REVIEW / SYNTHÈSE

Amphioxus molecular biology: insights into vertebrate evolution and developmental mechanisms[1]

Sebastian M. Shimeld and Nicholas D. Holland

Abstract: The cephalochordate amphioxus is the best available proxy for the last common invertebrate ancestor of the vertebrates. During the last decade, the developmental genetics of amphioxus have been extensively examined for insights into the evolutionary origin and early evolution of the vertebrates. Comparisons between expression domains of homologous genes in amphioxus and vertebrates have strengthened proposed homologies between specific body parts. Molecular genetic studies have also highlighted parallels in the developmental mechanisms of amphioxus and vertebrates. In both groups, a similar nested pattern of *Hox* gene expression is involved in rostrocaudal patterning of the neural tube, and homologous genes also appear to be involved in dorsoventral neural patterning. Studies of amphioxus molecular biology have also hinted that the protochordate ancestor of the vertebrates included cell populations that modified their developmental genetic pathways during early vertebrate evolution to yield definitive neural crest and neurogenic placodes. We also discuss how the application of expressed sequence tag and gene-mapping approaches to amphioxus have combined with developmental studies to advance our understanding of chordate genome evolution. We conclude by considering the potential offered by the sequencing of the amphioxus genome, which was completed in late 2004.

Résumé : L'amphioxus, un céphalochordé, est le meilleur modèle de remplacement disponible pour représenter le dernier invertébré à servir d'ancêtre commun des vertébrés. Au cours de la dernière décennie, on a beaucoup étudié la génétique du développement d'amphioxus à la recherche de perspectives nouvelles sur l'origine évolutive des vertébrés et sur le début de leur évolution. La comparaison des domaines d'expression des gènes homologues chez l'amphioxus et les vertébrés a appuyé les homologies proposées pour les différentes structures spécifiques du corps. Les études de génétique moléculaire ont aussi mis en lumière les parallèles qui existent entre les mécanismes du développement chez l'amphioxus et chez les vertébrés. Chez les deux groupes, un pattern emboîté semblable de l'expression du gène Hox est impliqué dans la structuration de la région rostrocaudale du tube neural et des gènes homologues semblent aussi être impliqués dans la structuration neurale dorsoventrale. De plus, des études de la biologie moléculaire de l'amphioxus laissent croire que l'ancêtre ptotochordé des vertébrés possédait des populations de cellules qui ont modifié leur voie génétique de développement durant la première évolution des vertébrés pour produire une véritable crête neurale et des placodes neurogènes. L'application des techniques de marqueurs de séquence exprimée et de cartographie génique à l'amphioxus combinée aux études sur le développement ont permis une meilleure compréhension de l'évolution du génome des chordés. Enfin, le séquençage du génome de l'amphioxus qui a été complété à la fin de 2004 offre un potentiel intéressant.

[Traduit par la Rédaction]

Received 30 March 2004. Accepted 17 November 2004. Published on the NRC Research Press Web site at http://cjz.nrc.ca on 16 April 2005.

S.M. Shimeld.[2,3] School of Animal and Microbial Sciences, University of Reading, Reading RG6 6AJ, UK.
N.D. Holland. Marine Biology Research Division, Scripps Institution of Oceanography, University of California San Diego, La Jolla, CA 92093-0202, USA.

[1]This review is one of a series dealing with aspects of the biology of the Protochordata. This series is one of several virtual symposia on the biology of neglected groups that will be published in the Journal from time to time.
[2]Corresponding author (e-mail: Sebastian.Shimeld@zoo.ox.ac.uk).
[3]Present address: Department of Zoology and Fellow of Balliol College, University of Oxford, The Tinbergen Building, South Parks Road, Oxford OX1 3PS, UK.

 doi: 10.1139/Z04-155

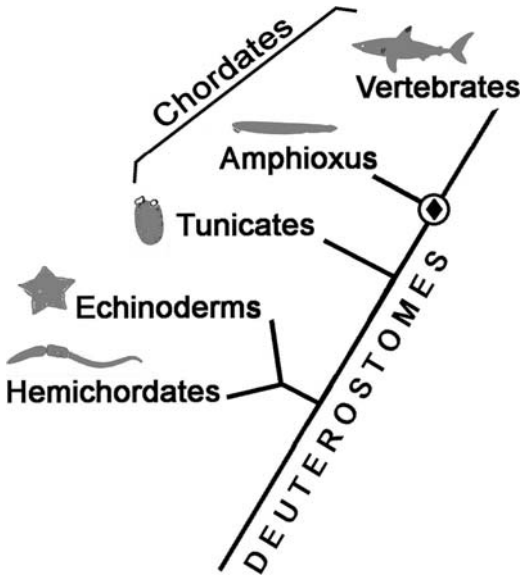
Introduction

The evolution of vertebrates from invertebrates is especially interesting because it is one of the big origin questions (cosmos, universe, life on Earth, etc.) that help us ascertain our place in nature (Huxley 1895). The identity of our last common invertebrate ancestor was a contentious subject during the 19th and much of the 20th century, when almost every invertebrate phylum was proposed, at one time or another, to be immediately ancestral to the vertebrates (Gee 1996). In recent decades, however, advances in molecular biology have progressively narrowed the field of contenders until amphioxus (also called the lancelet) has come to be widely accepted as the best available stand-in for the proximate invertebrate ancestor of all vertebrates. This winnowing has been helped most by the elucidation of gene family trees (Holland 2000; Ruvinsky et al. 2000; Schubert et al. 2000) and by the sequence-based restructuring of the phylogenetic tree of animal life (Winchell et al. 2002).

Figure 1 shows the phylogenetic arrangement of amphioxus, vertebrates, and other major groups within the deuterostome branch of the animal kingdom. The sister-group relation of amphioxus and vertebrates, by itself, permits two hypotheses about the common ancestor (diamond in Fig. 1): either (1) Dohrn's idea that the ancestor was vertebrate-like and degenerated into amphioxus in one line of descent or (2) Haeckel's view that the ancestor was amphioxus-like and became more complex in the vertebrate line of descent (reviewed in Stokes and Holland 1998). By now, however, the notion of amphioxus as a degenerate vertebrate has firmly been countered by data showing that the number of genes per gene family is markedly lower in amphioxus than in vertebrates. Such a pattern is readily explicable if small gene families in an amphioxus-like common ancestor enlarged by gene, chromosome, or genome duplications during early vertebrate evolution, but not in the amphioxus line of descent. The converse idea, that a large number of vertebrate genes in a vertebrate-like ancestor was reduced in the amphioxus lineage, would require a very improbable combination of gene removal events (Holland 2000).

Convincing fossils that closely approximate the last common invertebrate ancestor of the vertebrates have not yet come to light. Although the late-Cambrian *Pikaia gracilens* (Walcott, 1911) from the Canadian Burgess shale is widely reputed to be the ancestor of the vertebrates; this reputation is not justified by available data (Holland and Chen 2001). Similarly, chordate fossils recently described from the Chengjiang formation of the early Cambrian in south China, although of great significance, do not approximate an amphioxus-like creature particularly closely. One such fossil (*Haikouichthys ercaicunensis* Luo, Hu, and Shu, 1999) is almost certainly a vertebrate, and another (*Haikouella lanceolata* Chen, Huang, and Li, 1999) has been alternatively interpreted as either a vertebrate (Mallatt and Chen 2003) or a very basal deuterostome (Shu 2003). The absence of unequivocally amphioxus-like fossils from the early Palaeozoic makes it problematic to decide whether some amphioxus features are primitive or derived within chordates. Amphioxus and vertebrates have been evolving separately since they diverged from their common ancestor half a billion years ago, which is ample time for independent gain and (or) loss of characters.

Fig. 1. Phylogenetic relationships among the deuterostomes. The node marked with the diamond is the hypothetical last common ancestor of amphioxus and vertebrates.



Our present purpose is to review how recent advances in molecular biology have had an impact on ideas about chordate phylogeny and developmental mechanisms. Special emphasis is given to how the complete sequencing of the amphioxus genome (Gibson-Brown et al. 2003), completed in late 2004, can be expected to provide even deeper insights into vertebrate evolution and development at the dawn of chordate evolution.

Utility of developmental genetic data for assessing body-part homologies between amphioxus and vertebrates

The underlying logic of using molecular genetic data for hypothesising body-part homologies between distantly related animals is based on the remarkable conservation of developmental gene structure, as well as on similarities in the molecular machinery directing development over a wide spectrum of the animal kingdom. Many developmental genes encode proteins with highly conserved amino acid domains that can be used to identify homologous genes from different organisms (on this virtually everyone agrees). In addition, the developmental expression domains of homologous genes tend to be conserved in different kinds of animals. These conserved expression domains help suggest body-part homologies between distantly related species (without qualification, this statement is by no means acceptable to everyone, so some explanation is in order here). The chief problems with this approach are that a given gene may be used at more than one time and placed in the development of the same embryo, and there are unequivocal examples of dissociation between genotype and phenotype (Tautz 1998). Both of these problems are serious when one attempts to infer homologies between organisms with highly divergent body plans (e.g., when one tries to reconstruct a hypothetical an-

cestor for all bilaterian animals mainly on the basis of molecular genetic data). In contrast, as discussed in Holland and Holland (1999), these problems are much less critical when comparisons are between organisms with similar body plans, such as amphioxus and vertebrates.

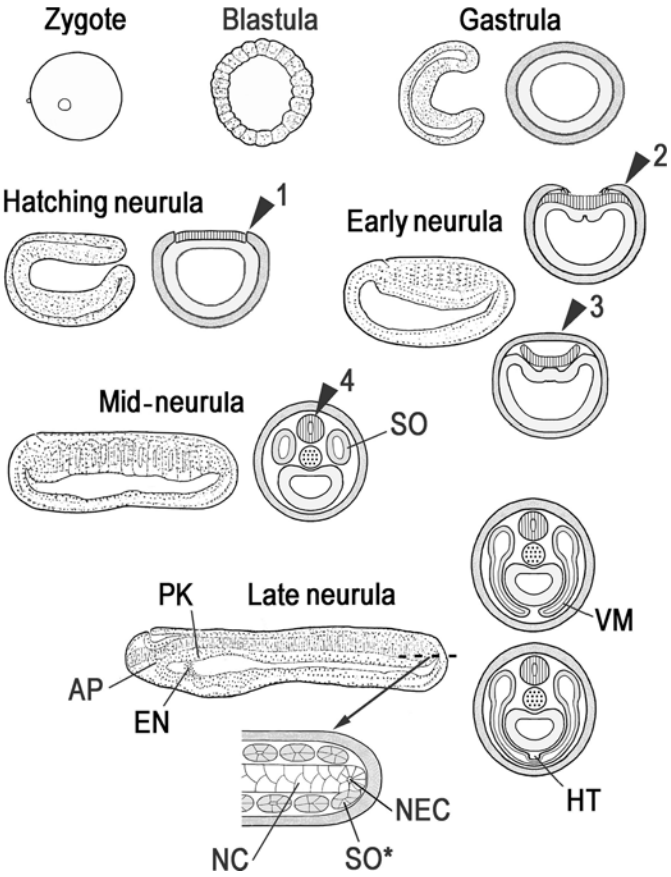
This section considers several comparisons between amphioxus and vertebrate structures that have been assessed with relatively extensive gene expression data. However, it should also be mentioned that more limited expression data have also given insights into many additional body-part homologies between amphioxus and vertebrates. Examples include the heart (Fig. 2, HT; Holland et al. 2003), pronephric kidney (Fig. 2, PK; Kozmik et al. 1999), frontal and pineal eyes (Glardon et al. 1997), gill slits (Holland et al. 1995*a*; Kozmik et al. 1999), notochord (Fig. 2, NC; Holland et al. 1995*b*; Shimeld 1997), tail-bud tissues associated with the neurenteric canal (Fig. 2, NEC; Schubert et al. 2001; Horton and Gibson-Brown 2003), adenohypophysis (Yasui et al. 2000; Boorman and Shimeld 2002; Wang et al. 2002), and muscular somites (Fig. 2, SO; Minguillón and Garcia-Fernàndez 2002; Gostling and Shimeld 2003; Minguillón et al. 2003; Schubert et al. 2003).

Proposed homology between the amphioxus endostyle and the vertebrate thyroid gland

In embryonic and larval amphioxus, the endostyle is a glandular portion of the endoderm on the right side of the pharynx (Fig. 2, EN). During metamorphosis, the endostyle is remodelled into a midventral pharyngeal groove. Although the amphioxus endostyle has no known endocrine functions (it produces food-capturing mucoidal secretions), its homology with the vertebrate thyroid gland was first proposed in Müller (1873) and has been gaining support ever since. Both structures derive from the pharynx, both metabolise iodine to form iodothyronines, and both synthesise similar thyroglobulins. Moreover, in developing lampreys, there is a transition between an endostyle-like structure in the larva and a definitive vertebrate thyroid gland in the adult (references in Holland and Holland 1999).

In addition there are striking parallels between genes directing vertebrate thyroid development and those directing amphioxus endostyle development. *Pax-8* is a thyroid marker in vertebrates (Plachov et al. 1990), and its amphioxus homolog (*AmphiPax-2/5/8*) is an endostyle marker in amphioxus (Kozmik et al. 1999). Similarly, the vertebrate thyroid marker *Nk2.1* (also called *TTF-1*, *thyroid transcription factor-1*; Lazzaro et al. 1991) has an amphioxus homolog (*AmphiNk2.1*) that is expressed in the developing endostyle (Venkatesh et al. 1999). In addition, the thyroid-specific thyroperoxidase gene is well conserved between vertebrates (Kimura et al. 1989) and amphioxus (Ogasawara 2000). Finally, *FoxE1* (also called *TTF-2*, *thyroid transcription factor-2*) is a vertebrate thyroid marker (Zannini et al. 1997) with an amphioxus homolog (*AmphiFoxE4*) that is expressed, if not in the endostyle itself, at least immediately adjacent to it in the enigmatic endodermal club-shaped gland (Yu et al. 2002*a*). The anomalous expression domain of *AmphiFoxE4* might be resolved if, at larval metamorphosis, part of the club-shaped gland were to be incorporated into the definitive adult endostyle, as tentatively suggested in Olsson (1983); this idea needs to be tested by a detailed study of pharyngeal remodelling during metamorphosis. In summary, data from recent gene expression studies have provided robust support for the homology between the amphioxus endostyle and the vertebrate thyroid gland.

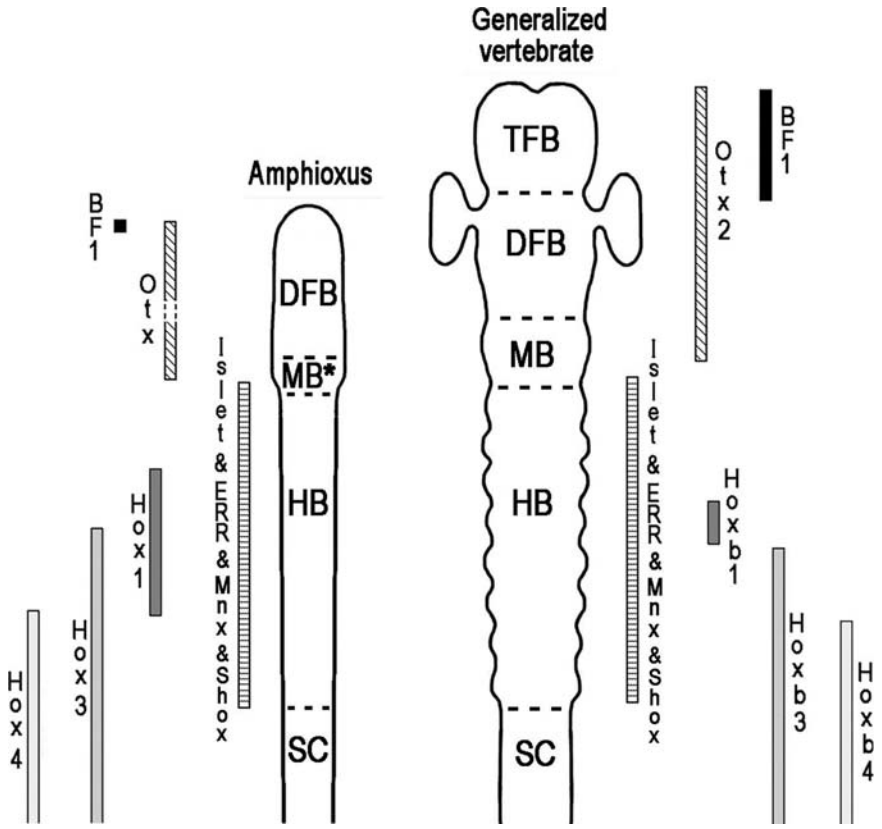
Fig. 2. Diagram of amphioxus embryology. The gastrula and neurula stages are shown in side and cross-sectional views (left and right of each pair, respectively). The broken line on the side view of the late-neurula stage indicates the level of the frontal section (marked by the arrow). Arrowheads 1–3 show migration of the non-neural ectoderm across the neuroectoderm (first phase of neurulation), and arrowheads 3 and 4 indicate the curling up of the neural plate into the neural tube (second phase of neurulation). During the mid-neurula stage, somites (SO) and notochord (stippled) arise from the gut by evagination and upfolding, respectively. For the late-neurula stage, the approximate positions of the endostyle (EN), pronephric kidney (PN), and rudiment of the possible adenohypophysis (AP) are indicated near the anterior end; the frontal section of the tail-bud region shows the notochord (NC), the neurenteric canal (NEC), and a nascent somite (SO*); the cross sections show the ventral mesoderm (VM) evaginating down from the somites and the formation of the heart tube (HT) midventrally between the gut and the visceral mesothelium.



Major anteroposterior subdivisions of the central nervous system: homologies between amphioxus and vertebrates

Beginning in the 19th century, there were three conflicting schools of thought about the central nervous system of amphioxus and, by extension, the brain of the proximate in-

Fig. 3. The anterior end of the developing central nervous systems of amphioxus and a generalised vertebrate in dorsal view with anterior toward the top. Abbreviations for brain regions are as follows: TFB, telencephalic forebrain; DFB, diencephalic forebrain; MB, midbrain (the asterisk indicates relatively weak support); HB, hindbrain; and SC, spinal cord. The anteroposterior expression domains of some key neural marker genes are shown (amphioxus genes to left and vertebrate genes to the right). Orthologous genes have matching textures; the diagram does not indicate that the *Shox*, *Islet*, *ERR*, and *Mnx* genes are expressed in periodic patterns in the hindbrain of amphioxus and vertebrates. The gene expression domains shown in this figure are taken from the following references: *Otx* (Williams and Holland 1996), *BF1* (Torreson et al. 1998), *Islet* (Jackman et al. 2000), *ERR* (P.-L. Bardet, personal communication), *Mnx* (Ferrier et al. 2001), *Shox* (Jackman and Kimmel 2002), *Hox3* (Holland et al. 1992), *Hox1* (Holland and Garcia-Fernàndez 1996), and *Hox4* (Wada et al. 1999).



vertebrate ancestor of the vertebrates: Huxley (1874) believed that the amphioxus brain was relatively large, Steida (1873) thought that it was quite small, and Schafer (1880) thought that it was completely absent. In recent years, studies of amphioxus neuroanatomy and developmental genetics have tended to support each other and support Huxley's suggestion that the amphioxus brain is a relatively large structure. Because Wicht and Lacalli (2005) cover amphioxus neuroanatomy, we will limit our discussion to studies of developmental gene expression along the anteroposterior and dorsoventral axes of the amphioxus central nervous system.

Numerous studies of developmental gene expression have been used to elucidate the anteroposterior regionalisation of the amphioxus central nervous system (Fig. 3). Combined, they strongly suggest that the dorsal hollow nerve cord of amphioxus, from anterior to posterior, comprises a diencephalic forebrain, a midbrain, a hindbrain, and a spinal cord. In other words, the telencephalic forebrain appears to be an evolutionary novelty that arose in early vertebrates. Although many of the amphioxus neural genes that have been studied fit well into this picture, some do not. A first example is amphioxus *Krox*, which is expressed in the developing forebrain and hindbrain (Knight et al. 2000; Jackman and Kimmel 2002) in patterns with no clear counterpart of the two persistent and distinct *Krox* expression stripes that are often used for landmarks in the developing vertebrate hindbrain. A second example is amphioxus *engrailed*, which is likewise expressed in the developing forebrain and hindbrain (Holland et al. 1997), but not at the midbrain–hindbrain boundary, a well-known expression domain of its vertebrate homologues. Because *engrailed* is expressed in the presumptive midbrain–hindbrain region of developing tunicates, the absence of an *engrailed* landmark may be a derived feature in amphioxus (Jiang and Smith 2002). In any event, current microanatomical and developmental genetic studies indicating the presence of a distinct midbrain in amphioxus are more suggestive than conclusive. However, even if, as seems likely, a midbrain is present, there are currently no indications that this region of the amphioxus neural tube acts as an anteroposterior organiser in the manner of the vertebrate midbrain–hindbrain junction (Williams and Holland 1998; Kozmik et al. 1999).

Conservation and divergence of the anteroposterior patterning mechanisms of chordate neural tubes

In vertebrates, mesodermal and posterior neural identity is

regulated by collinear *Hox* gene expression. *Hox* genes are expressed throughout the spinal cord and most of the hindbrain in a nested pattern, and at least three upstream regulators of *Hox* patterning have been identified: retinoic acid (RA), the *Cdx* homeobox genes, and FGF signalling. Studies have shown that most, if not all, of this basic molecular machinery is present in amphioxus. *Hox* gene expression in the amphioxus neural tube is collinear, with a two-segment periodicity that matches both the adjacent somites and the repetitive positioning of neuronal types marked by the expression of genes such as *Islet* and *Mnx* (Wada et al. 1999; Jackman et al. 2000; Ferrier et al. 2001). *Hox* expression is also regulated by RA in amphioxus so that the application of exogenous RA causes an anterior shift in the expression of *Hox1* (Holland and Holland 1996). Furthermore, while the expression and role of FGFs in amphioxus is currently unknown, the amphioxus *Cdx* ortholog, *AmphiCdx*, is posteriorly expressed in a manner compatible with the collinear regulation of *Hox* expression (Brooke et al. 1998).

These data suggest that the regulation of neural identity in the posterior nervous system is under the control of mechanisms similar to those in vertebrates. As discussed above, anterior neural tissue is marked by genes with expression patterns conserved between amphioxus and vertebrates (Fig. 3). In vertebrate embryos, early anterior development is regulated by cell populations adjacent to the anterior nervous system, including endoderm and prechordal mesoderm, although the precise roles of these tissues appear to vary between different vertebrate species (de Souza and Niehrs 2000). Whether such systems also operate in amphioxus is unknown, although in the gastrula and early neurula anterior endodermal tissue and presumptive anterior notochord are in close proximity to anterior neural tissue.

Despite some clear similarities there are also fundamental differences between the anteroposterior organisation of the amphioxus and vertebrate central nervous systems. These include the apparent absence from amphioxus (as described above) of the telencephalon and midbrain–hindbrain boundary, and the presence in vertebrates of overt hindbrain segmentation in the form of rhombomeres. Much has been learned about the developmental mechanisms of rhombomere segmentation in the vertebrate hindbrain (e.g., see Dupe and Lumsden 2001), and developmental gene expression in the amphioxus neural tube has been analyzed by comparison with these vertebrate studies. These comparisons suggest three key regulative differences between amphioxus and vertebrate neural development. First, the vertebrate midbrain–hindbrain boundary has an important organiser role in regulating anterior hindbrain segmentation, and the absence of a comparable midbrain organiser (as discussed above) correlates with the lack of morphologically obvious segmentation in amphioxus. Second, vertebrate *Hox* gene expression, under the control of RA synthesised by anterior somites, has an important role in regulating posterior rhombomeric segmentation in vertebrate embryos, in addition to its role in regulating segment identity. In amphioxus RA regulates neural identity, but not segmentation, leading Mazet and Shimeld (2002*a*) to propose that vertebrates had evolved a new role for RA and *Hox* in segmentation in addition to a primitive role in specification of anteroposterior identity. Third, critical genes required for establishing vertebrate hindbrain compartments prior to segmentation are not expressed appropriately in the amphioxus nervous system, suggesting that mechanisms downstream of the midbrain–hindbrain boundary and RA/*Hox* patterning also differ between these taxa (Knight et al. 2000; Jackmann and Kimmel 2002). Overall, these studies suggest that although amphioxus and vertebrates share a basic, conserved molecular ground plan in their nervous systems considerable complexity has been superimposed on this primitive state in vertebrates.

Conservation and divergence of dorsoventral organisation of chordate neural tubes

The dorsoventral (DV) axis of vertebrate neural tubes is organised by signals initially emanating from two sources (Liem et al. 1997; Briscoe and Ericson 1999). Ventral pattern is regulated by the floor plate, which lies at the ventral midline of the neural tube. Dorsal pattern is regulated by interactions with adjacent ectoderm. The key molecular components of dorsal and ventral patterning are, respectively, bone morphogenetic protein (BMP) and hedgehog (Hh) signalling. Numerous transcription factor genes lying downstream of these signals are involved in dividing the DV axis of the neural tube into a complex set of sub-domains that reflect the patterned development of multiple neuronal and glial cell types (Briscoe and Ericson 1999). (Note that in an embryological context the floor plate derives from the midline of the neural plate and is the most dorsal neural tissue in the embryo. The floor plate becomes the ventral neural tube by the process of neurulation. A similar, but opposite, reversal occurs for the dorsal neural tube, which prior to neurulation consists of lateral neural plate, and therefore is the most ventral part of the neural plate. For consistency with existing vertebrate developmental literature, we will continue to describe the floor plate as ventral).

In amphioxus larvae and adults there is clear DV organisation of cell types (Bone 1959, 1960; Wicht and Lacalli 2005), and this is reflected by similarities in the deployment of patterning genes in amphioxus embryos. At the ventral midline of the amphioxus neural tube lies a specialised set of glial cells (Lacalli et al. 1994), and in embryos these express genes that in vertebrates are expressed by floor-plate cells, including *HNF3/FoxA*, *Netrin*, and the ventral patterning gene *hedgehog* (Shimeld 1997, 1999, 2000). Similarly, amphioxus dorsal neural cells are potentially exposed to BMP signalling from adjacent ectoderm in which the *Bmp-2/Bmp-4* ortholog *AmphiBmp-2/4* is expressed, although amphioxus dorsal neural cells do not subsequently acquire expression of this gene family as do vertebrate dorsal neural cells (Panopoulou et al. 1998). These studies show that at least initially the amphioxus neural tube is exposed to the same extracellular signalling molecules as the vertebrate neural tube. Downstream of these signals, both similarities and differences between amphioxus and vertebrates have been uncovered. Similarities include the expression of genes such as *Msx*, *Dlx* and *Snail* by dorsolateral neural cells, and of motor-neuron markers such as *Islet* and *Mnx* by prospective neurons (Holland et al. 1996; Langeland et al. 1998; Sharman et al. 1999; Jackman et al. 2000; Ferrier et al. 2001). The similar expression patterns of these genes in

amphioxus and vertebrates clearly suggests that many aspects of patterning downstream of Hh and Bmp are conserved between these two groups of animals. A key difference is in the patterning of dorsal cells in the two taxa, particularly with regard to the development of neural crest cells in vertebrates. The neural crest is of such importance to vertebrate development that we consider this in detail below, in combination with ectodermal placodes, the development and subsequent differentiation of which is intimately associated with the neural crest.

Possible evolutionary precursors of definitive neural crest and placodes in amphioxus

Amphioxus, and by extension the proximate ancestor of all vertebrates, lacks the definitive neural crest, which is a quintessential feature of vertebrates (Gammill and Bronner-Fraser 2003). Even so, amphioxus has a cell population with properties (location, behaviour, and gene expression) which indicate that it represents the likely evolutionary source of definitive neural crest in vertebrates. This is an instance when it can be difficult to draw the line between homology and non-homology (our personal preference is to avoid the term homology here). As Braun and Northcutt (1997) have pointed out, "Morphological structures, and the ontogenies which produce them, do not simply arise from the dust of the earth…". Under this scenario, the protochordate ancestor of the vertebrates included a population of cells that modified their developmental genetic pathways early in vertebrate evolution to yield a characteristic end product, the definitive neural crest. The evidence for this is reviewed extensively in Holland and Holland (2001) and will only be briefly summarised and updated here.

During vertebrate development, the neural crest arises from cells located along the edges of the neural plate, and possibly from some immediately contiguous cells of the non-neural ectoderm. After these cells are internalised by neurulation, they migrate, mostly as individuals, within the embryonic body and differentiate into a wide variety of cell types that contribute to connective tissue, the peripheral and enteric nervous systems, melanocytes, etc. Nothing so dramatic happens during amphioxus development, but cells associated with the neural plate/non-neural ectoderm boundary do migrate during neurulation. The amphioxus neural plate separates at its edges (Fig. 2, arrowhead 1) from the non-neural ectoderm. The leading cells of the latter then develop lobopodia, crawl across the neural plate (Fig. 2, arrowhead, 2), and finally meet and fuse in the dorsal midline of the embryo (Fig. 2, arrowhead 3). Importantly, these migrating cells of amphioxus never move into the interior of the embryo and remain epidermal, evidently never differentiating into cell types other than the dorsal neurectoderm.

In developing amphioxus, the location of cells at the edge of the neural plate and their subsequent migration correspond in some ways to those of definitive vertebrate neural crest cells. However, these similarities become even more interesting in light of gene expression data. The amphioxus cells in question express a suite of genes whose homologs in vertebrates are key markers of premigratory and migratory neural crest cells. These genes include *AmphiDll* (Holland et al. 1996), amphioxus *snail* (Langeland et al. 1998), *AmphiPax3/7* (Holland et al. 1999), and *AmphiSox1/2/3* (Holland et al. 2000). The partially overlapping suite of genes expressed at the neural plate boundary of amphioxus and vertebrates suggests that comparably located cells in the protochordate ancestor had at least some of the genetic machinery needed for development of definitive neural crest.

These gene expression data raise the question of what additional genetic machinery is needed to be added to this basic platform to enable such cells to migrate within the embryo and differentiate into various cell types. Special interest, therefore, attaches to genes that are expressed in vertebrate neural crest cells, but not at the edge of the amphioxus neural plate. Such genes include amphioxus *twist* (Yasui et al. 1998), amphioxus *AP2* (Meulemans and Bronner-Fraser 2002), *AmphiFoxD* (Yu et al. 2002*b*), *AmphiZic* (Gostling and Shimeld 2003), and amphioxus *id* (Meulemans et al. 2003). Evidently, developmental gene networks including such genes were recruited to cells at the edge of the neural plate early in vertebrate evolution. The recruitment of novel gene activities was presumably enabled by the advent of new relationships among regulatory elements in the DNA. It is also likely that the conspicuous gene duplications during early vertebrate evolution facilitated such recruitment, because the resulting partial redundancy of gene functions among descendant paralogues presumably favoured their survival, and the evolution of novel regulatory elements (reviewed by Mazet and Shimeld 2002*b*).

In addition to neural crest, ectodermal placodes are also commonly considered to be a vertebrate innovation. However, as Begbie and Graham (2001) have observed, there are many kinds of placodes, and at least some of them (e.g., the olfactory placodes) may have had counterparts in the protochordate ancestor of the vertebrates. Placodes may be non-neurogenic (e.g., anlagen of the adenohypophysis, lens, and teeth) or neurogenic, and only the latter are considered in the present section. In vertebrate embryos, neurogenic placodes are first detected as thickened areas of ectoderm. Some placodal cells subsequently migrate into the embryo to become components of cranial ganglia, and others differentiate superficially to help form chemoreceptors (e.g., olfactory epithelium) or mechanoreceptors, most characteristically the neuromasts in the lateral line of anamniote vertebrates (Webb and Noden 1993). In amphioxus embryos, the earliest (type 1) sensory cells to differentiate are primary neurons, that is the cell body remains part of the epidermis but projects an axon into the central nervous system (reviewed by Holland and Yu 2002). Unfortunately, so far there have been no conclusive physiological studies of the sensory modalities of these cells.

In the larval epidermis of amphioxus, all of the type 1 sensory cells appear very similar to one another structurally, although their sensory modalities and central axonal connections might be diverse. Thus, it is possible that type 1 sensory cells in the anterior region of the body are comparable with the placodally derived olfactory neurons of vertebrates, which are also primary neurons. Some of the anterior epidermal cells of amphioxus express *AmphiPax6* (Glardon et al. 1997), a homolog of a general placode marker in vertebrates, as well as *AmphiMsx* (Sharman et al. 1999) and *AmphiNeurogenin* (Holland et al. 2000), which are more specific markers for vertebrate olfactory placodes. Comparisons between amphioxus type 1 sensory cells and the non-

olfactory, neurogenic placodes of vertebrates may be less firmly grounded, because epidermal sensory cells differentiating from the latter are secondary neurons, that is the epidermally located cell body lacks an axon and synapses basally with deeper elements of the nervous system. Moreover, Mazet et al. (2004) have recently demonstrated an amphioxus *Coe/Ebf* gene expressed in a subset of differentiating neurons all along the flanks of the body. Vertebrate homologues of this amphioxus gene are primarily expressed in the nervous system, including the olfactory placodes and in other differentiating sensory neurons (Dubois and Vincent 2001). Mazet et al. (2004) suggested that similar epidermal sensory cells in the last common invertebrate ancestor of the vertebrates might, at least in part, have been a source of neurogenic placodes during early vertebrate evolution.

The future of amphioxus as a developmental and evolutionary model: the role of genomics and post genomics

The sections above compare some key amphioxus and vertebrate structures, and summarise what has been learned about the molecular mechanisms regulating their development. We now consider the future prospects for amphioxus as a model system for the investigation of evolutionary and developmental biology. To date, there have been few direct, manipulative investigations of amphioxus development when compared with, for example, ascidians (see Cone and Zeller 2005), and consequently, much of our understanding of the molecular control of amphioxus development is inferred by examination of gene expression patterns and comparison with other taxa. This reflects the comparative difficulty of both obtaining and manipulating amphioxus embryos, although recent advances in manipulative techniques suggest that this bottleneck has now been passed (L.Z. Holland, unpublished data). An additional asset to such functional studies is provided via comparative genomics. Recent studies in the comparative genomics of amphioxus have addressed gene complement via expressed sequence tag (EST) screens and gene organisation via gene mapping.

EST studies of amphioxus

Two recent studies have used EST surveys to address questions of the gene complement in amphioxus and of gene duplication during vertebrate evolution. Mou et al. (2002) sequenced 5235 ESTs from the East Asian amphioxus *Branchiostoma belcheri* (Gray, 1847). The sequenced transcripts, which were derived from a neurula stage cDNA library, provided insight into the genes expressed at this developmental stage and identified amphioxus genes similar to vertebrate genes for which invertebrate homologs had not previously been identified (Mou et al. 2002). Panopoulou et al. (2003) sequenced 14 189 ESTs from the Florida amphioxus *Branchiostoma floridae* Hubbs, 1922. These ESTs were selected for sequencing on the basis of initial fingerprinting by oligonucleotide hybridisation, and as such contain less redundancy than would be expected from random sequencing. Analyses of these data were used to address the extent and timing of gene duplication in early vertebrate evolution, with the results compatible with the presence of at least one genome duplication in early vertebrate history (Panopoulou et al. 2003) and consistent with independent analyses (Furlong and Holland 2003; Horton et al. 2003).

EST studies are essentially non-targeted, in that they do not specifically examine a particular type of gene. In an amphioxus molecular developmental context, their utility is realised in two ways. First, they provide an invaluable resource of genes for molecular studies. Second, they can provide definitive evidence of the presence of a specific gene or gene family member in amphioxus, and in this context have proven a powerful base for molecular phylogenetic studies of vertebrate genes (Panopoulou et al. 2003). They are limited, however, in that they cannot prove that a gene is absent from the amphioxus genome.

Gene organisation in amphioxus

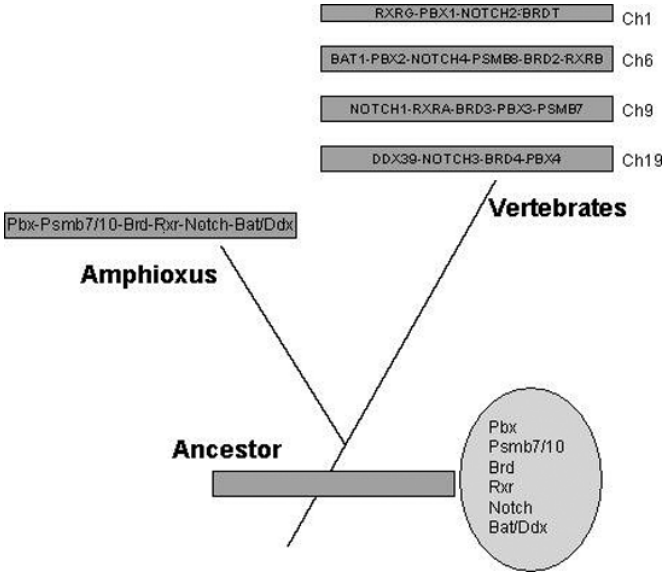
To date, studies of gene organisation in amphioxus have focused on two areas; the clustering of families of related genes and the relative positions of amphioxus genes whose orthologs are linked in vertebrate genomes. Clusters of related genes in amphioxus include the *Hox*, *ParaHox*, and *Nkx* homeobox genes (Garcia-Fernàndez and Holland 1994; Brooke et al. 1998; Luke et al. 2003). All these represent ancient gene clusters that have been preserved to varying degrees within the amphioxus lineage, and establishing their organisation has been a key step in unravelling the evolutionary history of vertebrate homeobox gene clusters. Furthermore, the expression of many members of these gene families in amphioxus embryos has been established. This has suggested a role for *Hox* genes in the development of anteroposterior organisation of the nervous system (see above; Wada et al. 1999), for *ParaHox* genes in the development of the gut and nervous system (Brooke et al. 1998), and for *Nkx* genes in the development of a variety of endodermal and mesodermal tissues (Holland et al. 1998, 2003; Venkatesh et al. 1999; Kozmik et al. 2001).

Recently, the application of fluorescent in situ hybridisation to chromosomes has been extended to include amphioxus (Castro and Holland 2002). This has allowed the relative positions of more distantly distributed genes to be determined, as it does not rely on the cloning of intervening genomic DNA to establish that two genes are physically linked. Castro and Holland (2003) used this technique to establish long-range linkage between several homeobox genes in the genome of the amphioxus *B. floridae*. Similarly, Castro et al. (2004) addressed the evolutionary origin of the MHC region on human chromosome 6, which has three paralagous regions found on chromosomes 1, 9, and 19, respectively. Using fluorescent in situ hybridisation, they showed that six amphioxus genes orthologous to genes from these vertebrate paralogy regions mapped to the same amphioxus chromosomal region (Fig. 4). This indicates that the vertebrate paralogy regions evolved by block duplication from a single ancestral chromosomal region, and furthermore, demonstrates that this region has remained relatively unchanged in amphioxus.

The future of amphioxus genomics

We have provided an indication of the current use of genome-level studies for investigating amphioxus biology. It is likely such applications will expand and be complimented by whole-genome sequencing (Gibson-Brown et al. 2003), a

Fig. 4. Evolution of the chordate MHC-linked chromosomal region, after Castro et al. (2004). In vertebrates, paralogs of several gene families (*Notch*, *Rxr*, *Brd*, *Psmb*, *Pbx*, *Bat/Ddx*) are found in four chromosomal regions associated with the MHC, as represented by human chromosomes 1, 6, 9, and 19. In amphioxus, single orthologs of these genes map to the same chromosome. This allows the inference that these chromosomal regions in the two lineages evolved from a single ancestral region in which these genes were localised (though it does not define the order of the genes, which has changed considerably presumably because of local inversions and translocations). In vertebrates this single ancestral region has duplicated to yield four copies.



project that was completed in late 2004. First, whole-genome sequencing will provide a catalogue of genes present in the amphioxus genome. This in turn will help establish when in chordate evolution specific genes and pathways evolved. This is an important issue for understanding amphioxus development and the evolution of vertebrate development, as directed cloning methods vary greatly in success depending upon the gene in question, making the absence of a gene impossible to prove in this manner. Second, the amphioxus genome will represent the best available proxy for the genome of the last common ancestor of the vertebrates. This will provide a baseline for inferring the organisation of the ancestral chordate genome and give important new insights into genome evolution during early vertebrate evolution. Of key importance in this respect will be the identification of regions of synteny between amphioxus and vertebrate genomes. A crucial question in vertebrate evolution concerns the extent and type of gene duplications inferred to have occurred in early vertebrate evolution, with a popular hypothesis suggesting that one or more genome duplications occurred during this time. Such genome duplications would form a genomic legacy carried by all subsequently diverging vertebrate lineages and would have profound implications for vertebrate evolution. Comparison of synteny maps between amphioxus and vertebrates is perhaps the only way that such a hypothesis can be conclusively tested (Horton et al. 2003). In retrospect, over a decade of molecular investigations into amphioxus development has yielded a wealth of data, providing considerable insight into the basic molecular ground plan of chordates, and how this has evolved in the vertebrate lineage. Future studies will soon extend this to the level of the genome, providing a comprehensive view of the genomic and developmental changes that separate us from our closest invertebrate relative.

Acknowledgements

S.M.S. thanks the Biotechnology and Biological Sciences Research Council, Medical Research Council, and Royal Society for their support, and N.D.H. thanks the US National Science Foundation (research grant IBN-00-78599). We are indebted to Linda Z. Holland and Jeremy Gibson-Brown for critical reading of the manuscript.

References

Begbie, J., and Graham, A. 2001. The ectodermal placodes: a dysfunctional family. Philos. Trans. R. Soc. Lond. B Biol. Sci. No. 356. pp. 1655–1660.

Bone, Q. 1959. The central nervous system in larval acraniates. Q. J. Microsc. Sci. **100**: 509–527.

Bone, Q. 1960. The central nervous system in amphioxus. J. Comp. Neurol. **115**: 27–64.

Boorman, C.J., and Shimeld, C.J. 2002. Pitx homeobox genes in *Ciona* and amphioxus show left-right asymmetry is a conserved chordate character and define the ascidian adenohypophysis. Evol. Dev. **4**: 354–365.

Braun, C.B., and Northcutt, R.G. 1997. The lateral line system of hagfishes Craniata: Myxinoidea. Acta Zool. (Stockh.), **78**: 247–268.

Briscoe, J., and Ericson, J. 1999. The specification of neuronal identity by graded hedgehog signalling. Semin. Cell Dev. Biol. **10**: 353–362.

Brooke, N.M., Garcia-Fernandez, J., and Holland, P.W.H. 1998. The ParaHox gene cluster is an evolutionary sister of the Hox gene cluster. Nature (Lond.), **392**: 920–922.

Castro, L.F., and Holland, P.W. 2003. Chromosomal mapping of ANTP class homeobox genes in amphioxus: piecing together ancestral genomes. Evol. Dev. **5**: 459–465.

Castro, L.F.C., and Holland, P.W.H. 2002. Fluorescent in situ hybridization to amphioxus chromosomes. Zool. Sci. (Tokyo), **19**: 1349–1353.

Castro, L.F., Furlong, R.F., and Holland, P.W.H. 2004. An antecedent of the MHC-linked genomic region in amphioxus. Immunogenetics, **55**: 782–784.

Cone, A.C., and Zeller, R.W. 2005. Using ascidian embryos to study the evolution of developmental gene regulatory networks. Can. J. Zool. **83**(1): 75–89.

de Souza, F.S.J., and Niehrs, C. 2000. Anterior endoderm and head induction in early vertebrate embryos. Cell Tissue Res. **300**: 207–217.

Dubois, L., and Vincent, A. 2001. The COE — Collier/Olf1/EBF — transcription factors: structural conservation and diversity of developmental functions. Mech. Dev. **108**: 3–12.

Dupe, V., and Lumsden, A. 2001. Hindbrain patterning involves graded responses to retinoic acid signaling. Development (Camb.), **128**: 2199–2208.

Ferrier, D.E.K., Brooke, N.M., Panopoulou, G., and Holland, P.W.H. 2001. The Mnx homeobox gene class defined by HB9,

MNR2 and amphioxus AmphiMnx. Dev. Genes Evol. **211**: 103–107.

Furlong, R.F., and Holland, P.W.H. 2003. Were vertebrates octaploid? Philos. Trans. R. Soc. Lond. B Biol. Sci. No. 357. pp. 531–544.

Gammill, L.S., and Bronner-Fraser, M. 2003. Neural crest specification: migrating into genomics. Nat. Rev. Neurosci. **4**: 795–805.

Garcia-Fernàndez, J., and Holland, P.W.H. 1994. Archetypal organization of the amphioxus Hox gene cluster. Nature (Lond.), **370**: 563–566.

Gee, H. 1996. Before the backbone: views on the origin of the vertebrates. Chapman and Hall, London.

Gibson-Brown, J.J., Osoegawa, K., McPherson, J.D., Waterson, R.H., De Jong, P.J., Rokhsar, D.S., and Holland, L.Z. 2003. A proposal to sequence the amphioxus genome. J. Exp. Zool. Mol. Dev. Evol. **300**: 5–22.

Glardon, S., Holland, L.Z., Gehring, W.J., and Holland, N.D. 1997. Isolation and developmental expression of the amphioxus *Pax-6* gene *AmphiPax-6*: insights into eye and photoreceptor evolution. Development (Camb.), **125**: 2701–2710.

Gostling, N.J., and Shimeld, S.M. 2003. Protochordate *Zic* genes define primitive somite compartments and highlight molecular changes underlying neural crest evolution. Evol. Dev. **5**: 136–144.

Holland, L.Z., and Holland, N.D. 1996. Expression of AmphiHox-1 and AmphiPax-1 in amphioxus embryos treated with retinoic acid: insights into evolution and patterning of the vertebrate nerve cord and pharynx. Development (Camb.), **122**: 1829–1838.

Holland, L.Z., and Holland, N.D. 2001. Evolution of neural crest and placodes: amphioxus as a model for the ancestral vertebrate? J. Anat. **199**: 85–98.

Holland, L.Z., Kene, M., Williams, N.A., and Holland, N.D. 1997. Sequence and embryonic expression of the amphioxus engrailed gene *AmphiEn*: the metameric pattern of transcription resembles that of its segment-polarity homolog in *Drosophila*. Development (Camb.), **124**: 1723–1732.

Holland, L.Z., Venkatesh, T.V., Gorlin, A., Bodmer, R., and Holland, N.D. 1998. Characterisation and developmental expression of *AmphiNK2-2*, an NK2 class homeobox gene from amphioxus. Dev. Genes Evol. **208**: 100–105.

Holland, L.Z., Schubert, M., Kozmik, Z., and Holland, N.D. 1999. *AmphiPax3/7*, an amphioxus paired box gene: insights into chordate myogenesis, neurogenesis, and the possible evolutionary precursor of definitive vertebrate neural crest. Evol. Dev. **1**: 153–165.

Holland, L.Z., Schubert, M., Holland, N.D., and Neuman, T. 2000. Evolutionary conservation of the presumptive neural plate markers *AmphiSox1/2/3* and *AmphiNeurogenin* in the invertebrate chordate amphioxus. Dev. Biol. **226**: 18–33.

Holland, N.D., and Chen, J.Y. 2001. Origin and early evolution of the vertebrates: new insights from advances in molecular biology, anatomy, and palaeontology. Bioessays, **23**: 142–151.

Holland, N.D., and Holland, L.Z. 1999. Amphioxus and the utility of molecular genetic data for hypothesizing body part homologies between distantly related animals. Am. Zool. **39**: 630–640.

Holland, N.D., and Yu, J.K. 2002. Epidermal receptor development and sensory pathways in vitally stained amphioxus *Branchiostoma floridae*. Acta Zool. (Stockh.), **83**: 309–319.

Holland, N.D., Holland, L.Z., and Kozmik, Z. 1995*a*. An amphioxus Pax gene, *AmphiPax1*, expressed in embryonic endoderm, but not in mesoderm: implications for evolution of Class 1 paired box genes. Mol. Mar. Biol. Biotechnol. **35**: 206–214.

Holland, N.D., Panganiban, G., Henyey, E.L., and Holland. L.Z. 1996. Sequence and developmental expression of *AmphiDll*, an amphioxus Distal-less gene transcribed in the ectoderm, epidermis and nervous system: insights into evolution of craniate forebrain and neural crest. Development (Camb.), **122**: 2911–2920

Holland, N.D., Venkatesh, T.V., Holland, L.Z., Jacobs, D.K., and Bodmer, R. 2003. *AmphiNk2-tin*, an amphioxus homeobox gene expressed in myocardial progenitors: insights into evolution of the vertebrate heart. Dev. Biol. **255**: 128–137.

Holland, P.W.H. 2000. Embryonic development of heads, skeleton and amphioxus: Goodrich revisited. Int. J. Dev. Biol. **44**: 29–34.

Holland, P.W.H., Holland, L.Z., Williams, N.A., and Holland, N.D. 1992. An amphioxus homeobox gene: sequence conservation, spatial expression during development and insights into vertebrate evolution. Development (Camb.), **116**: 653–661.

Holland, P.W.H., Koschorz, B., Holland, L.Z., and Herrmann, B.G. 1995*b*. Conservation of *Brachyury* (*T*) genes in amphioxus and vertebrates: developmental and evolutionary implications. Development (Camb.), **121**: 4283–4291.

Holland, P.W.H., and Garcia-Fernàndez, J. 1996. Hox genes and chordate evolution. Dev. Biol. **173**: 382–394.

Horton, A.C., and Gibson-Brown, J.J. 2003. Evolution of developmental functions by the Eomesodermin, T-brain1, Tbx21 subfamily of T-box genes: insights from amphioxus. J. Exp. Zool. Mol. Dev. Evol. **294**: 112–121.

Horton, A.C., Mahadevan, N.R., Ruvinsky, I., and Gibson-Brown, J.J. 2003. Phylogenetic analyses alone are insufficient to determine whether genome duplication(s) occurred during early vertebrate evolution. J. Exp. Zool. Mol. Dev. Evol. **299**: 41–53.

Huxley, T.H. 1874. On the classification of the animal kingdom. J. Linn. Soc. Lond. **12**: 199–226.

Huxley, T.H. 1895. Man's place in nature and other anthropological essays. MacMillan, London.

Jackman, W.R., and Kimmel, C.B. 2002. Coincident iterated gene expression in the amphioxus neural tube. Evol. Dev. **4**: 366–374.

Jackman, W.R., Langeland, J.A., and Kimmel, C.B. 2000. Islet reveals segmentation in the amphioxus hindbrain homolog. Dev. Biol. **230**: 16–26.

Jiang, D., and Smith, W.C. 2002. An ascidian *engrailed* gene. Dev. Genes Evol. **212**: 399–402.

Kimura, S., Hong, Y.S., Kotani, T., Ohtaki, S., and Kikkawa, F. 1989. Structure of human thyr0oid peroxidase gene: comparison and relationship to the human myeloperoxidase gene. Biochemistry, **28**: 4481–4489.

Knight, R.D., Panopoulou, G.D., Holland, P.W.H., and Shimeld, S.M. 2000. An amphioxus Krox gene: insights into vertebrate hindbrain evolution. Dev. Genes Evol. **210**: 518–521.

Kozmik, Z., Holland, N.D., Kalousova, A., Paces, J., Schubert, M., and Holland, L.Z. 1999. Characterization of an amphioxus paired box gene, *AmphiPax2/5/8*: developmental expression patterns in optic support cells, nephridium, thyroid-like structures and pharyngeal gill slits, but not in the midbrain–hindbrain boundary region. Development (Camb.), **126**: 1295–1304.

Kozmik, Z., Holland, L.Z., Schubert, M., Lacalli, T.C., Kreslova, J., Vlcek, C., and Holland, N.D. 2001. Characterization of Amphioxus AmphiVent, an evolutionarily conserved marker for chordate ventral mesoderm. Genesis, **29**, 172–9.

Lacalli, T.C., Holland, N.D., and West, J.E. 1994. Landmarks in the anterior central nervous system of amphioxus larvae. Philos. Trans. R. Soc. Lond. B Biol. Sci. No. 344. pp. 165–185.

Langeland, J.A., Tomsa, J.M., Jackman, W.R., and Kimmel, C.B. 1998. An amphioxus snail *gene*: expression in paraxial mesoderm and neural plate suggest a common role in patterning the embryo. Dev. Genes Evol. **208**: 569–577.

Lazzaro, D., Price, M., De Felice, M., and De Lauro, R. 1991. The transcription factor TTF-1 is expressed at the outset of thyroid and lung morphogenesis and in restricted regions of the foetal brain. Development (Camb.), **113**: 1093–1104.

Liem, K.F., Tremml, G., and Jessell, T.M. 1997. A role for the roof plate and its resident TGFB-related proteins in neuronal patterning in the dorsal spinal cord. Cell, **91**: 127–138

Luke, G.N., Castro, L.F., McLay, K., Bird, C., Coulson, A., and Holland, P.W. 2003. Dispersal of NK homeobox gene clusters in amphioxus and humans. Proc. Natl. Acad. Sci. U.S.A. **100**: 5292–5295.

Mallatt, J., and Chen, J.Y. 2003. Fossil sister group of craniates predicted and found. J. Morphol. **258**: 1–31.

Mazet, F., and Shimeld, S.M. 2002*a*. The evolution of chordate neural segmentation. Dev. Biol. **251**: 258–270.

Mazet, F., and Shimeld, S.M. 2002*b*. Gene duplication and divergence in the early evolution of vertebrates. Curr. Opin. Genet. Dev. **12**: 393–396.

Mazet, F., Masood, S., Luke, G.N., Holland, N.D., and Shimeld, S.M. 2004. Expression of *AmphiCoe*, an amphioxus *COE/EFB* gene, in the developing central nervous system and epidermal sensory neurons. Genesis, **38**: 58–65.

Meulemans, D., and Bronner-Fraser, M. 2002. Amphioxus and lamprey *AP-2* genes: implications for neural crest evolution and migration patterns. Development (Camb.), **129**: 4953–4962.

Meulemans, D., McCauley, D., and Bronner-Fraser, M. 2003. *Id* expression in amphioxus and lamprey highlights the role of gene co-option during neural crest evolution. Dev. Biol. **264**: 430–442.

Minguillón, C., and Garcia-Fernàndez, J. 2002. The single amphioxus *Mox* gene: insights into the functional evolution of *Mox* genes, somites, and the asymmetry of amphioxus somitogenesis. Dev. Biol. **246**: 455–465.

Minguillón, C., Jiménez-Delgado, S., Panopoulou, G., and Garcia-Fernàndez, J. 2003. The amphioxus hairy family: differential fate after duplication. Development (Camb.), **130**: 5903–5914.

Mou, C.Y., Zhang, S.C., Lin, J.H., Yang, W.L., Wu, W.Y., Wei, J.W., et al. 2002. EST analysis of mRNAs expressed in neurula of Chinese amphioxus. Biochem. Biophys. Res. Commun. **299**: 74–84.

Müller, W. 1873. Über die Hypobranchialrinne der Tunicaten und deren Vorhandensein bei Amphoxus und den Cyklostomen. Jena. Z. Med. Naturw. **7**: 327–332.

Ogasawara, M. 2000. Overlapping expression of amphioxus homologs of the thyroid transcription factor-1 gene and thyroid peroxidase gene in the endostyle: insight into evolution of the thyroid gland. Dev. Genes Evol. **210**: 231–242.

Olsson, R. 1983. Club-shaped gland and endostyle in larval *Branchiostoma lanceolatum* (Cephalochordata). Zoomorphology (Berl.), **103**: 1–13.

Panopoulou, G., Hennig, S., Groth, D., Krause, A., Poustka, A.J., Herwig, R., Vingron, M., and Lehrach, H. 2003. New evidence for genome-wide duplications at the origin of vertebrates using an amphioxus gene set and completed animal genomes. Genome Res. **13**: 1056–66.

Panopoulou, G.D., Clark, M.D., Holland, L.Z., Lehrach, H., and Holland, N.D. 1998. AmphiBMP2/4, an amphioxus bone morphogenetic protein closely related to *Drosophila* decapentaplegic and vertebrate BMP2 and BMP4: insights into evolution of dorsoventral axis specification. Dev. Dyn. **213**: 130–139.

Plachov, V., Chowdhury, K., Walther, C., Simon, D., Guenet, J.L., and Gruss, P. 1990. *Pax8*, a murine paired box gene expressed in the developing excretory system and thyroid gland. Development, (Camb.) **110**: 643–651.

Ruvinsky, I., Silver, L.M., and Gibson-Brown, J.J. 2000. Phylogenetic analysis of T-box genes demonstrates the importance of amphioxus for understanding evolution of the vertebrate genome. Genetics, **156**: 1249–1257.

Schafer, E.A. 1880. Some teachings of development. Q. J. Microsc. Sci. **20**: 202–218.

Schubert, M., Holland, L.Z., Holland, N.D., and Jacob, D.K. 2000. A phylogenetic tree of the Wnt genes based on all available full-length sequences, including five from the cephalochordate amphioxus. Mol. Biol. Evol. **17**: 1896–1903.

Schubert, M., Holland, L.Z., Stokes, M.D., and Holland, N.D. 2001. Three amphioxus *Wnt* genes (*AmphiWnt3*, *Amphiwnt5*, and *AmphiWnt6*) associated with the tail bud: the evolution of somitogenesis in chordates. Dev. Biol. **240**: 262–273.

Schubert, M., Meulemans, D., Bronner-Fraser, M., Holland, L.Z., and Holland, N.D. 2003. Differential mesodermal expression of two amphioxus MyoD family members *AmphiMRF1* and *AmphiMRF2*. Gene Expr. Patterns, **3**: 199–202.

Sharman, A.C., Shimeld, S.M., and Holland, P.W.H. 1999. An amphioxus Msx gene expressed predominantly in the dorsal neural tube. Dev. Genes Evol. **209**: 260–263.

Shimeld, S.M. 1997. Characterization of amphioxus *HNF-3* genes: conserved expression in the notochord and floor plate. Dev. Biol. **183**: 74–85.

Shimeld, S.M. 1999. The evolution of the hedgehog gene family in chordates: insights from amphioxus hedgehog. Dev. Genes Evol. **209**: 40–47.

Shimeld, S.M. 2000. An amphioxus netrin gene is expressed in midline structures during embryonic and larval development. Dev. Genes Evol. **210**: 337–344.

Shu, D.G. 2003. A paleontological perspective of vertebrate origin. Chin. Sci. Bull. **48**: 725–735.

Steida, L. 1873. Studien über den *Amphioxus lanceolatus*. Mem. Acad. Imp. Sci. St. Petersb. Ser. VII, **19**: 1–70.

Stokes, M.D., and Holland, N.D. 1998. The lancelet. Am. Sci. **86**: 552–560.

Tautz, D. 1998. Debatable homologies. Nature (Lond.), **398**: 17–18.

Torreson, N.A., Martinez-Barbera, J.P., Beardsley, A., Caubit, X., and Kraus, S. 1998. Conservation of *BF-1* expression in amphioxus and zebrafish suggests evolutionary ancestry of anterior cell types that contribute to the vertebrate telencephalon. Dev. Genes Evol. **208**: 431–439.

Venkatesh, T.V., Holland, N.D., Holland, L.Z., Su, M.T., and Bodmer, R. 1999. Sequence and developmental expression of *AmphiNk2-1*: insights into the evolution of the vertebrate thyroid gland and forebrain. Dev. Genes Evol. **209**: 254–259.

Wada, H., Garcia-Fernàndez, J., and Holland, P.W.J. 1999. Collinear and segmental expression of amphioxus Hox genes. Dev. Biol. **213**: 131–141.

Wang, Y., Zhang, P.J., Yasui, K., and Saiga, H. 2002. Expression of *Bblhx3*, a LIM-homeobox gene, in the development of amphioxus *Branchiostoma belcheri tsingtauense*. Mech. Dev. **117**: 315–319.

Webb, J.F., and Noden, D.M. 1993. Ectodermal placodes: Contributions to the development of the vertebrate head. Am. Zool. **33**: 434–447.

Wicht, H., and Lacalli, T.C. 2005. The nervous system of amphioxus: structure, development, and evolutionary significance. Can. J. Zool. **83**(1): 122–150.

Williams, N.A., and Holland, P.W.H. 1996. Old head on young shoulders. Nature (Lond.), **383**: 490.

Williams, N.A., and Holland, P.W.H. 1998. Molecular evidence of the brain of chordates. Brain Behav. Evol. **52**: 177–185.

Winchell, C.J., Sullivan, J., Cameron, C.B., and Swalla, B.J. 2002. Evaluating hypotheses of deuterostome phylogeny and chordate evolution with new LSU and SSU ribosomal DNA data. Mol. Biol. Evol. **19**: 762–776.

Yasui, K., Zhang, S.C., Uemura, M., Aizawa, S., and Ueki, A. 1998. Expression of a *twist*-related gene, Bbtwist, during the development of a lancelet species and its relation to cephalochordate anterior structures. Dev. Biol. **195**: 49–59.

Yasui, K., Zhang, S.C., Uemura, M., and Saiga, H. 2000. Left–right asymmetric expression of BbPtx, a Ptx-related gene, in a lancelet species and the developmental left-sidedness of deuterostomes. Development (Camb.), **127**: 187–195.

Yu, J.K., Holland, L.Z., Jamrich, M., Blitz, I.L., and Holland, N.D. 2002*a*. *AmphiFoxE4*, an amphioxus winged helix/forkhead gene encoding a protein closely related to vertebrate thyroid transcription factor-2: expression during pharyngeal development. Evol. Dev. **4**: 9–15.

Yu, J.K., Holland, N.D., and Holland, L.Z. 2002*b*. An amphioxus winged helix/forkhead gene, *AmphiFoxD*: insights into vertebrate neural crest evolution. Dev. Dyn. **225**: 289–297.

Zannini, M., Avantaggiato, V., Biffali, E., Arnone, M.I., Sato, K., Pischetola, M., et al. 1997. TTF-2, a new forkhead protein, shows a temporal expression in the developing thyroid which is consistent with a role in controlling the onset of differentiation. EMBO (Eur. Mol. Biol. Organ.) J. **16**: 3185–3197.