles were used. The 3' RACE PCR was carried out in two steps with an initial 30-cycle PCR being used as the template for a second 30-cycle PCR with nested adapter and gene-specific primers. Amplification of the 5' ends of zebrafish dlx5 and dlx6 and the 5' and 3' ends of zebrafish Dlx1, Dlx7, and Dlx8 was carried out using a Marathon cDNA amplification kit (Clontech) according to the manufacturer's instructions, and essentially as described (64).

**Cloning and Sequencing.** PCR products of interest (identified by size or by Southern blotting with a labeled oligonucleotide) were cloned into pBluescript SK+ (Stratagene) by standard methods (32). Double-stranded plasmid DNA was sequenced on an Applied Biosystems model 373 automated sequencer. Errors inherent in the sequencing of cloned PCR products were minimized by sequencing multiple clones of each PCR product so that each nucleotide of the coding regions was determined from at least five different clones representing both strands of the DNA. Sequences were manually translated and compared using the programs in the DNASTAR package (DNASTar, Madison, WI).

**Phylogenetic Analyses.** Amino acid sequences of the following Dlx genes were obtained from GenBank data base: nematode C. elegans Dlx (accession no. Z32683); Drosophila Dil (17); tunicate (Ciona intestinalis) Dil-A and Dil-B (33); zebrafish dlx2, dlx3, and dlx4 (18); Xenopus Xdil (20); X-DLL1 (22); Xdil-2 (23); X-dil2, X-dil3, and X-dil4 (21); newt (Nototritalus viridescens) NtwB (34); rat (Dlx5) Dbcl and Dms (19); chicken (Dlx6) Dms (25); mouse Dlb6 (25); Dlx2 (24, 26); Dlx3 (27, 28); and Dlx7 (64); and human Dlx4, Dlx5, Dlx6, Dlx14, Dlx2 (29), and Dlx7 (64). Members of the related Mxs (36) and Barx1 (37) gene families were used as outgroups to root phylogenetic trees. The necessary assumption that these genes are outside of the Dlx family is based on homoeodomain amino acid identities of at least 67% within the Dlx family (77% when only Drosophila and vertebrate members are considered) compared with identities of 49–62% for Mxs and Dlx genes and 48–51% for Barx1 and Dlx genes.

Because it was not possible to obtain reliable alignments of all Dlx and outgroup amino acid sequences outside of the homoeodomain, phylogenetic analyses of the entire gene family were conducted on the homoeodomains alone. Alignment of these 61 amino acid regions did not require the addition of gaps. Pairwise distances were calculated as percent similarities (p-values) and neighbor-joining trees were constructed using the program **MEGA** (38).

We attempted to use phylogenetic information from regions outside the homoeodomain that are conserved among many of the vertebrate Dlx genes to provide greater resolution of the relationships among vertebrate Dlx genes. Alignments of complete Dlx amino acid sequences were constructed using **CLUSTALW** (39). Unsatisfactory results from simultaneous multiple alignment of the entire data set led us to construct independent multiple alignments for each of the strongly supported clades from the phylogenetic analysis of homoeodomains. The profile alignment feature of **CLUSTALW** was used to assemble these subalignments sequentially into a global alignment. At each stage of the alignment process, manual adjustments were made. The final alignment is available from the authors on request.

Alignment of the sequence upstream of the homeodomain was more difficult than downstream of it because some sequences were incomplete in the former region and there is evidence for alternative splicing upstream of the homeodomain and not downstream of it (ref. 64 and this study). For this reason we report a phylogenetic analysis using only the homeodomain and the sequence downstream of it. However, analysis of the complete sequence gave similar results. Phylogenetic analyses were as described above except that distances were γ-corrected (a = 2.0; ref. 38).

**RESULTS**

**Dlx Genes Detected in the Mouse and Zebrafish.** We sequenced ~75 independent homeobox clones from the mouse. We did not detect any new Dlx genes, but found clones matching Dlx1, Dlx2, Dlx3, Dlx5, and Dlx6. The cloning, but not the sequence, of the latter two genes had been reported previously, and the homoeodomain amino acid sequences we determined were identical to the human genes of the same name (14). The only known mammal Dlx gene that was not detected in our survey was Dlx7 (64). We determined the complete cDNA sequence of Dlx7 and the sequence of most of the homeobox and 3' end of Dlx6.

Sequencing a total of ~60 homeobox clones from zebrafish larvae and ovary revealed all three previously reported Dlx genes (dlx2, dlx3, and dlx4; ref. 18) as well as five new ones. We determined the complete cDNA sequence of the five new genes and have named them based on the phylogenetic analyses reported below. dlx1 and dlx6 are obvious orthologues of the mammal genes of the same name (14, 25). We have designated one new gene dlx5 that is a member of a clade containing mammal Dlx2, Dlx3, and Dlx5. The previously reported zebrafish dlx4 is the likely orthologue of mammal Dlx5, but we have not attempted to revise the nomenclature at this point. We have designated one new gene dlx7 despite some difficulty in inferring its orthology from sequence analyses because it is linked to dlx3 (D.L.E. et al., unpublished), as is mammalian Dlx7 (64). The remaining gene was named dlb6 by accession.

We obtained multiple transcripts differing in sequences upstream of the homeodomain for dlx5, dlx6, dlx7, and dlx8 (data not shown). In the case of dlx8, where 5' sequences were obtained from both larvae and adult ovary, multiple transcripts were found in the larvae and all of these differed from the type found in the ovary. In all cases, the differing transcripts predict amino acid sequence differences upstream of the homeodomain. We have reported the sequences that match most closely other vertebrate Dlx genes, but it is possible that alternative splicing accounts for some of the difficulties in aligning Dlx genes upstream of the homeodomain. Nakamura et al. (64) found evidence for alternative splicing upstream of the homoeodomain of mouse Dlx7.

**Number of Dlx Paralogues in Vertebrates.** Phylogenetic analyses of the homoeodomain alone and the homoeodomain along with downstream amino acid sequences are shown in Figs. 1 and 2, respectively. There are six strongly supported groups of vertebrate Dlx genes, each containing a single type of mammalian Dlx gene. These groups designated by their mammalian member(s) and their bootstrap support (from Fig. 1; from Fig. 2) are Dlx1 (not determined because of identical sequences; 99%), Dlx2 (86%; 99%), Dlx3 (91%; 100%), Dlx5 (83%; 100%), Dlx6 (56%; 100%), and Dlx7 (36%; 83%). Xenopus Dlx genes fall into four of these six groups, and two of the groups (Dlx2 and Dlx3) contain two Xenopus members. In both cases, these are highly similar sequences that are most
Relationship of Vertebrate Dlx Genes to Protochordate and Invertebrate Genes. Phylogenetic analysis of potential invertebrate and protochordate homologues of vertebrate Dlx genes (Fig. 1) shows that the nematode (Caenorhabditis elegans) gene C28A5.4 clusters outside the remaining eucoelomate Dlx genes. This result is not strongly supported (49% of the bootstrap trees), but matches the phylogenetic relationships of the organisms. Drosophila DII and one of the tunicate genes (Dil-A) form a weakly supported cluster (33% of bootstrap trees) that itself clusters weakly (25%) with the vertebrate Dlx1-Dlx6-Dlx7 clade. The remaining tunicate gene (Dil-B) clusters outside all other Dlx genes except that of C. elegans with moderately high support (74%).

**DISCUSSION**

Tandem Duplication of Dlx Genes Followed by Cluster Duplication. Fig. 3 presents a hypothesis of the evolutionary history of the vertebrate Dlx gene family, the evidence for which is discussed below. An important clue to understanding Dlx gene evolution is provided by linkage data. The six mammalian Dlx genes are arranged as three pairs of closely linked (10–30 kb), convergently transcribed genes (14, 64). Each of these clusters is linked to one of the four Hox clusters. This linkage to Hox clusters is relatively distant where known. The DLX3/DLX7 cluster is 1–2 Mb from HOXB in humans (64) and the DLX5/DLX6 cluster and HOXA are on opposite chromosome arms (5, 14). The DLX1/DLX2 and DLX3/DLX7 clusters are known to be on the side of the posteriorly expressed members of their respective linked Hox clusters (15, 64). The convergently transcribed configuration of Dlx genes

**Fig. 1.** Phylogenetic analysis of Dlx and related homeodomains. Neighbor-joining tree rooted with msh-like and Barxl homedomain-like sequences. The scale bar indicates a p-distance of 0.05. Numbers at nodes indicate bootstrap support (1000 replications). Left set of brackets indicate six orthologous groups of Dlx genes and the numbers indicate the single enalll members of each group. Right set of brackets highlight the clustering of vertebrate Dlx genes into two major groups. Dotted lines indicate adjustments to the tree that are necessary to make it consistent with the scenario for Dlx evolution depicted in Fig. 3.

A *Fundamental Split Among Vertebrate Dlx Genes.* Fig. 1 shows that vertebrate Dlx genes fall into two major clades, one of which is characterized by mammal Dlx2, Dlx3, and Dlx5, and the other of which contains mammal Dlx1, Dlx6, and Dlx7. Bootstrap support for these groupings is moderate to low (68% for the former and 44% for the latter). The phylogenetic analysis in Fig. 2 provides strong support (98%) for the existence of an internal branch separating these two groups, but the absence of an outgroup leaves open the possibility that one (but only one) of the two groups is monophyletic. As discussed below, linkage arrangements of Dlx genes provide a strong indication that both of these groups are monophyletic. The relationships of the three parologue groups within each of the two major divisions is unresolved because of low bootstrap support and contradictory results between the analyses in Fig. 1 and Fig. 2.

**Fig. 2.** Phylogenetic analysis of homeodomain and carboxy terminus of vertebrate Dlx genes. Neighbor-joining tree is rooted according to Fig. 1. The scale bar indicates a γ-corrected distance of 0.1. Numbers indicate bootstrap support (1000 replications). Brackets indicate orthologous groups as described for Fig. 1. Arrows indicate evidence for independent duplications of Dlx genes in zebrafish depicted in Fig. 3.
Fig. 3. Scenario for the evolutionary origin of multiple pairs of Dlx genes linked to Hox clusters. Hypothesized genomic arrangement of Hox and Dlx genes are diagrammed at different stages of metazoan evolution. Open boxes, filled boxes, and x's represent Hox genes, Dlx genes, and Dlx gene losses, respectively. Broken lines represent the distant linkage of Hox and Dlx genes. A and P refer to anteriorly expressed and posteriorly expressed Hox genes, respectively. Numbers indicate evolutionary events as follows: 1, ancestral condition of a single Dlx gene distantly linked to the posteriorly-expressed side of a small Hox cluster (drawn as that of C. elegans); 2, tandem duplication of Dlx genes along a single Hox cluster-containing chromosome; 3, disruption of the HOM/Hox cluster and translocation of Dll to a different chromosome; 4, duplication of large genomic regions to produce four Hox clusters linked to four pairs of Dlx genes (drawn as the Hox clusters of mammals); 5, loss of Dlx pair (alternatively these genes remain to be isolated); and 6, two independent Dlx duplications lead to the eight locus condition in zebrafish. The linkage relationships cannot be predicted. Alternatively the “extra” zebrafish genes may represent a retention of the condition resulting from step 4.

also applies to at least some of the eight zebrafish genes; zebrafish dlx1 and dlx2 form a closely linked pair with this arrangement, as do dlx4 and dlx6 (D.L.E. et al., unpublished observations). Analysis of zebrafish/mouse somatic hybrids (41) has indicated that the zebrafish dlx3 and dlx7 genes are present on the same chromosome as the Hoxa cluster (G. Giroux and M.E., unpublished) although the distance separating dlx3 and dlx7 has yet to be determined precisely.

Each of the three mammalian Dlx linkage groups contains a member of both of the major divisions of Dlx genes. This pattern is consistent with a tandem duplication along a chromosome to form a cluster of two Dlx genes, followed by chromosomal duplication to give multiple pairs of linked Dlx genes with the same relative orientation. This pattern of duplication resembles that proposed for Hox genes (5, 6). The linkage of Dlx genes to chromosomes containing Hox clusters, along with evidence for Hox cluster duplication involving large chromosomal regions (6), makes it reasonable to assume that Dlx cluster duplication occurred during these same genomic events. Phylogenetic analyses of Dlx sequences do not provide strong support for a particular order of cluster duplication; the trees in Figs. 1 and 2 yield conflicting results with respect to relationships of paralogous groups within the two major Dlx clades. To date, it has also been difficult to reconstruct the order of chromosomal duplication using data from the Hox genes (refs. 5 and 6; W. Bailey, and F.H.R., unpublished).

The timing of the initial tandem duplication of Dlx genes can be estimated by considering the Dlx genes of invertebrates and protochordates. It is likely that the nematode C. elegans contains a single Dlx orthologue. It has been suggested that this orthologue is ceh-23, which is closely linked to the HOM/ Hox cluster (6, 15, 42, 43). However, this gene, whose homeodomain is 50% identical to Dll, is actually slightly closer (52%) to Drosophila Ems (42). More recent genomic sequencing in C. elegans has revealed a gene (C28A5.4) with a homeodomain 74% similar to that of Dll. This gene is distantly linked to the HOM/Hox cluster and, like ceh-23, is located on the side of the cluster containing the most posteriorly expressed gene (A Celegans Data Base on the worldwide web). The extensive sequence available flanking both the HOM/Hox cluster and C28A5.4 (ref. 43; ACEDB) does not contain any other potential Dlx homologues (c.f., the close linkage of vertebrate Dlx gene pairs). The presence of a single Dlx locus, a single HOM/Hox cluster and the phylogenetic result presented here suggest that all Dlx duplications occurred after the divergence of nematodes and eucelomates.

A single Dlx homologue has been described to date in Drosophila. Dll is located on a separate chromosome from the homeotic genes, but these latter genes themselves have been separated during evolution by approximately 1 Mb (5, 44). The vertebrate Hox cluster duplications appear to have occurred after the divergence of insects and vertebrates (5, 8). The presence of the single Drosophila Dll gene is consistent with all Dlx duplications occurring after this divergence. In the phylogenetic tree presented in Fig. 1, however, Dll groups with the Dlx1-Dlx6-Dlx7 clade. This result is not strongly supported and because it would require evolutionary loss of or experimental failure to detect an additional Drosophila Dlx gene, we tentatively conclude that the tandem duplication of Dlx genes occurred after insects and vertebrates diverged.

The duplication of Hox clusters has been proposed to have occurred after the divergence of amphioxus and vertebrates (8), and consistent with this, the tunicate Ciona intestinalis appears to have a single Hox cluster (8, 33). Di Gregorio et al. (33) isolated two Dlx genes from Ciona intestinalis from a single λ phage clone. This linkage arrangement and the presence of a single Hox cluster is consistent with the tandem duplication of Dlx genes occurring before the divergence of vertebrates and tunicates. This scenario would predict the tunicate genes to be partitioned among the two main groupings of vertebrate Dlx genes. One of these genes, Dll-A, does cluster specifically with the Dlx1-Dlx6-Dlx7 grouping (Fig. 1), but
Dlx-B clusters outside of all chordate Dlx genes and that of Drosophila. These results would require that Ciona intestinalis has lost a Dlx2-Dlx3-Dlx5 homologue and that Drosophila and vertebrates have independently lost a homologue of Dlx-B (probably from multiple chromosomes in the case of vertebrates). Because it is difficult to obtain reliable phylogenetic trees with the 61 amino acid homeodomain alone, especially with respect to the root which plays a large role in these conflicting results, and because of the additional duplications and losses required, we feel that it is more reasonable to assume that the phylogenetic tree is in error and that the Dlx tandem duplication occurred once in a common ancestor of vertebrates and tunicates.

Dlx Gene Loss in the Mouse Versus Additional Duplications in the Zebrafish. The proposed Dlx cluster duplications tied to that of the Hox clusters might suggest the presence of an additional pair of mammalian Dlx genes linked to Hoxc (only six Dlx genes have been cloned to date, exclusive of Dlx4, which is likely to be artificial; ref. 27). Because the origin of four Hox clusters has been proposed to predate the divergence of zebrafish and mammals (45), the five new zebrafish Dlx loci that we have reported in combination with the three previously described ones would be more consistent with the hypothetical early formation of ancestral states in fish and tetrapods. The clustering of zebrafish dlx2 with dlx5 and dlx7 with dlx8 in our phylogenetic analyses, however, suggests that these two pairs of genes may be the result of two independent duplication events along the lineage leading to zebrafish. The grouping of zebrafish dlx7 and dlx8 more closely with the new NvhBox-5 than with mammalian Dlx7, contradicting the phylogenetic relationship of the organisms, would indicate that seven of the predicted ancestral eight groups of Dlx paralogues have been isolated from modern vertebrates. It is also possible, however, that the rapid rate of mammalian Dlx7 evolution postulated by Nakamura et al. (64) has caused an artificial clustering (46) of newt and zebrafish genes to the exclusion of mammal Dlx7. Linkage of both mammalian and zebrafish Dlx7 genes to Dlx3 (ref. 64; G. Giroux and M.E., unpublished) does support the orthology of the former genes. The possibility that zebrafish dlx5 and dlx8 represent the fourth pair of Dlx genes formed early in vertebrate history (contradicted in our tree) may be tested by determining the linkage arrangements of these genes relative to Hox clusters.

Leaving aside the question of the orthology of the Dlx7 genes in mammals and zebrafish, it is intriguing that Dlx3 is linked to Hoxb in mammals (64), and Hoxa in zebrafish (G. Giroux and M.E., unpublished; D.L.E., et al., unpublished). This may be an example of a translocation between "homologous" chromosomes formed during the polyploidization events postulated to have occurred early in vertebrate evolution. Morizot (47) suggested that such translocations have occurred for a number of families of metabolic enzymes and proposed a mechanism based on pairing of homologous chromosomes during meiosis.

Interestingly, zebrafish appear to have a larger number of members of several additional developmentally important gene families than the mouse—e.g. Msx (48), Hh (49), En (50), and perhaps Evx (51). One possible explanation is tetraploidization, which has occurred in a number of members of the family Cyprinidae (52), to which the zebrafish belongs. However, the chromosome number of zebrafish is very similar to that of most diploid teleosts (53). The greater number of Dlx genes in zebrafish than in mammals may therefore be related to the genomes of teleosts as a group; Morizot (47) lists a number of metabolic enzymes encoded by more gene family members in teleosts than in mammals.

Significance of Dlx–Hox Gene Linkage. The members of the Hox clusters are relatively closely linked to each other (~10 kb) and the linkage conservation among species is believed to be at least partly the result of shared regulatory regions (4–6). It is difficult to apply this argument as an explanation for the conservation of Dlx–Hox linkage. Mouse Evx-1 and Evx-2 are more closely linked to Hoxa (50 kb) and Hoxd (8 kb), respectively, than are the Dlx gene pairs and they are located on the same side (54). Dollé et al. (54) showed that while aspects of Evx-2 expression are coordinately regulated with the Hox cluster, coordinate regulation of Evx-1 and Hoxa was not detected. In the case of the HOM/Hox complex of C. elegans and mammalian Hoxb and Hoxd, the Dlx genes are located on the side of the most posteriorly expressed Hox genes, while Dlx genes are expressed more anteriorly in the CNS than are any of the Hox genes (14, 21, 24–26, 48, 55, 56). Ruddle et al. (6) have noted the clustering of a number of additional developmentally important gene families around the Hox clusters, but the significance of these conserved linkage relationships remains elusive.

Functional Significance of Duplicated Dlx Genes. Dlx genes have been proposed to participate in developmental "codes" for specifying distinct regions of the developing brain (55–57), branchial arches (18), fins (18), and dentition (28, 58). It has been argued that in an analogous "Hox code" for the branchial region of the head, there is greater functional redundancy between trans-paralogues (related genes on different chromosomes) than between cis-paralogues (linked genes; ref. 3). More recent analyses of mice deficient for multiple Hox genes suggest functional redundancy among cis- and nonparalogous Hox genes as well (59–61). Because the linkage and sequence relationships of Dlx genes resemble a smaller version of the Hox complexes, it is interesting to compare cis- and trans-paralogues for their functional similarity. Some functional redundancy probably does exist because the inactivation of mouse Dlx2 did not affect all regions in which the gene is expressed (30). The few comparisons of expression that can be made among Dlx paralogues actually seem to show greater similarities among cis-paralogues. In the brain, mouse Dlx1 and Dlx2 (cis-paralogues) have very similar expression patterns (55), while Dlx3 (a trans-paralogue of Dlx2) is not expressed at all (27). Dlx5 and Dlx6 (cis-paralogues) have similar expression patterns throughout the embryo and share the unique feature of being expressed in most developing skeletal elements (14). There is clearly a need for more comparisons of expression and especially function among Dlx paralogues.

Dlx Nomenclature. The orthologous relationships among vertebrate Dlx genes proposed here and by other researchers (18, 21, 62) would be best represented by changes to the nomenclature. This could be accomplished by renaming the Xenopus and zebrafish genes according to their mouse orthologues. In the case of Xenopus, this would require a designation for genes related by recent tetraploidization as well. Adjusting the zebrafish nomenclature would require fewer changes than that of Xenopus—i.e., changing dlx4 to dlx5 and giving a different designation to the dlx5 gene reported here. Given the similarity of genomic organization to that of the Hox clusters, a new system reflecting cis- and trans-paralogue relationships analogous to that in use for the Hox genes (63) might be useful—e.g., Dlx1a and Dlx2a for mouse Dlx5 and Dlx6. Because of the possibility of translocations of Dlx clusters relative to Hox clusters, however, such a system should not be undertaken until a better understanding of Dlx gene linkage in the zebrafish is obtained.

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