Origins of anteroposterior patterning and *Hox* gene regulation during chordate evolution

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All chordates share a basic body plan and many common features of early development. Anteroposterior (AP) regions of the vertebrate neural tube are specified by a combinatorial pattern of *Hox* gene expression that is conserved in urochordates and cephalochordates. Another primitive feature of *Hox* gene regulation in all chordates is a sensitivity to retinoic acid during embryogenesis, and recent developmental genetic studies have demonstrated the essential role for retinoid signalling in vertebrates. Two AP regions develop within the chordate neural tube during gastrulation: an anterior 'forebrain–midbrain' region specified by *Otx* genes and a posterior 'hindbrain–spinal cord' region specified by *Hox* genes. A third, intermediate region corresponding to the midbrain or midbrain–hindbrain boundary develops at around the same time in vertebrates, and comparative data suggest that this was also present in the chordate ancestor. Within the anterior part of the *Hox*-expressing domain, however, vertebrates appear to have evolved unique roles for segmentation genes, such as *Krox-20*, in patterning the hindbrain. Genetic approaches in mammals and zebrafish, coupled with molecular phylogenetic studies in ascidians, amphioxus and lampreys, promise to reveal how the complex mechanisms that specify the vertebrate body plan may have arisen from a relatively simple set of ancestral developmental components.

Keywords: *Hox*; retinoic acid; zebrafish; lamprey; amphioxus; ascidian

1. INTRODUCTION

The cellular and genetic events that lead to patterning along the anteroposterior (AP) body axis begin early in embryonic development. Regional differences in structure arise through a diversification of developmental programmes at different AP locations, and changes in these programmes have almost certainly been central in the evolution of body plans. All chordates have a similar basic plan, with an anterior end containing an expanded neural tube and pharyngeal slits, and a posterior end containing a thinner dorsal nerve cord, notochord and muscle segments. But between chordate groups there are major differences in the types of structures that form at different AP levels. Vertebrates, in contrast to their closest relatives, develop a large brain and skull housing elaborate sense organs. One key innovation in vertebrate evolution from a protochordate (non-vertebrate chordate) ancestor appears to have been the acquisition of a new head with the emergence of neural crest and placodes during development (Northcutt & Gans 1983). There is now evidence that at least some of this innovation involved the elaboration of existing head structures, as suggested by the spatial patterns of expression of many early developmental regulatory genes in protochordates (Williams & Holland 1996). The dorsal nerve cord in urochordates and cephalochordates, however, displays few anatomical features along the AP axis that can be compared with the vertebrate forebrain, midbrain or segments of the hindbrain, with some exceptions (Fritsch & Northcutt 1993; Lacalli *et al.* 1994; Lacalli 2001). At one extreme, the urochordate neural tube is extremely simple, in some species containing fewer than 400 cells, among which fewer than 100 are neurons. Vertebrates also show extensive variation in brain structure as well as jaws, limbs and internal organs along the body axis. Thus to understand the evolution of the vertebrate body plan it is important to determine the ancestral and derived molecular mechanisms that control how cells are allocated to different fates along the AP axis.

Genetic analyses in mice and more recently in zebrafish have begun to reveal how developmental regulatory genes control AP patterning in vertebrates (for reviews, see Cappechi 1997; Schilling 1997; Trainor *et al.* 2000). Comparative studies more broadly across chordates are also leading to new ideas about gene functions and their ancestral roles (Holland & Holland 1999). In this article we first introduce the molecules involved in AP patterning and draw together the recent genetic findings. We then present phylogenetic studies that address two major evolutionary issues: (i) how conserved are these molecules across chordates?; and (ii) how conserved are their functions and modes of regulation? Functional divergence and redundancy arising from gene duplications obscure the roles of these genes during chordate evolution (Holland 1999). Determination of gene copy number in chordates, combined with gene expression analysis can give us clues to their roles in the most recent common ancestor. Here we discuss studies in three chordate groups that are emerging as model systems for answering such questions: ascidians or tunicates (urochordates); amphioxus (a cephalochordate); and lampreys

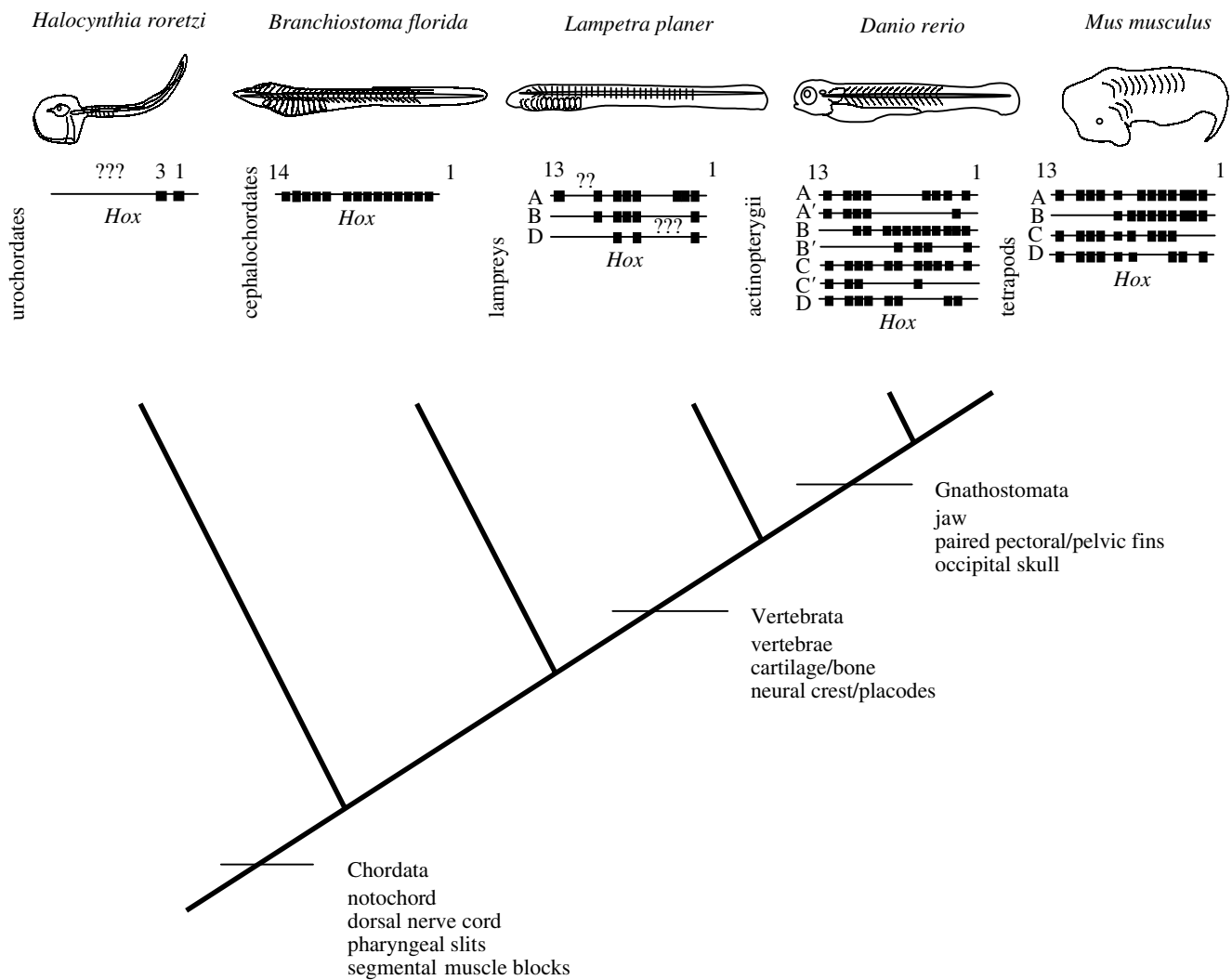[*]Author and address for correspondence: (tschilli@uci.edu).

Figure 1. Chordate phylogeny and *Hox* cluster organization. Cladogram (adapted from Holland 1999) showing major branch points of groups that contain model developmental systems. Diagrams of larval forms of these animals are shown at the top and underneath each are schematic diagrams of their *Hox* clusters, in some cases hypothetical ones, in which lines indicate chromosomes and black boxes indicate individual *Hox* genes in clusters A–D. Chordata comprises the Urochordata, sister group to all other chordates, and the Cephalochordata, sister group to the vertebrates, both of which are characterized by the morphological features listed and the apparent presence of only one cluster. Vertebrata includes the lampreys, sister group to the gnathostomes, characterized most notably by the lack of a jaw, paired appendages and apparently fewer *Hox* clusters. Gnathostomata includes all the jawed vertebrates and apparently at least four *Hox* clusters.

(agnathans) (figure 1). All three occupy key phylogenetic positions, the first two as invertebrate chordate outgroups to the vertebrates and the latter as an agnathan outgroup to the jawed vertebrates (gnathostomes). Two of these, ascidians and lampreys, offer not only an opportunity to compare gene expression but also to test gene function (see Sordino *et al.* 2001). Work in these systems is changing the way we think about the developmental constraints that have governed the evolution of the vertebrate nervous system.

When is an AP axis first established during vertebrate development? Studies since the early part of the century, primarily in *Xenopus*, have suggested that signals from mesoderm of Spemann's organizer induce AP patterning in the neural ectoderm during gastrulation (for reviews, see Weinstein & Hemmati-Brivanlou 1999; Gamse & Sive 2000). These subdivide the neural axis progressively, beginning with broad regions that do not correspond

precisely to the classical notions of forebrain, midbrain, hindbrain and spinal cord. Anterior neural tissue is the first to be induced during early gastrulation, and is specified by expression of the homeobox transcription factor *Otx2* (an orthologue of *Drosophila orthodenticles*, *otd*). This region probably includes future forebrain and part of the midbrain (for a review, see Bally-Cuif & Boncinelli 1997). Subsequently, a more posterior domain, corresponding roughly to future hindbrain and spinal cord, becomes defined by expression of relatives of *Drosophila* homeotic genes or *Hox* genes. Thus, by mid-gastrulation there is already a clear AP axis within the neural ectoderm before it narrows to form the neural tube. A third region that will form the midbrain and midbrain–hindbrain boundary (MHB) is also specified at this stage, between the other two, through the activities of molecules such as Pax, Engrailed (En), Wnt and fibroblast growth factor (FGF). Of these broad domains, some are further
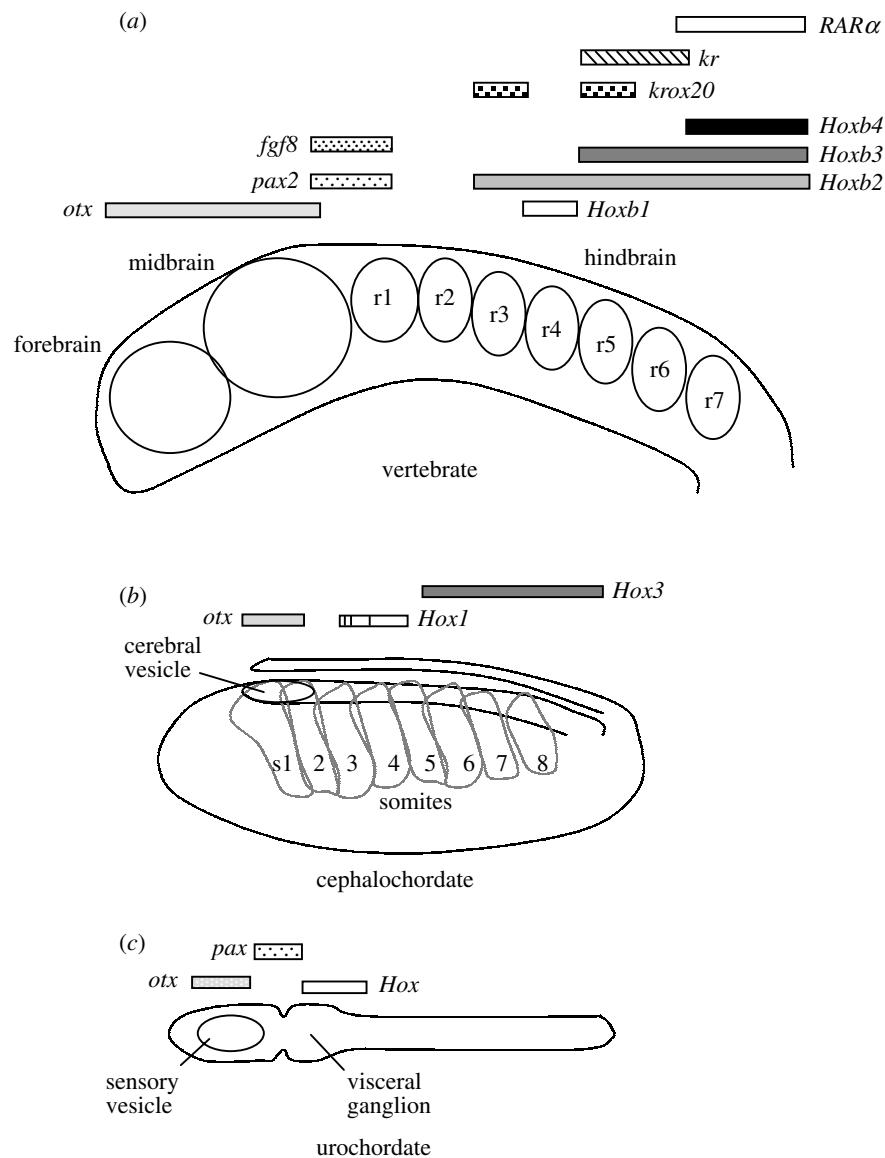
Figure 2. Conserved genetic control of AP patterning among chordates. Diagrams illustrate embryonic morphology and patterns of gene expression in: (*a*) the embryonic zebrafish head; (*b*) amphioxus and (*c*) an ascidian. Shaded bars above each diagram indicate the AP extent of expression for each gene. Expression of *Pbx* genes are not depicted here as those that have been described are ubiquitous. (*a*) Left-side view of the zebrafish head, anterior to the left, indicating the major brain regions including forebrain, midbrain and rhombomeres (r) 1–7 of the hindbrain. (*b*) Left-side view of a whole amphioxus embryo during somitogenesis, anterior to the left, indicating the segmental somites (s) and dorsal nerve cord, which contains the cerebral vesicle at its anterior end. (*c*) Outline of a larval ascidian, dorsal view, anterior to the left. The sensory vesicle expresses *otx* and the visceral ganglion expresses *hox* genes.

subdivided, most notably the hindbrain into a series of segments (rhombomeres). It is then the *Hox* genes expressed in different rhombomeres that finally give cells their AP identities (figure 2*a*).

Five classes of transcription factors directly regulate *Hox* expression in the hindbrain: RARs and RXRs, Krox-20, Kreisler (Kr/mafB), Pbx (and related 3-amino-acid loop extension transcription factors) and the Hox proteins themselves (figure 2*a*). The RARs and RXRs are receptors for retinoic acid (RA), a vitamin A derivative that promotes posterior cell character in the hindbrain. By contrast, Krox-20 and Kr/mafB have more restricted functions in maintaining *Hox* expression in subsets of hindbrain rhombomeres. Pbx transcription factors (relatives of *Drosophila extradenticles*, *exd*) form complexes with Hox proteins, thereby modifying their

regulation of target gene transcription. Though probably incomplete, this set of genetic interactions now provides a framework for understanding how cells in the hindbrain acquire their AP characters. As we discuss below, major differences in neural tube patterning between vertebrates and their chordate relatives may reflect relatively simple modifications in this genetic hierarchy.

The idea that *Hox* genes are the ultimate determinants of AP cell identities in the hindbrain is complicated by the fact that cells remain plastic and responsive to extracellular signals after *Hox* expression domains are established. Testing plasticity (or a cell's 'commitment' to an identity) requires challenging cells with foreign environments, and this was done recently with transpositions of individual hindbrain cells both in mouse and zebrafish embryos (Trainor & Krumlauf 2000*a*; Schilling *et al.*

2001). These studies uncovered a considerable degree of plasticity with respect to patterns of *Hox* gene expression, which depends on the size of the cell community. Transplanted cells that remain in coherent groups maintain their AP character in a cell-autonomous manner while individual transplanted cells do not. This implies that single cells in ectopic locations lack the neighbouring signals needed to reinforce their AP identity. Consistent with this idea, *Hox* expression is often maintained in grafts of whole rhombomeres in the chick, which presumably carry along these reinforcing signals (for a review, see Trainor & Krumlauf 2000*b*). Thus models of AP patterning must consider not only the secreted factors that establish major neural territories during gastrulation, but a much longer period in which such factors influence local cell fate decisions in the neural tube. A prime candidate for the source of such signals is the paraxial mesoderm (Muhr *et al.* 1997, 1999).

2. THE ROLE OF RETINOIC ACID IN PATTERNING THE AP AXIS

Candidates for the signals involved in early AP patterning include members of the FGF and Wnt families (Pownall *et al.* 1996, 1998; McGrew *et al.* 1997) and RA, all of which 'posteriorize' embryos, transforming anterior tissue into more posterior fates. For RA this is based on a long history of exogenous treatments of vertebrate embryos, which results in truncations of the head, loss of eyes and brain structures (Durston *et al.* 1989) (figure 3*b*). Exogenous RA given to mouse embryos *in utero* causes some *Hox* genes, such as *Hoxb-1* (normally expressed in rhombomere 4, r4), to be expressed more anteriorly in r1−3 and homeotic transformations in the hindbrain and craniofacial skeleton consistent with posteriorization. *Hoxa-1*, *Hoxb-1*, *Hoxb-4* and *Hoxd-4* all have RA response elements in their upstream regulatory regions which are bound by RARs and RXRs to regulate transcription (Marshall *et al.* 1994; Dupe *et al.* 1997; Gould *et al.* 1998). These studies have led to the idea that RA acts as a graded, posteriorizing signal, with long-range effects on *Hox* gene expression and cell fates in the central nervous system (CNS) (Maden 1999).

What has been missing in studies of RA signalling, however, are loss-of-function data demonstrating a requirement for RA in AP patterning. One approach has been to deplete RA in quail or mouse embryos by depriving their mothers of the RA precursor, vitamin A (figure 3*c*) (Maden *et al.* 1996; White *et al.* 2000). In these vitamin A deficient (VAD) animals, in contrast to RA treatments, the hindbrain fails to become 'posteriorized' and anterior identity persists, as judged by the expression of *Hox* genes and other rhombomere markers. Another way to disrupt RA signalling is by interfering with RARs and RXRs. These nuclear hormone receptors heterodimerize and also interact with co-activators and co-repressors to regulate target gene transcription. Dominant negative forms of RARs in *Xenopus*, which can dimerize but do not activate transcription, for example, fail to posteriorize the hindbrain as seen in VAD embryos (Blumberg *et al.* 1997). Direct evidence that the receptors are essential has been more difficult to obtain from mutations in mice, presumably due to receptor redundancy,

but the data so far are largely consistent with the model for RA as a posteriorizing factor (for a review, see Mark *et al.* 1999).

Recent attention has turned to enzymes that synthesize RA, the retinaldehyde dehydrogenases (RALDHs). One of these in particular, RALDH2, is expressed posteriorly in early vertebrate embryos (Swindell *et al.* 1999). Loss-of-function mutations in mouse *Raldh2* disrupt hindbrain segmentation but, surprisingly, do not affect forebrain and midbrain development (Neiderreither *et al.* 2000) (figure 3*c*). In *Raldh2*−/− mutants, *Hoxb-1* expression in the hindbrain expands posteriorly outside its normal domain in r4, *Krox-20* is expressed in a single broad domain rather than r3 and r5, and *Kr/mafB* expression is undetectable in r5−r6, all consistent with anteriorization. These results suggest that in the absence of RALDH2, the posterior hindbrain takes on characteristics of r4. A similar phenotype is observed in the *neckless* mutant in zebrafish, which disrupts an orthologue of *Raldh2* (Begemann *et al.* 2001). Interestingly, in both fish and mouse the requirement for *Raldh2* is confined to the posterior hindbrain, as are defects in embryos with VAD. If RALDH2 and RA are the major sources of retinoids in the embryo this argues that the posteriorizing role of retinoids may be restricted to the head–trunk boundary instead of having a graded influence throughout the CNS, as suggested by the classical studies with RA. This idea is consistent with previous studies showing that RA does not induce posterior *Hox* genes *in vitro* (Simeone *et al.* 1991) or when used to treat *Xenopus* embryos *in vivo*, and that FGFs may provide a posteriorizing influence in these more caudal regions via regulation of *Cdx* genes (Pownall *et al.* 1996, 1998; Isaacs *et al.* 1998).

RA signalling is highly conserved among chordates; both amphioxus and ascidians are anteriorized by exogenous RA though they do not display any rhombomeric organization in the hindbrain. RA treatments of amphioxus embryos cause *AmphiHox-1* expression to expand anteriorly in the nerve cord (Holland & Holland 1996) (figure 3*d*). This was recently shown to be a direct effect, similar to that seen in vertebrates, through transgenic experiments in mouse and chick (Manzaneres *et al.* 2000). Likewise, in the ascidian *Halocynthia roretzi*, RA expands the anterior boundary of *Hox-1* (*HrHox-1*) expression and reduces the expression domain of *Otx* (*Hroth*) (Katsuyama *et al.* 1995; Katsuyama & Saiga 1998) (figure 3*e*). Not surprisingly, lamprey embryos are also sensitive to exogenous RA during gastrulation, which leads to head truncations and defects in brain morphology (Kuratani *et al.* 1998).

Given the conservation of RA sensitivity it is interesting to ask how conserved are the genes encoding the RA receptors and synthesis enzymes among chordates. To date, only a few receptors have been cloned from protochordates. Ascidians have both RARs and RXRs, and these function as RA-dependent transcriptional activators like their vertebrate relatives (Hisata *et al.* 1998; Kamimura *et al.* 2000). Only partial RAR sequences have been described from amphioxus and lampreys, making it difficult to draw informative molecular phylogenetic conclusions about their interrelationships (Laudet 1997). The only relative of an RA signalling component found outside the chordates to date appears to be an RXR from a
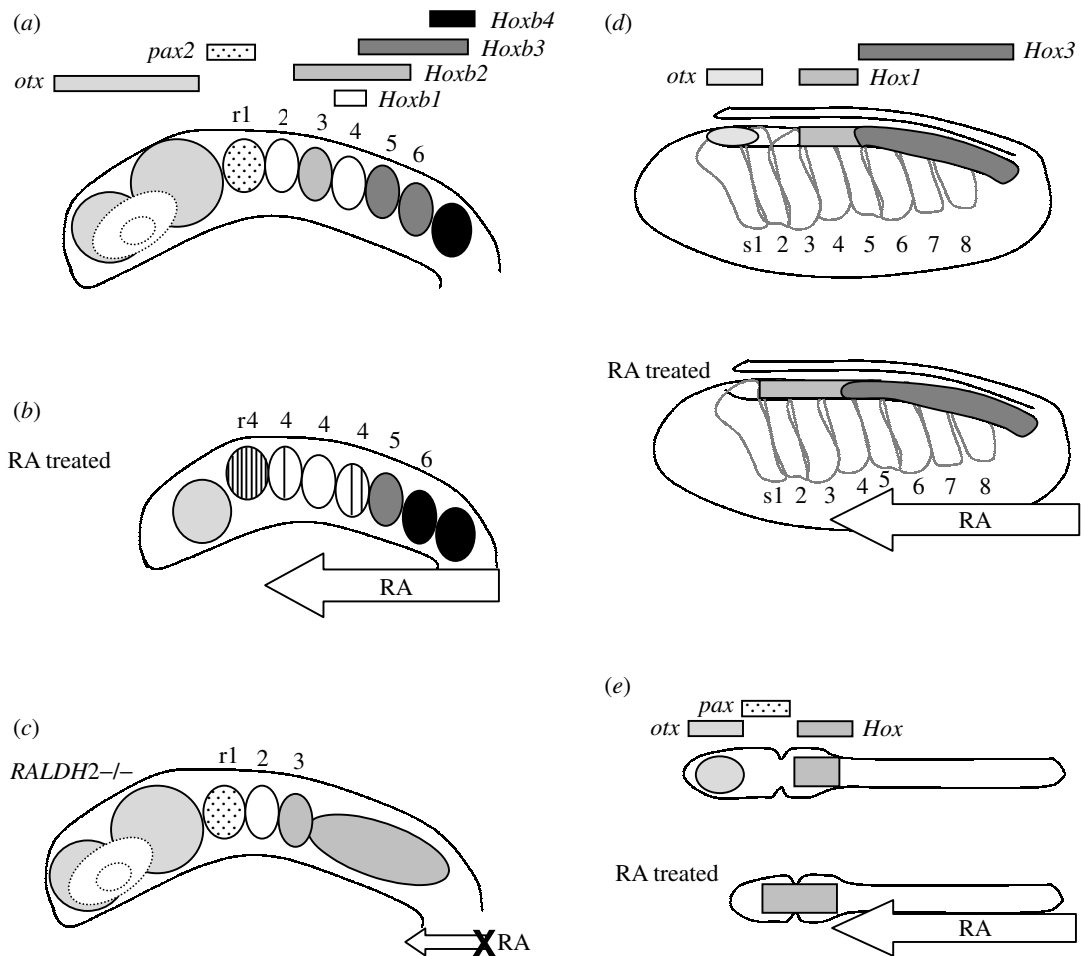
Figure 3. Conserved role of retinoic acid (RA) in AP patterning among chordates. Diagrams illustrate embryonic morphology and patterns of gene expression in (*a,b,c*) the embryonic zebrafish head, (*d*) amphioxus and (*e*) ascidians. Shaded bars above each diagram indicate the AP extent of mRNA expression for each gene. Open arrows indicate RA treatments or disruption. (*a*) Left-side view of the zebrafish head, anterior to the left, indicating the major brain regions, including forebrain, midbrain and rhombomeres (r) 1–7 of the hindbrain. (*b*) Left-side view of an embryo after treatment with 10^{-7} M all-trans-RA. (*c*) Left-side view illustrating the phenotypes of both mouse and zebrafish embryos mutant for *retinaldehyde dehydrogenase 2* (*RALDH2*, known as *neckless* in zebrafish) in which RA synthesis is disrupted. (*d*) Left-side views of whole amphioxus embryos during somito-genesis, anterior to the left, untreated or treated with 10^{-7} M RA. (*e*) Larval ascidians, anterior to the left, untreated or soaked in 10^{-7} M RA.

crustacean, though the relationship of this to vertebrate RXRs is unknown (Chung *et al.* 1998). Thus while RA signalling appears to be a primitive chordate mechanism of AP patterning, it may also be a chordate innovation.

3. *Otx* GENES AND ANTERIOR PATTERNING

Members of the *Otx* homeobox gene family in vertebrates are required for specification of the anterior brain (for reviews, see Bally-Cuif & Boncinelli 1997; Acampora & Simeone 1999). Loss-of-function mutations in mouse *Otx2* result in embryos that lack both forebrain and midbrain (Acampora *et al.* 1995) while the essential functions of its orthologue *Otx1* are more restricted to cortical and sense organ development (Acampora *et al.* 1996) (figure 4*a,b*). Similar patterns of expression in the anterior neuroectoderm have been described for *Otx* genes in all other vertebrates that have been examined.

Otx function in anterior specification appears to be an ancient feature of both vertebrates and invertebrates

given that *otd* in *Drosophila* is also required for anterior neural patterning. Relatives cloned from both *Halocynthia*, *Hroth* and amphioxus, *AmphiOtx*, are expressed in anterior neuroectoderm (Wada *et al.* 1998; Williams & Holland 1998) (figure 2*b,c*). In *Halocynthia*, *Hroth* misexpression causes ectopic formation of anterior neuroectoderm and repression of epidermis indicating a similar function to vertebrate *Otx2* in specifying the anterior head (Wada & Saiga 1999). An *Otx* orthologue has also been cloned from a hemichordate, the sister group of echinoderms, and is expressed anteriorly indicating an ancestral deuterostome function (Harada *et al.* 2000).

Using the available protein sequences, we have re-analysed the relationship between *Otx* genes among the chordates. *Halocynthia roretzi Otx* (*Hroth*), *Branchiostoma floridae Otx* (*AmphiOtx*), *Lampetra japonica Otx A* and *B* (*LjOtxA*, *LjOtxB*), and *Petromyzoan Otx* (*PmOtx*) were aligned with zebrafish, mouse and human *Otx* (*Zf Otx*, *Mus Otx*, *Hs Otx*, respectively) using CLUSTALX and then edited by eye (figure 5). A maximum likelihood (ML)
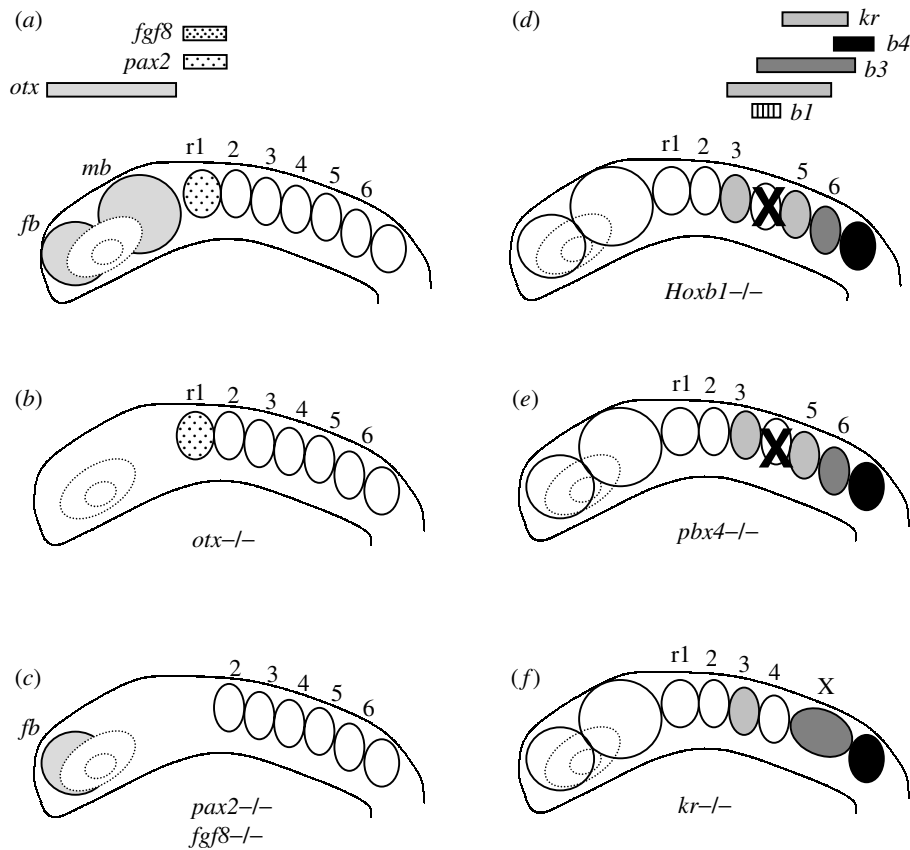
Figure 4. Mutations in mouse and zebrafish that disrupt AP patterning of the brain. Diagrams illustrate embryonic morphology and patterns of gene expression in both mouse and zebrafish mutants in the genes indicated below each diagram. Shaded bars above each diagram indicate the AP extent of mRNA expression for each gene. (*a*) Left-side view of head in wild-type, anterior to the left. (*b*) *Otx*−/− in mice lack forebrain and midbrain. (*c*) *Pax2*−/− mutants in mice (known as *no isthmus* in zebrafish) lack the midbrain and cerebellum. *Acerebellar* (*Fgf8*−/−) mutants in zebrafish lack a cerebellum. (*d*) *Hoxb1*−/− mutants in mice show defects in r4. (*e*) *Lazarus* (*Pbx4*−/−) mutants in zebrafish show defects in r4. (*f*) *Kr*/ mutants in mice (known as *valentino* in zebrafish) show defects in r5/r6 specification and instead develop one larger hindbrain territory known as rX.

tree was constructed from these aligned sequences by quartet puzzling using one invariant and eight variable rates of substitution to compensate for rate heterogeneity, and rooted by *Hroth*. In this tree, amphioxus and ascidian *Otx* form an outgroup to all of the vertebrate *Otx* family members. Zebrafish *Otx3* groups with the *Otx1* subfamily, suggesting it arose through a gene duplication event within the teleost lineage. Lamprey *Otx* proteins fall outside the gnathostome *Otx* family in our tree, in contrast to Ueki *et al.* (1998) who grouped LjOtxA with gnathostome *Otx2*. Our tree agrees with Tomsa & Langeland (1999), and suggests that the gene duplication event that gave rise to the gnathostome *Otx* genes occurred following the divergence of lampreys and jawed vertebrates. The *Petromyozon Otx* clusters with *Lampetra OtxB* in our tree, while *LjOtxA* falls basal to all of the vertebrate *Otx* family. Tomsa & Langeland (1999) suggested that the two *Lampetra* genes arose from a gene duplication after the divergence of the *Lampetra* and *Petromyozoan* lineages, but we find no support for this in our trees. However, in support of this suggestion, *PmOtx* is expressed in the same spatial and temporal patterns as both *Lampetra Otx* genes, indicating that the *Lampetra* genes have split an ancestral *Otx* function between them, following a duplication of the ancestral *Otx* gene within this lineage.

4. THE MIDBRAIN–HINDBRAIN BOUNDARY

In vertebrates, a region corresponding to the MHB is also specified during gastrulation and plays a central role in the local interactions that pattern the neural plate after its initial polarity is established. These interactions induce tectal and cerebellar structures, some of which are derived from the MHB itself. This region appears to be specified slightly later and in a region sandwiched between the early domains specified by *Otx* and *Hox* genes (figure 2). Molecules involved in MHB specification include a large and ever-growing list such as members of the Pax, En, Gbx, Lmx, Wnt and Fgf families, all of which are expressed at some stage in cells of the MHB (for a review, see Joyner 1996). The exact sequence of events in this specification remains unclear, though the earliest expressed genes appear to be *Pax2, Fgf8* and *Wnt1*. This is further complicated by the presence of multiple family members expressed in the MHB, including two different *Fgf* genes, *Fgf8* and *Fgf18*, and two *Pax* genes, *Pax2* and *Pax5*. Such multiple family members, expressed in similar patterns, emphasizes the need to understand their molecular phylogeny, as discussed below.

Cells of the MHB have unique organizing properties, such that when transplanted anteriorly in the chick they reorganize forebrain tissue and induce a mirror-image
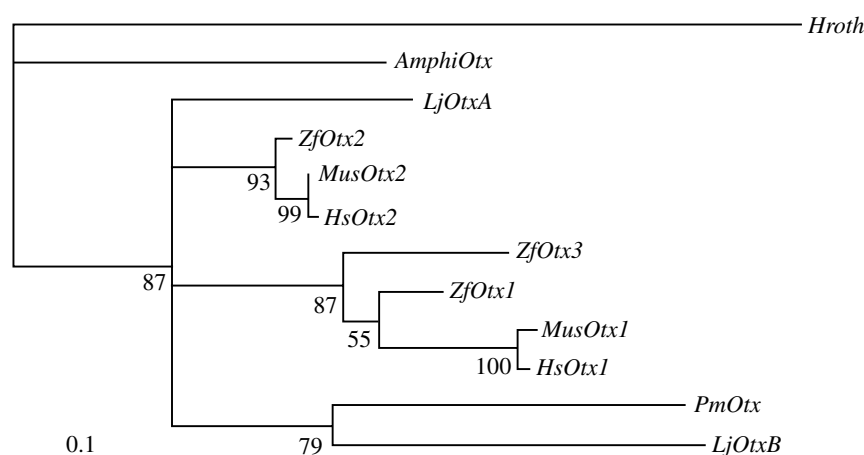
Figure 5. Phylogenetic relationships among *Otx* genes in chordates: *Halocynthia roretzi Otx* (*Hroth*), *Branchiostoma floridae Otx* (*AmphiOtx*), *Lampetra japonica Otx A and B* (*LjOtxA, LjOtxB*), *Petromyzon Otx* (*PmOtx*), zebrafish (*ZfOtx*), mouse (*MusOtx*) and human (*Hs Otx*). Alignments used CLUSTALX and were edited by eye to construct a ML tree by quartet puzzling using one invariant and eight variable rates of substitution to compensate for rate heterogeneity. The tree is rooted by *Hroth*.

duplicate of the midbrain. Both Fgf8 and Wnt1 are potential secreted mediators of this organizing activity. Fgf8, in particular, when misexpressed as protein coated onto beads and placed into the forebrain or hindbrain, can mimic the organizer activity of an ectopic MHB (Crossley *et al.* 1996). This includes repression of *Hoxa-2*, and formation of ectopic midbrain and cerebellar structures (Irving & Mason 2000). Fgf8 therefore is thought to act as a planar signal within the neural ectoderm that polarizes the midbrain and anterior hindbrain.

Loss-of-function mutations have pinpointed the essential components of this complex patterning situation at the MHB. In mice, mutations in *Pax2* disrupt formation of the entire posterior midbrain and cerebellum (Favor *et al.* 1996), while mutants in its close relative, *Pax5*, which is expressed slightly later in the same region, show only minor brain defects (Urbanek *et al.* 1994) (figure 4*c*). Similar to the mouse, a mutant in zebrafish *pax2.1*, called *no isthmus* (*noi*), lacks the MHB region as early as the gastrula stages (Lun & Brand 1998). However, *wnt1* and *fgf8* expression are initiated normally in *noi*/*pax2.1* mutants suggesting that *pax2, wnt1* and *fgf8* are activated independently during MHB development. Homozygous mutations in *Fgf8* in mice are early lethals, due to defects in primitive streak formation, and thus are not informative in terms of the role of *Fgf8* in MHB formation. Loss-of-function mutations in zebrafish complement the targeted studies in mice in this case, overcoming the problem of early lethality. Mutations in zebrafish *fgf8* are called *acerebellar* (*ace*) (Reifers *et al.* 1998) (figure 4*c*). Here, unlike in mice, mutants gastrulate normally but lack a MHB and subsequently lack the cerebellum. Consistent with the model of independent pathways, *pax2.1* expression is initiated but not maintained in *ace*/*fgf8* mutants. Interestingly, these studies also show that *fgf8* and *pax2.1* expression is spatially distinct at first, in separate but adjacent cells of the MHB, then later overlap. Finally, the *ace*/*fgf8* phenotype reveals that *fgf8* functions relatively late, after gastrulation, to polarize the midbrain.

Among the chordates, comparative data on genes in the *Pax, En, Wnt* and *Fgf* families are preliminary. Based on cloning and expression of one *Pax* gene from *Halocynthia* (*HrPax-2/5/8*), Wada *et al.* (1998) hypothesized the existence of an MHB-like region in the anterior neural ectoderm (figure 2*c*). *HrPax-2/5/8* expression in the embryonic neural tube abuts *Hroth* expression anteriorly and *HrHox-1* posteriorly. However a *Pax2/5/8* orthologue cloned from amphioxus (*AmphiPax-2/5/8*) shows no comparable neuroectodermal expression abutting *AmphiHox-1* and *AmphiOtx*; at early stages the only neural tube expression is posterior to the anterior limit of *AmphiHox-1* (Kozmik *et al.* 1999) (figure 2*b*).

An amphioxus orthologue of *En* (*AmphiEn*), is expressed in the anterior neuroectoderm (Holland *et al.* 1997) but in a domain that more probably coincides with part of the forebrain, based on both anatomical and comparative gene expression analyses (Lacalli *et al.* 1994). An amphioxus *Wnt1* (*AmphiWnt1*) has been cloned (Schubert *et al.* 2000) but its expression pattern has not been published—and to date no amphioxus *Fgf8* orthologue has been described. Thus the comparative gene expression data cannot resolve definitively whether the MHB is an evolutionarily primitive chordate character, but do suggest that it is likely to be conserved. If, as suggested by the data from *HrPax-2/5/8* and *AmphiPax-2/5/8* expression, a MHB is present in ascidians but not in amphioxus, then we can hypothesize that this feature may have been secondarily lost in the lineage leading to cephalochordates.

Agnathans, both lampreys and hagfish, exhibit intriguing anatomical differences at the MHB that make comparative molecular studies of this region particularly interesting. Both lack a well-developed cerebellum, which arises from rl as part of the MHB. A rhombic lip in the lamprey forms at this level but appears to lack the stereotypical descending spinal projections, Purkinje cells, and many other aspects of neuronal architecture typical of the gnathostome cerebellum (Nieuwenhuys 1977). Of the genes known to be involved in MHB development, two *En*-related genes have been cloned from lampreys, and immunohistochemical studies have demonstrated that lamprey En proteins are expressed in the MHB, though it is unclear exactly which structures this will form
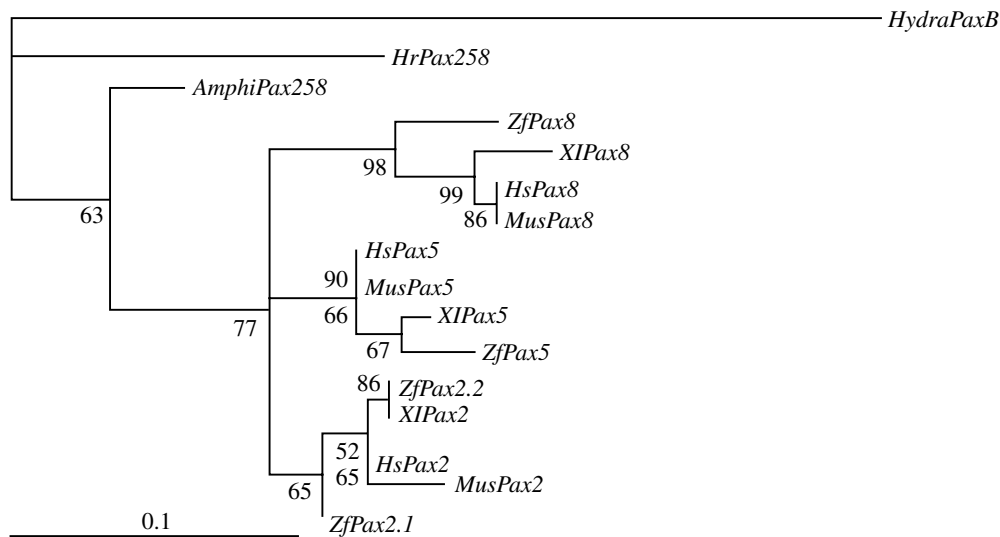
Figure 6. Phylogenetic relationships among members of the *Pax2/5/8* family in chordates: *Hydra littoralis* (*PaxB*), ascidian (*Halocynthia roretzi*), amphioxus (*Branchiostoma floridae*), zebrafish (Zf), mouse (Mus) and human (Hs). Alignments used CLUSTALX to construct a maximum likelihood tree, as for the *Otx* genes. The tree is rooted using *Hydra PaxB*.

(Holland & Williams 1990; Holland *et al.* 1993). Thus, further resolution of the nature of MHB development in agnathans awaits characterization of lamprey relatives of *Pax2* and *Fgf8*.

Molecular phylogenetic studies suggest that, like *Otx* genes, *Pax* genes have undergone duplications in the chordate lineages leading to vertebrates (figure 6). We have reanalysed the available protein sequences to determine their relationships. Members of the *Pax2/5/8* family from *Hydra littoralis* (*PaxB*), ascidian (*Halocynthia roretzi*), amphioxus (*Branchiostoma floridae*), zebrafish (Zf), mouse (Mus) and human (Hs) were aligned using CLUSTALX and then edited by eye. A ML tree was constructed as for the *Otx* family. The tree is rooted using *Hydra PaxB* and shows that amphioxus *Pax2/5/8* groups with vertebrate *Pax2/5/8*, indicating that they arose from the same ancestral gene in the most recent common ancestor of amphioxus and vertebrates. Two zebrafish *Pax2* relatives cluster with tetrapod *Pax2*, although they do not show a sister relationship as described by Kozmik *et al.* (1999) and Pfeffer *et al.* (1998). The expression profiles of *pax2.1* and *pax2.2* suggests that they are paralogous in that they have similar spatial patterns of expression. Mapping of these two genes to paralogy regions will resolve if they arose by gene duplication within the teleost lineage. The gene duplication that gave rise to the *Pax2/5/8* genes occurred after the divergence of cephalochordates and vertebrates. The characterization of *Pax2/5/8* genes from other non-chordates will clarify whether *Pax2/5/8* genes have an ancestral function in AP patterning of the neural tube and whether there was a MHB in the chordate ancestor.

5. *Hox* GENES AND POSTERIOR PATTERNING

By far the best-studied components of vertebrate AP patterning are the *Hox* genes (see figure 1). These determine AP identities of cells throughout much of the animal kingdom. *Hox* mutations in *Drosophila* and in vertebrates alike give homeotic transformations, in which one body region takes on the characteristics of another. Furthermore, the conserved clustered arrangement of these genes within the genome and the temporal and spatial patterns of *Hox* expression are similar in all major bilaterian groups.

Segmental identity in the vertebrate neural tube has been postulated to reflect a combinatorial *Hox* code, in which unique sets of *Hox* genes expressed in each segment control regional identity. Mammals have a total of 39 *Hox* genes that lie in four clusters (figure 1). These show overlapping AP domains of expression in the neural tube and recent studies have begun to address the problem of their redundancy. How do such similar genes expressed in the same regions differ in function? Interesting examples are the *Hox-1* genes, expressed in the anterior hindbrain. Mutations in either *HoxA-1* or *HoxB-1* alone show defects in r4 of the hindbrain (for a review, see Cappechi 1997) (figure 4*d*). Double mutants in both genes show a more severe loss of r4 and r5, which may result from an early mis-specification followed by apoptosis (Rossel & Cappechi 1999). These results suggest that *Hox-1* paralogues in different clusters do not differ so much in function as in the kinetics of their expression. A similar double mutant approach has revealed apparent redundancy in *HoxA-3* and *HoxD-3* (Greer *et al.* 2000). One recent approach to testing redundancies between *Hox* genes within a cluster has been to delete whole clusters, or at least the majority of genes within them. Surprisingly, large deletions of either *HoxB1–9* or *HoxC1–9* survive until birth (Medina-Martinez *et al.* 2000; Suemori & Noguchi 2000). Homozygous animals in which the *HoxB* cluster is deleted show combinatorial phenotypes of the individual mutants for each gene. These results suggest that there is considerable redundancy in the roles of paralogous *Hox* genes in early AP patterning.

Both ascidians and amphioxus lack an overtly segmented hindbrain, yet their *Hox* genes are expressed in nested, anteriorly restricted expression patterns in the neural tube like their vertebrate counterparts. *Hox* genes have now been isolated from two different ascidians,

Ciona (*CiHox3*, *5* and *8*) and *Halocynthia* (*HrHox1*) (Di Gregorio *et al.* 1995; Wada *et al.* 1999; Locascio *et al.* 1999), and an entire *Hox* cluster has been examined in detail in one amphioxus species *Branchiostoma floridae* (Garcia-Fernandez & Holland 1994; Ferrier *et al.* 2000). The results of these studies of *Hox* expression in protochordates show that the role of these genes in specifying AP identity in the neural tube is an ancestral chordate feature.

Given the crucial roles of *Hox* genes in AP patterning, one might predict that changes in the total number of *Hox* genes played an important evolutionary role in the diversification of animal body plans. Comparisons of *Hox* gene number between chordates suggest major *Hox* gene duplication events in vertebrates after their divergence from an invertebrate chordate ancestor (figure 1). Mammals, including humans, have four *Hox* clusters, each with up to 13 genes, and this appears to be the primitive condition for gnathostomes. A first indication that the number of *Hox* genes might have been an important factor in the origin of vertebrates came from the realization that the genes in *Branchiostoma* all occupy a single cluster (Garcia-Fernandez & Holland 1994) and at least the first 10 (*AmphiHox1–10*) are orthologues of the vertebrate *Hox 1–10* genes. Recently, additional members of this cluster, *AmphiHox11–14*, have been identified and were all shown to cluster in the same physical order as the vertebrate genes (Ferrier *et al.* 2000). Anteriorly-expressed *Hox* genes in *Branchiostoma* embryos specify overlapping AP domains of the neural tube (except *AmphiHox-2* which is not expressed in neural ectoderm), with *AmphiHox-1* the most anteriorly expressed, then *AmphiHox-3* and *AmphiHox-4*, as in vertebrates (Holland & Holland 1996; Wada *et al.* 1999). There is now similar evidence for AP regionalization of *Hox* gene expression in ascidians (Gionti *et al.* 1998) and echinoderms (Popodi *et al.* 1996), which are a sister group to the chordates. These results suggest that the four *Hox* clusters thought to be primitive for vertebrates arose through large-scale gene duplications, perhaps whole genome duplications, that occurred in the vertebrate ancestor. This is supported by the presence of clusters of paralogous genes throughout the vertebrate genome, many of which show conserved gene order or synteny. These results also suggest that an increase in *Hox* gene number could have contributed to the diversification of vertebrate body plans in which additional paralogues acquired new functions (Ohno 1999).

One important test of these ideas is to examine *Hox* cluster organization in jawless vertebrates such as the lamprey. Lampreys, along with the hagfishes, are the only living relatives of what were most probably jawless ancestors of all living gnathostomes. Lamprey–hagfish relationships are still debated (Gursoy *et al.* 2000), but together they form an outgroup to the gnathostomes and occupy a pivotal position in determining the primitive vertebrate condition. Morphologically, lampreys show many features thought to be intermediate between cephalochordates and gnathostomes, and thus we might predict an intermediate genome organization and *Hox* gene number (Fritsch & Northcutt 1993). Interestingly, the first attempts at identifying lamprey *Hox* genes suggest that they may only have three clusters. A total of only 21 *Hox* genes were isolated from *Lampetra planeri*, including

members of paralogue groups 1–10, plus one group 13 *Hox* gene (Sharman & Holland 1998) (figure 1). Since these results are based only on their homeobox sequences, the authors conclude that at this point they cannot resolve whether this organization arose through the loss of a fourth cluster or, alternatively, an independent cluster duplication within the lamprey lineage.

Although the number of species sampled is small, these studies in amphioxus and in lampreys are consistent with the idea that an increase in *Hox* genes allowed the diversification of vertebrate body plans. What is becoming clear, however, is that such correlations do not always hold. In zebrafish, *hox* genes form seven clusters, probably reflecting an ancient genome duplication in the common ancestor of the teleost fishes since both the Japanese medaka, *Oryzias latipes*, and pufferfish have a similar number of clusters (Amores *et al.* 1998; Postlethwait *et al.* 1998; Prince *et al.* 1998; Taylor *et al.* 2001). Subsequent gene loss has led to a total of 51 *hox* genes in zebrafish, only 12 more than in mammals, for instance, suggesting that there may be selection for certain numbers of some *Hox* gene classes. Yet, despite the increase in both cluster and gene number in these teleosts there is arguably no significant elaboration of AP patterning along the body axis as compared with mammals. This could be because the genome duplication in teleosts occurred relatively soon after a previous genome duplication in the vertebrate ancestor, such that the paralogous genes did not have time to undergo extensive functional divergence and many of the newly duplicated genes were lost with little consequence.

6. *Pbx* GENES AND HINDBRAIN SEGMENTATION

Changes in *Hox* regulation, rather than gene number, may have also played a role in vertebrate origins. There are few comparative studies of the regulatory regions that control *Hox* expression between chordates. Recently, *cis*-regulatory elements from amphioxus *Hox* genes *AmphiHox-1* to *AmphiHox-3* were shown to drive spatially restricted reporter gene expression in the hindbrain both in transgenic mice and, transiently, in chick embryos, using focal electroporation (Manzanares *et al.* 2000). For example, elements 3′ of *AmphiHox-3* mediated expression in both mouse and chick neural tubes with an anterior limit between r5 and r6. This pattern is similar but offset by one rhombomere from the endogenous expression pattern of the vertebrate group three *Hox* genes. Furthermore, these amphioxus elements show RA-dependent activities that resemble their vertebrate paralogues. These results demonstrate a conserved tissue specificity in *Hox* regulation among chordates, but reveal subtle differences, some of which may be accounted for by differences in other regulatory factors, such as Krox-20, Kr/mafB and Pbx, between amphioxus and vertebrates (as discussed below).

In *Drosophila*, specificity in the case of the *ultrabithorax* and *antennapedia* complexes depends on the presence of co-operative DNA-binding partners such as *exd* (for a review, see Mann & Chan 1996), *polycomb* and *trithorax*. Mutations in these genes can cause homeotic transformations without corresponding changes in *Hox* expression, indicating a downstream affect on *Hox* signalling (Peifer

& Weischauss 1990). In mice, *polycomb*-related genes regulate *Hox* genes by controlling access to retinoic acid response elements within *Hox* promoters, and mutations in these disrupt *Hox* expression and AP patterning (Bel-Vialar *et al.* 2000). Three *Pbx* genes have been identified in mammals but, to date, targeted mutations of these have not been particularly informative. *Exd* and *Pbx* genes encode TALE-homeodomain proteins that interact with Hox proteins, and are thought to modulate expression of their downstream targets. Recently an additional set of homeodomain proteins (Prep1, Meis, HTH), which are capable of dimerizing with Pbx, have also been implicated in controlling rhombomere-specific expression of *Hox* genes, and these will certainly be a focus of future genetic analyses (Jacobs *et al.* 1999; Ferretti *et al.* 2000).

The first evidence that *Pbx* genes are required for vertebrate development came recently from a zebrafish mutant called *lazarus* (*lzr*) (Popperl *et al.* 2000) (figure 4*e*). The *lzr* gene encodes a novel *pbx* gene (zebrafish *pbx4*) required for AP patterning throughout the hindbrain and anterior trunk. *lzr/pbx4* function is essential for the activities of multiple *hox* genes including group 1–4 *hoxb* genes as well as *hoxa-2*, and for rhombomere boundary formation. Regulatory interactions between these *hox* genes also appear to be disrupted in the absence of *lzr/pbx4* activity. Morphologically, interneurons fail to form in r4 in *lzr/pbx4* mutants and cranial motor neurons of the facial nerve fail to migrate from r4, consistent with a role for *lzr/pbx4* in specification of this segment. *lzr/pbx4* is expressed earlier than other *pbx* genes in zebrafish and thus other members of the *pbx* family may not compensate for its function. Given similarities in expression and binding properties of Pbx in mice and zebrafish, their biochemical functions are likely to be conserved, but differences in temporal and spatial patterns of expression may underlie differences in their *in vivo* functions.

If *Pbx* and *polycomb*-related genes are so important for vertebrate *Hox* gene regulation and function perhaps evolutionary changes in the roles of these genes can account for some of the differences in AP patterning among chordates. To date, no *Pbx* or *polycomb* relatives have been identified in ascidians or in amphioxus. Furthermore, the recent experiments by Manzanares *et al.* (2000) suggest that at least some of their roles in *Hox* regulation may not be conserved. Amphioxus *cis*-regulatory elements 3′ to *AmphiHox-1* drive reporter-gene expression in transgenic mouse and chick embryos in patterns similar to endogenous *Hoxa-1* and *Hoxb-1*, with one important difference: there is no r4-restricted domain, which in vertebrates is controlled by a group 1 *Hox/Pbx*-dependent autoregulatory loop. Furthermore, the *AmphiHox-1* regulatory element lacks the consensus binding sites for Hox/Pbx. These results suggest that the elaboration of segment-specific patterns of *Hox* expression vertebrates involved the addition of new regulatory elements.

7. NEW ROLES FOR *kreisler* AND *krox* GENES IN VERTEBRATES

Two additional components of the AP patterning model are the so-called segmentation genes, *Kr/mafB* and *Krox-20*, both of which are regulated by RA and, in turn,

regulate *Hox* gene expression. Little is known about their developmental roles outside mammals, or their evolutionary history.

In mice, *Kr/mafB* specifies a pair of adjacent hindbrain rhombomeres, r5 and r6. Loss-of-function mutations in *Kr/mafB* disrupt *Krox-20* and *Hox* gene expression in r5/6 (McKay *et al.* 1994; Manzanares *et al.* 1997). *Kr/mafB* directly regulates *Hoxb4* expression. More recently, the cellular requirements for *Kr/mafB* were revealed by studies of a zebrafish mutation in a *Kr/mafB* orthologue called *valentino* (*val*) (Moens *et al.* 1996, 1998) (figure 4*f*). Mosaic studies were performed using cell transplantation to introduce *val/kr* mutant cells into a wild-type hindbrain, and vice versa. These showed that mutant cells are unable to contribute to the r5/6 region, presumably due to differences in their adhesive properties. Based on these results, combined with the changes in gene expression in *val/kr*, Moens *et al.* (1996) proposed that *val/kr* specifies a 'protorhombomere', the precursor of r5/6 which they called rX, and is required for its subdivision. Such a process may be a common mechanism of progressive subdivision throughout other regions of the CNS.

In contrast, the zinc finger gene, *Krox-20*, is expressed in and required for the development of rhombomeres 3 and 5 (r3 and r5) in the vertebrate hindbrain (Wilkinson *et al.* 1989). Mice mutant for *Krox-20* lack these rhombomeres and show corresponding defects in cranial nerve growth and neural crest migration (Schneider-Manoury *et al.* 1993, 1997). *Krox-20* has a conserved function in vertebrates in activating and maintaining *Hox-2* group gene expression in r3 and r5 (Nonchev *et al.* 1996). It is also required for the expression of the chicken ephrin receptor EphA4 in r3, implicating it as a key gene in defining segmental identity in the vertebrate hindbrain and regulating segment morphogenesis (Theil *et al.* 1998). *Krox-20* is a C2H2 zinc finger transcription factor and is a member of the *Egr* gene family; this includes the vertebrate genes *Egr-1* (*Krox-24*, *NGFI-A*, *Zif268*), *Egr-2* (*Krox-20*), *Egr-3* (*Pilot*) and *Egr-4* (*NGFI-C*) as well as *Drosophila Stripe*. In vertebrates, *Krox-20* is the earliest acting member of this family during development.

Given the conservation of nested *Hox* expression along the AP axis among chordates, are the roles of *Kr/mafB* and *Krox-20* in segmental control of *Hox* gene expression in the vertebrate neural tube ancestral chordate features? To answer this question, Knight *et al.* (2000) recently cloned an amphioxus orthologue (*AmphiKrox*) of the vertebrate *Egr* gene family (figure 7). Phylogenetic analysis showed that this amphioxus orthologue is equally related to the four vertebrate *Egr* genes, suggesting that they are all descended from the same gene in the most recent common ancestor of vertebrates and cephalochordates. *AmphiKrox* expression was examined throughout embryogenesis and found in the cerebral vesicle and in the club-shaped gland but not in the neural tube, nor is it co-expressed with *Hox-2* group genes. This suggests that the function of *Krox-20* as a hindbrain segmentation gene arose in the vertebrates after the divergence of cephalochordates and amphioxus. It is possible that the expression of *AmphiKrox* reflects derived features of amphioxus and that the ancestral function of this gene was in the neural tube; expression analysis of orthologous *Krox* genes in other invertebrate chordates, such as the appendicularian *Oikopleura*, may clarify this.
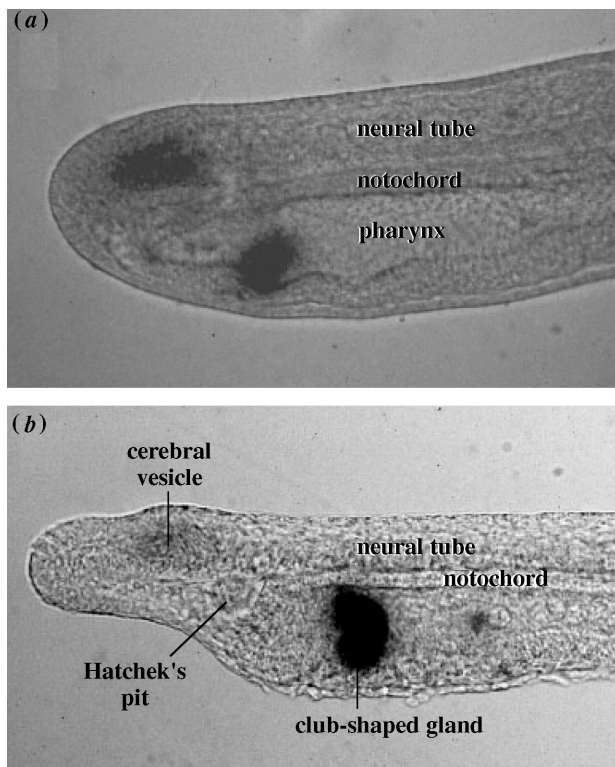
Figure 7. Expression of *AmphiKrox* during embryogenesis. Lateral views. (*a*) *AmphiKrox* mRNA is detected in the anterior neural tube and a localized region of the pharynx of the embryo. (*b*) Soon thereafter expression is detected in the cerebral vesicle and in the club-shaped gland associated with the pharynx.

In support of this idea, experiments by Manzanares *et al.* (2000) suggest that at least some of the roles of *Krox-20* and *Kr/mafB* in *Hox* regulation also may not be conserved. Amphioxus *cis*-regulatory elements 5′ to *AmphiHox-3* drive reporter-gene expression in transgenic mouse and chick embryos in a pattern that is offset by one rhombomere from the pattern of endogenous group 3 *Hox* gene expression. There is no r5-restricted domain, which in vertebrates is controlled by a *Kr/mafB*-dependent segmental enhancer. Likewise, amphioxus regulatory elements surrounding *AmphiHox-2* show no activity equivalent to the conserved r3/r5 *Krox-20*-dependent enhancers of *Hoxa-2* and *Hoxb-2*, though they do drive reporter-gene expression in the neural crest. These results suggest that the elaboration of segment-specific patterns of *Hox* expression vertebrates involved the addition of new regulatory elements.

A key question concerning the evolution of hindbrain segmentation in the vertebrate hindbrain by *Krox-20* regards the phylogenetic relationships of chordate *Egr* genes. We have reanalysed their relationships in the chordates based on the available protein sequences (figure 8). *Egr* family members from vertebrates were aligned with a *Branchiostoma floridae* orthologue (*AmphiKrox*) using CLUSTALX, and the highly conserved zinc finger region was used for constructing a ML tree by quartet puzzling, as described for the Otx and *Pax2/5/8* families, and rooted using *AmphiKrox*. This tree shows that vertebrate *Egr2, 3* and *4* all group together in contrast to neighbour joining (NJ) trees; in trees constructed using NJ, *Egr4* falls outside of an *AmphiKrox* (*Egr1,2,3*) clade (Hughes 1999). A quartet puzzling tree with the related C2H2 zinc finger protein, WT-1, as an outgroup to the *Egr*s (and including *Drosophila* and *C. elegans* orthologues), shows the same topology as the tree displayed here (Knight *et al.* 2000). Furthermore, Martin (2000) found that *Egr4* grouped with *Egr1, 2* and *3*, which is consistent with our results. This indicates that *Egr4* genes have undergone a period of rapid evolution, such that molecular phylogenetic analyses tend to place them more basally to other vertebrate *Egr* genes. This is supported by the long branch length for *Egr4* relatives in the puzzling tree. *AmphiKrox* falls basally to vertebrate Egrs in our tree, but its position relative to Egr1 is unresolved, probably due to the high conservation of the zinc finger region used in generating this alignment. Other conserved domains present in vertebrate *Egr*, and absent from amphioxus, *Drosophila* and *C. elegans* orthologues, support the hypothesis that the vertebrate *Egr* genes arose from a single ancestral gene in the vertebrate lineage. In addition, the mammalian *Egr1, 2* and *3* genes are all located in well-defined paralogy regions, indicating that
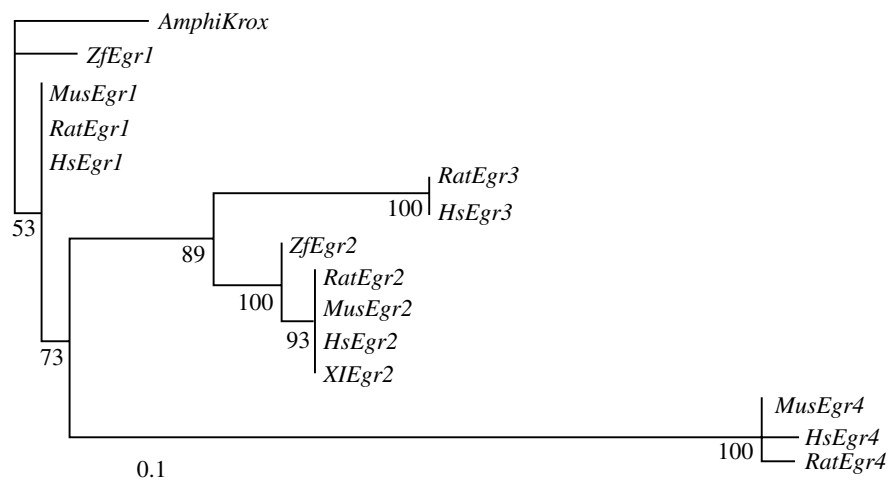

Figure 8. Phylogenetic relationships among *Krox* genes in chordates. Vertebrate *Egr* genes were aligned with a *Branchiostoma floridae* orthologue (*AmphiKrox*) using CLUSTALX, and the highly conserved zinc finger region was used for constructing a maximum likelihood tree by quartet puzzling, and rooted using *AmphiKrox*.

they arose through large-scale duplications of the ancestral vertebrate genome. There appears to be no conserved function of Egr proteins between invertebrates and vertebrates, suggesting that the function of the vertebrate *Krox-20* (*Egr-2*) in hindbrain segmentation arose in the vertebrate lineage, concordant with at least two gene duplication events in the vertebrate *Egr* family.

8. RELATIONSHIPS BETWEEN EARLY AP PATTERNING AND NEURONAL DIFFERENTIATION

The expression patterns of the genes we have discussed along the AP axis in vertebrates correlate with the specific types of motor neurons and interneurons that develop in different regions, and in many cases have been shown to be required for neuronal identities. Thus, mutations in mouse *Hoxa-2* cause the motor neurons of rhombomeres 2 and 3 (r2 and r3), which normally exit r2 to innervate the mandibular arch, to turn posteriorly and exit the hindbrain from r4, the normal facial nerve exit point (Gavalas *et al.* 1997). Misexpression of *Hox* genes, both in chick and in zebrafish, also causes homeotic transformations of rhombomeric identities (Alexandre *et al.* 1996; Yan *et al.* 1998; Bell *et al.* 1999). Interestingly, misexpression of *Hoxa-2* or *Hoxb-1* outside their normal expression domains, in r1 which does not normally express *Hox* genes, leads to the generation of motor neurons in this territory. Moreover, the type of motor neuron generated appears to the depend on the specific *Hox* gene they express: *Hoxa2*-expressing cells become trigeminal-like, while *Hoxb1*-expressing cells appear facial-like. These data support the idea that expression of a *Hox* gene is both necessary and sufficient to specify motor neuronal type along the AP axis of the hindbrain.

As discussed in much further detail by Lacalli (2001, and see Lacalli *et al.* 1994), both ascidians and amphioxus larval CNS are patterned on a tiny scale that can be compared with the axial layout of the vertebrate brain and spinal cord. When combined with gene expression studies, these suggest that amphioxus has a hindbrain homologue that does not appear to be overtly segmented as it is in vertebrates where there are clear segmental groups of motor neurons. To address this issue further, Jackman *et al.* (2000) cloned an amphioxus relative of the vertebrate LIM−homeodomain transcription factor *islet-1* (*Amphi-islet*). This gene is expressed in segmentally arranged cells in the putative hindbrain that are aligned next to the somite boundaries, which the authors call putative motor neurons. These correlate with patterns of *Hox* expression that indicate some degree of segmentation, in that the *AmphiHox1, AmphiHox3* and *AmphiHox4* expression domains have anterior boundaries separated by two-somite intervals. These results suggest that the hindbrain of the common ancestor of cephalochordates and vertebrates had reiterated segmentation but lacked clear differences between different AP regions of the neural tube and their derivatives in the homologues of the vertebrate hindbrain and spinal cord. This accords well with studies by Bone (1960) and supports the hypothesis of Fritsch & Northcutt (1993), who proposed, based on anatomical evidence, that amphioxus has no direct homologues of the vertebrate cranial nerves. Rather, vertebrate cranial and spinal nerves may have arisen independently from serially reiterated nerves of the chordate ancestor.

9. SUMMARY AND OUTLOOK

The mechanisms that pattern the body axis begin with signals that induce AP polarity in the neural ectoderm. Targeted mutagenesis in mice and random mutant screens in the zebrafish have begun to unravel the genetic pathways that organize the vertebrate nervous system along its AP axis. A number of new mutations in zebrafish, in particular, have helped to define the roles of RA, Pax2, Fgf8, Kr/mafB and Pbx in AP patterning and the regulation of *Hox* genes. This article is intended not only to summarize these new findings, but also to speculate on how we can use them in a comparative context to begin to learn how gene functions may have diverged to lead to the evolution of the complex vertebrate nervous system that we find today.

Protochordates have orthologues of many of the genes controlling vertebrate development, but vertebrates clearly have many more of each type. Extensive gene duplication, possibly due to polyploidy after the divergence of vertebrates and invertebrate chordates, makes comparisons of orthologous genes complicated. Amphioxus and ascidians are useful for inferring ancestral function since they diverged from the vertebrate stem lineage prior to these extensive gene duplications. Comparisons of the expression of ascidian and amphioxus genes indicate that the primitive chordate brain contained at least three regions, an anterior one corresponding to the vertebrate forebrain and possibly the midbrain, a posterior domain corresponding to the hindbrain and spinal cord, and an intermediate region at the MHB. We have seen how genes involved in vertebrate AP patterning in these chordates, such as *Otx* and *Hox* genes as well as several of the genes known to regulate *Hox* expression, reveal patterns of gene divergence after duplication. The molecular phylogenetic comparisons of these gene families will allow us to compare orthologous genes accurately between distantly related chordate species. In the future, these genes and their regulatory regions can be tested in vertebrates for conservation of function. The recent experiments by Manzanares *et al.* (2000) demonstrate the power of such an approach. Furthermore, ascidians and lampreys offer the possibility of investigating gene functions through injecting DNA or RNA constructs into fertilized eggs or early blastomeres in order to assay their developmental consequences. Together, these comparisons are beginning to unravel the primitive and derived features of AP patterning.

Perhaps the comparisons we are making, however, between distantly related chordate groups in which there are major developmental and genome differences, will in some cases be too distant to resolve until we have more information about close relatives. Teleost fishes, in which there are several groups that have become polyploid relatively recently, such as salmonids, may offer an alternative approach. One may need to choose species in which polyploidization and functional divergence of paralogous genes have occurred, but sufficiently recently that regulatory regions can be compared. Similarly, one might compare closely related species, one of which has

undergone polyploidy, in order to understand how recently duplicated paralogues share ancestral functions and gain new ones. Combining such comparative genomic and developmental approaches, one small step at a time, may be the clearest way to identify the gain of novel gene functions during vertebrate evolution.

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