Gnathostome vertebrates have multiple members of the Dlx family of transcription factors that are expressed during the development of several tissues considered to be vertebrate synapomorphies, including the forebrain, cranial neural crest, placodes, and pharyngeal arches. The Dlx gene family thus presents an ideal system in which to examine the relationship between gene duplication and morphological innovation during vertebrate evolution. Toward this end, we have cloned Dlx genes from the lamprey Petromyzon marinus, an agnathan vertebrate that occupies a critical phylogenetic position between cephalochordates and gnathostomes. We have identified four Dlx genes in P. marinus, whose orthology with gnathostome Dlx genes provides a model for how this gene family evolved in the vertebrate lineage. Differential expression of these lamprey Dlx genes in the forebrain, cranial neural crest, pharyngeal arches, and sensory placodes of lamprey embryos provides insight into the developmental evolution of these structures as well as a model of regulatory evolution after Dlx gene duplication events.

Extant vertebrates consist of gnathostomes and lampreys. Cladistic analyses based on comparing morphological characteristics of vertebrates with hagfish, amphioxus, and ascidians suggest a stepwise progression for the developmental evolution of the vertebrate body plan (Fig. 1). Craniate and vertebrate origins are thus characterized by the acquisition and diversification of neural crest, placodes, and endoskeletal elements, as well as the reorganization and elaboration of the brain (1–3). Gnathostome origins are characterized by the subsequent elaboration of this body plan to include bone, teeth, paired appendages, and jaws (4).

A major goal of the field of evolutionary developmental genetics is to correlate specific morphological innovations with discrete genetic events. In the chordate lineage, most studies addressing this issue have focused on comparing genes and developmental processes of gnathostomes with either cephalochordates or ascidians (reviewed in refs. 3 and 5). One of the lessons of these studies has been that, in addition to their greater morphological complexity, gnathostome vertebrates also have a greater genomic complexity than protochordates. The now-classic example of this is the Hox genes, which are present in a single cluster in the cephalochordate amphioxus (6) and ascidians (7) but are present in four to seven clusters in gnathostomes (8). Such expansions of gene families are widespread (5), and they support the longstanding hypothesis that genome duplications near the time of vertebrate origins facilitated their greater morphological complexity (9). However, it is unclear whether such duplications in fact correlate with vertebrate origins because very few studies have addressed the copy number of developmental regulatory genes in agnathan vertebrates such as lampreys.

Lampreys are generally considered to be the sister group to gnathostome vertebrates (Fig. 1). They are jawless, lack paired appendages, and have a relatively simple axial morphology. Yet, as vertebrates, they share several morphological features with gnathostomes, including multiple brain divisions, neural crest and its derivatives, placodes, pharyngeal arches, and a cartilaginous endoskeleton (10, 11). Hagfish are a second group of extant jawless fish whose relationship with lampreys remains controversial (see refs. 12 and 13). Because of their phylogenetic position, and their multiple shared-derived characters with gnathostomes, lampreys have long been viewed as the best living proxy for the vertebrate ancestor. By comparing the developmental genetics of lampreys with their gnathostome and protochordate cousins, it should be possible to more precisely reconstruct the developmental evolutionary history of early vertebrates.
The Dlx family of transcription factors presents a compelling case for a comparative analysis in lampreys. First, studies in multiple gnathostome species indicate that Dlx genes are expressed in the forebrain, cephalic neural crest, otic vesicle, olfactory placodes, pharyngeal arches, limb buds, and teeth (14–34). Gene-knockout experiments in mice support a critical role for Dlx genes in the development of many of these characters (28, 35, 36). Because these sites of Dlx expression and function are major morphological innovations along the chordate and vertebrate lineage (see Fig. 1), examination of Dlx gene expression in lampreys should allow a fuller understanding of vertebrate developmental evolution. Second, Dlx genes are also interesting from the perspective of genomic organization. In extant gnathostomes, there are six distinct Dlx families that fall into two superfamilies (37). The cephalochordate amphioxus has but a single Dlx ortholog (38). Examination of the number of Dlx genes in lampreys and their phylogenetic relationships to gnathostome Dlx genes should provide significant insights into the evolution of the vertebrate Dlx gene family and the relationship between gene duplication and morphological innovation.

We have identified four Dlx genes in the lamprey Petromyzon marinus, whose orthology with gnathostome Dlx genes provides a model for how this gene family evolved in the vertebrate lineage. We suggest that a tandem duplication of an ancestral Dlx gene predated the divergence of lampreys from gnathostomes and was followed by independent duplication events in each lineage. Differential expression of these lamprey Dlx genes in several lamprey tissues provides a model for regulatory evolution after these gene duplication events.

Materials and Methods

Cloning of Lamprey Dlx Homologs. Degenerate oligonucleotide primers were used to PCR amplify 147-bp Dlx homeodomain fragments from P. marinus embryonic cDNA libraries (39). Upper primer, 5'-CCGAATTCACRCNMGNACNATHTA3'; lower primer, 5'-CCGGATCCRTTYTGRAACCADA-TYT-T3'. PCR products were cloned, and 26 independent plasmids were sequenced. Four distinct Dlx homologs were identified on the basis of an alignment of DNA and inferred amino acid sequences.

Homeoboxes from each of these four groups were used to screen embryonic cDNA libraries (39) by hybridization at moderate stringency (final washes 0.1× SSC/0.1% SDS at 60°C). From each probe, multiple independent cDNAs were obtained; cDNAs with the longest insert size from each group were fully sequenced. Sequences of the four Dlx genes have been submitted to GenBank under accession nos. AY010116, AY010117, AY010118, and AY010119.

Sequence Analysis. Nucleotide sequences were converted to amino acid sequences and used in a CLUSTAL X sequence alignment with previously described Dlx homologs obtained from GenBank: amphioxus AmphiDI (accession no. P53772); zebra fish dlx1, dlx2, dlx3, dlx4, dlx5, dlx6, and dlx8 (P50574, P50576, U67842, U67843, U67844, U67845, and U67846); Xenopus Xdl1, X-DLL1, Xdl2, X-dll2, X-dll3, and X-dll4 (P53773, A56570, P53774, I51409, P54655, and P53775); chicken Dlx5 (P50577); mouse Dlx1, Dlx2, Dlx3, Dlx5, and Dlx6 (Q64317, P40746, Q64205, U67840, and U67841). We first constructed independent multiple alignments for each of the strongly supported gnathostome clades as identified by Stock et al. (37). These were then aligned with the lamprey Dlx sequences and the amphioxus DI sequence by using the profile alignment feature of CLUSTAL X. This global alignment was manually edited to remove regions with no clear sequence similarities to produce an edited alignment consisting of the entire homeodomain region plus approximately 110 amino-terminal residues and 60 carboxyl-terminal residues of each protein. For edited alignment, see Fig. 8, which is published as supplemental data on the PNAS web site, www.pnas.org). Trees were constructed from the entire edited alignment by using the neighbor-joining method on PAUP 4.0, as well as by using the maximum-likelihood analysis on TREE-PUZZLE 4.0.2. Trees were rooted against the mouse Msx1 gene (AAAB35456), which shares 62% homeodomain sequence similarity with vertebrate Dlx genes, and is thus clearly outside the Dlx family. The two phylogenetic methods produced nearly identical topologies.

In Situ Hybridizations. Lamprey embryos were staged and fixed as previously described (39). Antisense digoxigenin-dUTP riboprobe sequences were synthesized from each cDNA; to avoid possible cross-hybridization between the various lamprey Dlx probes, only 3’ untranslated regions (UTRs) were used for riboprobe synthesis. In situ hybridization was carried out as described (39), and embryos were mounted whole in Permount or embedded in Polybed 812 (Polysciences, Warrington, PA) for thick (7.5-μm) sectioning.

Results

Four P. marinus Dlx Homologs. Four P. marinus Dlx homologs were identified by PCR and hybridization screening of embryonic cDNA libraries. The longest cDNA clones for each homolog have the following characteristics: DlxA is 2,468 nucleotides with a 400-amino acid ORF and a 1,000-nucleotide 3’ UTR. DlxB is 2,054 nucleotides with a 280-amino acid ORF (the amino terminus is missing from all DlxB cDNAs) and a 1,200-nucleotide 3’ UTR. DlxC is 2,416 nucleotides with a 342-amino acid ORF and a 800-nucleotide 3’ UTR. DlxD is 2,390 nucleotides with a 247-amino acid ORF and a 1,500 nucleotide 3’UTR. An alignment of the inferred homeodomain regions of the four P. marinus Dlx genes with those of the six murine Dlx genes is shown in Fig. 2. DlxA, DlxB, DlxC, and DlxD share 97%, 90%, 93%, and 93% sequence similarity, respectively, with the consensus vertebrate Dlx homeodomain region.

Phylogenetic Relationships of Lamprey and Gnathostome Dlx Genes. The inferred amino acid sequences of the four P. marinus Dlx genes were used to create phylogenetic trees of chordate Dlx genes (Fig. 3). Gnathostome Dlx genes form two distinct clades consisting of the Dlx2, Dlx3, and Dlx5 genes in one clade, and the Dlx1, Dlx6, and Dlx7 genes in a second clade (see also ref. 37). Mouse Dlx7 was excluded from our phylogenetic analysis because of an apparently rapid rate of evolution, particularly within the homeodomain. P. marinus Dlx genes are more closely related to all of the gnathostome Dlx genes than is the Dll gene from the cephalochordate amphioxus. Lamprey and gnathostome Dlx genes thus form a vertebrate-specific clade. The four P. marinus Dlx genes also form two distinct groups, but none of them are strict orthologs of any of the gnathostome Dlx genes. Instead,
three of the P. marinus Dlx genes group with the gnathostome Dlx2/3/5 clade, but do not group with Dlx2, Dlx3, or Dlx5 separately. Likewise, the other P. marinus Dlx gene groups with the gnathostome Dlx1/6/7 clade, but does not group with Dlx1, Dlx6, or Dlx7. Amphioxus Dll is an outgroup to all vertebrate Dlx genes.

**Dlx Gene Expression During Lamprey Embryogenesis.** Dlx expression first becomes pronounced around 9 days after fertilization (Fig. 4), which corresponds to Piavus (40) stage 12. At this time, DlxA (Fig. 4 A and D) and DlxD (Fig. 4 C and F) transcripts are expressed in very similar patterns. Transverse sections reveal that both of these genes are expressed in the dorsal aspect of the neural tube, in a domain consistent with premigratory neural crest, as well as in mesenchymal cells that are consistent with migratory neural crest. Whole mounts reveal that these DlxA- and DlxD-expressing putative neural crest cells are present throughout the cephalic neural tube and have relatively sharp posterior boundaries (Fig. 4 A and C, arrows). DlxC is also expressed at this stage (Fig. 4 B and E). Transverse sections reveal that expression is consistent with only migratory neural crest, and whole mounts indicate that this domain has much more narrow anterior and posterior limits. DlxA, DlxC, and DlxD are all expressed strongly in the ectoderm surrounding the mouth. DlxB expression is not detected at all at this stage.

By 15 days after fertilization (Piavus stage 14) all four lamprey Dlx genes are strongly expressed in various head tissues (Fig. 5). The most prominent Dlx-expressing tissues are the pharyngeal arches, where all four genes are expressed in each arch. Horizontal sections reveal that DlxA, DlxB, DlxC, and DlxD transcripts are in cells of the ectomesenchyme of each arch (Fig. 5 F, G, I, and K, arrows), in domains consistent with their being cartilage precursors derived from cephalic neural crest. All four transcripts are also strongly expressed in the ectoderm surrounding the mouth. Three of the Dlx genes (DlxA, DlxB, and DlxC) are expressed in discrete domains within the forebrain. DlxA (Fig. 5 A and E) and DlxC (Fig. 5 C and H) are each expressed in two bilaterally paired stripes that flank the ventral boundary of the diencephalon and telencephalon. DlxD (Fig. 5 D and J) is expressed in a single bilaterally paired stripe in the ventral diencephalon. DlxA, DlxC, and DlxD are each expressed in the olfactory placode at this stage, whereas only DlxA remains strongly expressed in the otic vesicle (both DlxC and DlxD are expressed transiently in the otic vesicle at earlier stages; data not shown). DlxB transcripts are never detected outside of the pharyngeal arches.

In addition to the head-specific expression described above, subsets of the lamprey Dlx genes are also expressed in more posterior regions. DlxA, DlxC, and DlxD are expressed transiently in the dorsal fin fold, whereas DlxB is not detected in the fin fold (data not shown). Intriguingly, DlxA and DlxD are expressed in the ventrolateral edge of trunk somites (data not shown).

**Discussion**

The Phylogeny of Vertebrate Dlx Genes. Gnathostome Dlx genes fall into six families and two superfamilies (ref. 37; Fig. 3). As gene nomenclature is inconsistent within these families, we employ the nomenclature devised by Stock et al. (37) that identifies gnathostome families on the basis of the name of the mouse gene within each family. In cases where physical linkage has been determined (31, 41–43), individual paralogs within a superfamily are each located adjacent to a member from the other superfamily, and are linked to a Hox gene cluster (see ref. 8). This
The cephalochordate amphioxus has a single *Dlx* homolog (38), which, by our phylogenetic analyses, falls as a moderately well supported outgroup to all of the vertebrate *Dlx* genes. We thus place the relevant tandem duplication event as having occurred after the divergence of cephalochordates and gnathostomes (Fig. 6).

The four lamprey *Dlx* genes that we identified also fall into the two superfamilies, but none of them are strict orthologs of any of the gnathostome *Dlx* families. Instead, following the terminology of Sharman (44), *P. marinus* *Dlx A, Dlx B* and *Dlx C* constitute a trans-homologous group to the gnathostome *Dlx 2*, *Dlx 3*, and *Dlx 5* genes. Likewise, *P. marinus* *Dlx D* is a trans-homolog of the gnathostome *Dlx 1*, *Dlx 6*, and *Dlx 7* genes. The simplest model that can explain these phylogenetic relationships is that lampreys and gnathostomes underwent independent duplications of a tandem pair of *Dlx* genes that was present in the vertebrate ancestor (Fig. 6).

There are two anomalies in our phylogenetic tree. First, lampreys have three members of one *Dlx* superfamily (*Dlx A, Dlx B, Dlx C*), but only one member of the other superfamily (*Dlx D*). While it is possible that there are additional, undiscovered *P. marinus* *Dlx* genes, we feel this is unlikely because our extensive cDNA screening uncovered the same four genes multiple times, and independent screening for genomic *Dlx* clones uncovered only these same four genes (S. Irvine, personal communication). We suggest instead that lampreys lost members of the second superfamily after chromosomal or genome duplications. A second, more puzzling, anomaly is that lamprey *Dlx B* groups more closely with gnathostome *Dlx 2/Dlx 3/Dlx 5* than it does with lamprey *Dlx A* or *Dlx C*. This grouping would suggest either that gnathostomes lost homologs of lamprey *Dlx A/Dlx C* or that our tree is in error at this node. Further resolution of this model could result from an analysis of the genomic organization of lamprey *Dlx* and *Hox* genes, as well as the analysis of *Dlx* and *Hox* genes in basal gnathostomes such as sharks.

If our model of independent duplications of *Dlx* genes in gnathostomes and lampreys is correct, it has important implications for vertebrate developmental evolution. It has long been speculated that duplications of genes, or even the whole genome, near the time of vertebrate origins facilitated their greater morphological complexity (5, 9). The findings presented here suggest that only the initial tandem duplication of *Dlx* accompanied vertebrate origins, and that widespread developmental regulatory gene duplications were not a prerequisite to vertebrate morphological innovations. This conclusion has support from previous studies of lamprey *Otx* (39) and is consistent with surveys of lamprey *Hox* genes (45, 46),...
but it requires further phylogenetic and genomic analyses in lampreys.

**Dlx Expression in Lampreys and Gnathostomes.** Because there is no strict orthology between the lamprey Dlx genes described here and the gnathostome Dlx genes, it is not meaningful to compare the expression of Dlx genes on a one-to-one basis. Rather, we must compare the composite of Dlx expression in lampreys with the composite of Dlx expression in gnathostomes.

**Cranial Neural Crest and Sensory Placode Expression.** Neural crest and placodes are thought to be vertebrate innovations whose derivatives facilitated the transition from passive filter feeding to active predation (1, 47, 48). We have shown that, like their gnathostome homologs, lamprey Dlx genes are expressed in cranial neural crest. This assertion is based on analysis of fixed tissue alone and requires confirmation by lineage tracing. Lamprey cranial neural crest is initially produced at all neuraxial levels except for the rostral-most area (49), and we find DlxA and DlxD transcripts throughout the dorsal aspect of the cranial neural tube. In addition, DlxA, DlxC, and DlxD are all expressed in putative migratory cranial neural crest. These results suggest that Dlx genes were functionally recruited into cranial neural crest near the time of vertebrate origins. Also like their gnathostome homologs, lamprey Dlx genes are expressed in the otic placode and otic vesicle, reflecting an ancient role for Dlx genes in the evolution of these structures.

**Pharyngeal Arch Expression.** In gnathostomes, Dlx-expressing cranial neural crest cells give rise to pharyngeal cartilages that form jaws and gill supports (28, 50). Lampreys also have cartilaginous gill supports that are derived from cranial neural crest (51, 52). Despite these similar embryological origins, it has remained controversial whether gnathostome and lamprey gill supports are homologous, an issue that has direct bearing on the hypothesis that gnathostome jaws evolved from gill supports (11, 53).

DlxA and DlxD transcripts are located in premigratory and migratory neural crest, and they persist into the pharyngeal arches. DlxC is expressed only in migratory neural crest, and it also persists into the pharyngeal arches. DlxB is not detected in any early stage of cranial neural crest, but is expressed in pharyngeal arches. Lamprey pharyngeal arches contain a caudally located muscle plate and a rostromedially located cartilage bar (54, 55). Like their gnathostome homologs, all lamprey Dlx genes are expressed in a domain consistent with where cartilage condensations will form. This finding provides deeper evidence for the homology of gnathostome and lamprey gill supports. However, the gnathostome Dlx genes are expressed in distinct overlapping dorsal-ventral domains within each arch, and mutational analyses suggest that they are responsible for patterning the pharyngeal arches along the dorsal-ventral axis (28). We discern no such overlapping expression domains of lamprey Dlx genes. Since gnathostome jaws and gill supports have moveable ventral segments (see ref. 56), which are not present in the lamprey branchial basket (57), we suggest that Dlx-mediated dorsoventral patterning of pharyngeal arches is a gnathostome innovation, and may have been a necessary prerequisite for the origin of jaws.

**Forebrain Expression.** Gnathostome Dlx genes are expressed in two well-conserved forebrain domains (14–16, 22, 23, 26, 27, 31, 41, 58). One domain is in the diencephalon and extends from the boundary separating the dorsal thalamus and the ventral thalamus to just behind the optic stalk. The other domain consists of most of the basal telencephalon. Lamprey brains are simpler than those of gnathostomes, but lamprey DlxA and DlxC are expressed in patterns that are nearly identical to their gnathostome homologs. DlxD is expressed only in the diencephalic domain, whereas DlxB is not detected at all in the forebrain. These results suggest that lampreys and gnathostomes share fundamental mechanisms of forebrain patterning, and they are consistent with the prosomeric model of forebrain organization, which postulates that true segmentation extends through all regions of the vertebrate brain (59–61).

**Regulatory Evolution of Dlx Genes.** Our comparisons of the sequence and embryonic expression of lamprey Dlx genes with those of gnathostomes and protochordates suggest a model for Dlx gene regulatory evolution (Fig. 7). We hypothesize that a tandem duplication event occurred before the origin of vertebrates, along with the evolution of several enhancer elements driving expression in cranial neural crest, pharyngeal arches, placodes, and the dorsal fin fold. This was followed by independent chromosomal or genomic duplications in both the lamprey and gnathostome lineages, accompanied by enhancer gain and/or loss in each lineage. The result is that lampreys and gnathostomes have several independently derived Dlx genes, but the composite expression of Dlx genes is very similar in lampreys and gnathostomes. These expression relationships support the duplication–degeneration–complementation (DDC) model for regulatory evolution after gene duplication (62), which predicts that the sum of expression sub-functions of duplicate genes will equal the total expression sub-functions in the (unduplicated) ancestral gene.

While the number of cases examined is limited, lampreys appear not to have undergone the widespread gene/genome duplications characteristic of gnathostomes. Given that much of their morphology is shared—derived with gnathostomes, examination of additional developmental regulatory genes in lampreys should provide continued important insights into the developmental and molecular evolution of early vertebrates.

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