

Expression of *AmphiHox-1* and *AmphiPax-1* in amphioxus embryos treated with retinoic acid: insights into evolution and patterning of the chordate nerve cord and pharynx

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SUMMARY

Excess all-*trans* retinoic acid (RA) causes severe craniofacial malformations in vertebrate embryos: pharyngeal arches are fused or absent, and a rostral expansion of *Hoxb-1* expression in the hindbrain shows that anterior rhombomeres are homeotically respecified to a more posterior identity. As a corollary, neural crest migration into the pharyngeal arches is abnormal. We administered excess RA to developing amphioxus, the closest invertebrate relative of the vertebrates and thus a key organism for understanding evolution of the vertebrate body plan. In normal amphioxus, the nerve cord has only a slight anterior swelling, the cerebral vesicle, and apparently lacks migratory neural crest. Nevertheless, excess RA similarly affects amphioxus and vertebrates. The expression domain of *AmphiHox-1* (homologous to mouse *Hoxb-1*) in the amphioxus nerve cord is also extended anteriorly. For both the amphioxus and mouse genes, excess RA causes either (1) continuous expression throughout the preotic hindbrain (mouse) and from the level of somite 7 to the anterior end of the nerve cord (amphioxus) or (2) discontinuous expression with a gap in rhombomere 3 (mouse) and a gap at the posterior end of the cerebral vesicle (amphioxus). A comparison of these expression patterns suggests that amphioxus has a homolog of the vertebrate hindbrain, both

preotic and postotic. Although RA alters the expression of *AmphiHox-1* expression in the amphioxus nerve cord, it does not alter the expression of *AmphiHox-1* in presomitic mesoderm or of *alkali myosin light chain* (*AmphiMlc-alk*) in somites, and the axial musculature and notochord develop normally. The most striking morphogenetic effect of RA on amphioxus larvae is the failure of mouth and gill slits to form. In vertebrates effects of excess RA on pharyngeal development have been attributed solely to the abnormal migratory patterns of *Hox*-expressing cranial neural crest cells. This cannot be true for amphioxus because of the lack of migratory neural crest. Furthermore, expression of *Hox* genes in pharyngeal tissues of amphioxus has not yet been detected. However, the absence of gill slits in RA-treated amphioxus embryos correlates with an RA-induced failure of *AmphiPax-1* to become down-regulated in regions of pharyngeal endoderm that would normally fuse with the overlying ectoderm. In vertebrates, RA might similarly act via *Pax-1/9*, also expressed in pharyngeal endoderm, to impair pharyngeal patterning.

Key words: pattern formation, retinoic acid, amphioxus, *Hox*, chordate evolution, *Pax*, *myosin light chain*

INTRODUCTION

The origin of the vertebrate body plan has long been controversial. Many attempts to reconstruct the vertebrate ancestor have begun with their closest living invertebrate relative, the lower chordate amphioxus. Typically, these analyses have been based on body part homologies between amphioxus and the vertebrates. The body plan of amphioxus is similar to, but much simpler than that of vertebrates. Although amphioxus apparently lacks some vertebrate features such as neural crest and an axial skeleton, it has pharyngeal gill slits, a dorsal nerve cord, a notochord and a segmented axial musculature, all of which appear homologous to similar structures in vertebrates. Even so, anatomical and embryological differences make homologies uncertain. For example, the dorsal nerve cord of amphioxus has only a small anterior swelling, the cerebral

vesicle, sometimes called the brain, which however, lacks obvious divisions into fore-, mid- and hindbrain. Thus, opinions vary concerning what part, if any, of the amphioxus nerve cord is homologous to the vertebrate brain. In the 19th century, some anatomists (like Rathke, 1841) thought amphioxus to be brainless, while others claimed that the cerebral vesicle was homologous to all or part of the vertebrate brain (Owsjannikow, 1868; Stieda, 1873; Willey, 1893). More recently, Gans and Northcutt (1983) proposed that the central nervous system of the proximate vertebrate ancestor consisted of a spinal cord and a postotic hindbrain – the preotic hindbrain, midbrain and forebrain being vertebrate innovations. However, Lacalli et al. (1994) concluded from serial electron microscopic sections that amphioxus may have a homolog of the vertebrate diencephalon.

A new approach for inferring homologies between

amphioxus and the vertebrates is the use of the expression patterns of developmental genes as phenotypic characters. For example, amphioxus has one cluster of *Hox* genes compared to four in vertebrates (Garcia-Fernández and Holland, 1994), and a comparison of the expression of *AmphiHox-3* in the amphioxus nerve cord and its homolog *Hoxb-3* in the vertebrate brain strongly suggests that amphioxus has a counterpart of the vertebrate hindbrain (Holland et al., 1992). Similarly, the expression of *AmphiPax-1* (Holland et al., 1995b) and its vertebrate homologs *Pax-1* and *Pax-9* (Neubüser et al., 1995; Peters et al., 1995) in pharyngeal endoderm, suggests that class-I paired box genes originally functioned in endodermal patterning and supports the homology of the gill slits in amphioxus and vertebrates (Holland et al., 1995b).

The apparent lack of neural crest in amphioxus has led to the widely accepted proposal that much of the complexity of anterior parts of the vertebrate head is due to the evolution of neural crest in the earliest vertebrates (Gans and Northcutt, 1983). Neural crest cells migrate from the dorsal neural fold to contribute anteriorly to cranial ganglia and to mesenchyme of the pharyngeal arches. Hindbrain neural crest expresses the same *Hox* genes as the rhombomeres from which it originates and, after arriving in the branchial arches, induces the expression of these genes in the overlying ectoderm (Hunt et al., 1991). Thus, in vertebrates, the *Hox* code expressed in the hindbrain appears to have been co-opted for the new function of patterning of the branchial arches.

To understand the mechanisms of patterning of the vertebrate head, teratogens, including all-*trans* retinoic acid (RA), have been used to perturb morphogenesis and developmental gene expression in anterior structures. Excess RA administered before neurulation causes abnormalities chiefly in ectodermally or mesodermally derived structures such as the vertebrate brain, somites, pharyngeal arches and facial structures (Sive et al., 1990; Holder and Hill, 1991; Hofmann and Eichele, 1994). The extent of these abnormalities is concentration-dependent, stage-dependent and, to some extent, species-dependent (Durston et al., 1989; Sive et al., 1990; Holder and Hill, 1991; Wood et al., 1994; Lee et al., 1995). Application of RA during gastrulation often results in partial or complete loss of the forebrain and midbrain (Durston et al., 1989). In all vertebrates, administration of RA before somitic segmentation results in loss of anterior hindbrain structures due to respecification of the genetic identity of much of the preotic hindbrain (rhombomeres 1-4) to rhombomere 4. This is reflected in an anterior shift in the expression domains of the most 3' *Hox* genes (e.g. *Hoxb-1*) (Marshall et al., 1992; Wood et al., 1994).

A corollary of RA-induced alteration of the anterior-posterior patterning of the brain is that pathways of neural crest migration are abnormal (Lee et al., 1995). Pharyngeal arch abnormalities have been attributed to the abnormal migration and concomitant abnormal pattern of expression of anterior *Hox* genes in the neural crest-derived mesenchyme of the pharyngeal arches (Morriss-Kay et al., 1991). The possibility that other factors might be involved in RA effects on pharyngeal patterning has not previously been considered.

To what extent effects of RA on the vertebrate nerve cord are direct or mediated through mesodermal components such as notochord and somites has been controversial. Recent iden-

tification of binding sites for retinoic acid receptors (RAR) in promoter regions of anterior *Hox* genes indicates that RA can regulate *Hox* gene expression directly (Marshall et al., 1994; Studer et al., 1994; Ogura and Evans, 1995). On the other hand, anterior *Hox* genes are expressed in embryonic mesoderm as well as ectoderm, and RA can interfere with mesodermal induction and patterning (Cho and De Robertis, 1990; Ruiz i Altaba and Jessell, 1991).

In the present paper, we have correlated the temporal and concentration-dependent effects of excess RA on the morphology of embryos of amphioxus (*Branchiostoma floridae*) with the expression patterns of three amphioxus genes: *AmphiHox-1*, a homolog of vertebrate *Hoxb-1* and *Hoxa-1*; *AmphiMLC-alk*, a homolog of vertebrate *alkali myosin light chain* genes; and *AmphiPax-1*, a homolog of vertebrate *Pax-1* and *Pax-9*. In normal neurulae, *AmphiHox-1* is expressed in the dorsal nerve cord at the level of somites 3.5-5 and in posterior mesoderm (Holland and Garcia-Fernández, 1996). *AmphiMLC-alk* is expressed in the somites and notochord, and later in the axial and other muscles (Holland et al., 1995a). Expression of *AmphiPax-1* is restricted to the pharyngeal endoderm in neurulae and early larvae (Holland et al., 1995b). The results, compared to the effects of RA on expression of homologous genes in vertebrates, give insights into the evolution and patterning of the vertebrate nerve cord and pharynx. They suggest that the common ancestor of amphioxus and the vertebrates had an extensive hindbrain homologous to both the preotic and postotic hindbrain of vertebrates. In addition, we suggest that a role for *Hox*-expressing neural crest cells in pharyngeal patterning of vertebrates has been superimposed on an ancestral patterning mediated by homologs of the *Pax-1* gene.

MATERIALS AND METHODS

Amphioxus collection and rearing of larvae

Amphioxus adults (*Branchiostoma floridae*) were collected from Old Tampa Bay, Florida by shovel and sieve. Spawning was induced by electrical shock and developmental stages were reared at 24°C as previously described (Stokes and Holland, 1995).

In situ hybridization

Fixation of larvae and whole-mount in situ hybridization were as in Holland et al. (1992, 1996). Anti-sense riboprobes were transcribed from cDNA clones in pBluescript SK (Stratagene, Inc., La Jolla, CA, USA). Two *AmphiHox-1* riboprobes were combined, one corresponding to 800 bp of the 3' untranslated end (3'UTR) and the other to 750 bp at the 5' end of the cDNA. The *AmphiPax-1* riboprobe corresponded to 1.2 kb of the 3' UTR (Holland et al., 1995b) and the *AmphiMLC-alk* riboprobe to 739 bp of the 3' UTR plus 203 bp of 3'-coding sequence (Holland et al., 1995a).

Scanning electron microscopy (SEM)

After 2.5 days of development 160 experimental and control larvae were processed for SEM (Stokes and Holland, 1995). To view the opposite sides of the larvae after photography of one side, they were removed from the stubs by soaking in xylene for 4 minutes, teased from the surrounding metal coating, transferred to 100% ethanol, critical-point dried and remounted with the uncoated (light) side up.

Retinoic acid treatment

Stock solutions of all-*trans* retinoic acid (Sigma Chemical Co., St.

Louis MO) were 10^{-3} M, 10^{-4} M and 10^{-6} M in DMSO and stored at -20°C . Embryos were raised at 24°C . Only embryos with a high percentage of normal cleavage were used. To approximately 500-700 embryos in 3 ml seawater, 3 μl of stock RA solution or DMSO alone was added with rapid mixing. An untreated control sample was allowed to develop. RA or DMSO-exposure was terminated by dilution at 12 hours (hatched neurula). About 40 larvae were fixed for in situ hybridization at times indicated in the figure legends. At 36 hours, the larvae were fed with a phytoplankton mixture consisting of *Tetraselmis*, *Isochrysis*, *Monochrysis* and *Ellipsoidion*. At 2.5 days, the remaining larvae were fixed for SEM.

RESULTS

The effect of RA concentration and time of application on amphioxus development

Normal amphioxus larvae have an extended asymmetrical stage. The mouth and the ciliated pit, an external opening to a coelomic cavity, open on the left, and the primary gill slits open just to the right of the ventral midline. The mouth opens and feeding begins at 38 hours (the larvae are filter feeders eating unicellular algae). The first gill slit to form (about 24 hours) is the most anterior. Additional gill slits are added sequentially posterior to the first. During metamorphosis, the mouth shifts to the midline, secondary gill slits form, the gill slits become bilaterally symmetrical and metapleural folds fuse ventrally to form the atrial cavity (Stokes and Holland, 1995).

At 8-9 hours, there is a neural plate and, by 16-18 hours, a neural tube, containing the first pigment spot at the level of somite 5. The anterior portion of the nerve cord at the level of somites 1 and 2 has a slightly greater diameter than the more posterior nerve cord and is called the cerebral vesicle. The second pigment spot forms between 2.5 and 3.5 days at its anterior tip.

The effect of RA on the morphology of amphioxus larvae depends on both the time of application and the concentration. The earlier RA is applied and the higher the concentration, the higher the percentage of abnormal larvae (Fig. 2). However, regardless of the RA concentration or application time, the types of morphological abnormalities vary little. Defects are first apparent at approximately 25 hours of development and are obvious by 2.5 days. They include (1) a small or absent mouth (2) no gill slits, (3) no ciliated pit, and (4) a smaller than normal anus (Fig. 1). DMSO-control larvae (Fig. 1A,B,E) are indistinguishable from untreated larvae (see Stokes and Holland, 1995): on the left, the ciliated pit opens anterior to the mouth (Fig. 1A,B). On the right, the primary gill slit is open, the second gill slit is beginning to form and the anus is open at the base of the tail (Fig. 1E). In larvae treated with RA, the mouth is either absent (Fig. 1C) or when present, is smaller (Fig. 1D) and rounder than in controls. The ciliated pit is occasionally absent. It may be present without the mouth (Fig. 1C) or the mouth may be present without the ciliated pit (Fig. 1D). The anterior end of the larva is sometimes slightly reduced (Fig. 1F). If either the mouth or ciliated pit is affected, the gill slits are absent (Fig. 1F). The anus is reduced (Fig. 1G) or absent. Larvae lacking mouths cannot begin to feed as usual at 38 hours. They have little yolk and do not grow as rapidly as controls. Their anterior ends become bent and shriveled (Fig. 1C,F), and they die by about 4 days. This anterior deformity

and death appear due to starvation and not to RA treatment, since the same defects and early death occur in untreated larvae which are not fed.

In the light microscope, the morphology of the developing nerve cord appears unaffected by RA before the mouth opens about 38 hours, except perhaps for a slight shortening of the cerebral vesicle (Fig. 4F). The first pigment spot forms normally, but the anterior pigment spot, which in controls is present by 3.5 days (in 9/10 DMSO controls) usually fails to form (absent in 31/35 larvae treated with 1×10^{-6} M RA). We do not know whether the absence of this pigment spot is directly due to RA or to retarded growth due starvation.

Because of starvation (Fig. 4C,F), effects of RA on the cerebral vesicle of larvae older than 38 hours could not be determined. At this time, cells in the normal cerebral vesicle are still generally cuboidal and do not yet express such early markers of differentiation as serotonin (Holland and Holland, 1993). Thus, possible effects of RA on the differentiation of nerve cells or axonal outgrowth can never be known.

Nevertheless, all RA-treated larvae, even those most severely affected, swim normally with undulatory muscular movements beginning at about 20 hours. Therefore, RA treatment during the blastula stage, well before the onset of somitogenesis, does not affect the development of functional axial muscles. Amphioxus axial muscles send muscle tails to contact the nerve cord. Thus, innervation of these muscles does not depend on neuronal outgrowth.

Fig. 2 shows the time- and concentration-dependence of RA treatment. At the lowest concentration (10^{-9} M), only 10% of the larvae were abnormal when RA was applied at the early blastula. At later stages, there was no effect (Fig. 2). At 10^{-7} M RA, about 15% of larvae lacked mouth and gill slits when RA was applied at mid-blastula (4.5 hours), but later application had no effect. At the highest concentration (10^{-6} M), 60% of the larvae lacked mouth and gill slits when RA was applied at the mid-gastrula stage (7 hours) and 15% were similarly affected when application was at the mid-neurula (9 hours). Thus, RA treatment even at the neurula can severely affect amphioxus development.

Effect of RA on expression of *AmphiHox-1*

Fig. 3A-M show the effect of excess RA on the expression pattern of *AmphiHox-1*. In 15-hour controls *AmphiHox-1* is expressed in the posterior presomitic mesoderm and in the neural tube with the anterior and posterior limits of expression respectively adjacent the posterior half of somite 3 and the anterior half of somite 5 (Fig. 3A). RA treatment does not affect expression of *AmphiHox-1* in the posterior mesoderm or the posterior limit of expression in the nerve cord (compare Fig. 3A and D). However, RA treatment extends the anterior limit of expression in the nerve cord forward. Although, at any given stage, the anterior limit of expression varies somewhat, in general it moves more rostrally with increasing time of development up to about 24 hours. For example, in 15-hour embryos, the anterior limit of *AmphiHox-1* expression ranges from the level of the somite 1/2 boundary (Fig. 3C) to nearly as far rostrally as the neuropore (Fig. 3D). At this stage in more weakly stained embryos, the expression of *AmphiHox-1* in the nerve cord is faintly striped, most clearly so at the anterior end of the expression domain (Fig. 3C). Whether or not this striping is due to RA treatment is difficult to determine because

of the restricted domain of *AmphiHox-1* expression in controls. Between 15 and 18 hours, about 4 new somites are added posteriorly. In 18-hour controls, the anterior limit of *AmphiHox-1* expression in the nerve cord remains at the level of the posterior half of somite 3, while the posterior limit has extended posteriorly to approximately the level of somite 7; expression in the posterior mesoderm is no longer detectable (Fig. 3E). In RA-treated embryos, *AmphiHox-1* expression typically extends nearly to the neuropore (Fig. 3F).

By 24 hours the cerebral vesicle has formed (Fig. 3G-K). Although some 24- to 28-hour embryos treated with RA have slightly shortened anterior ends and correspondingly shorter (by about 25%) cerebral vesicles (compare Fig. 4D and F); most do not have obviously shorter anterior structures (compare Fig. 3G and H,J). Even with RA treatment begun during the blastula, before somite formation, the morphology of the notochord appears unaffected (Fig. 3K).

In 24-hour controls, *AmphiHox-1* is expressed in a band in the nerve cord with the anterior limit remaining at the level of about somite 3 (Fig. 3G). RA treatment causes two patterns of *AmphiHox-1* expression – continuous and discontinuous (diagrammed in Fig. 5A). In both, the posterior limit of expression is similar to that of the controls and the anterior limit is at the anterior tip of the cerebral vesicle. However, in the continuous pattern, all cerebral vesicle cells express *AmphiHox-1* (Fig. 3H,I), while in the discontinuous pattern, there is a gap in expression at the posterior end of the cerebral vesicle about the level of somite 2 (Figs 3J,K, 5). The patterns of *AmphiHox-1* expression in controls (Fig. 3L) and in RA-treated embryos (Fig. 3M) remain unchanged throughout the next 4 hours. Thus, excess RA applied up to the early neurula stage appears to cause a homeotic transformation in the amphioxus nerve cord, in which the expression domain of *AmphiHox-1* is shifted anteriorly.

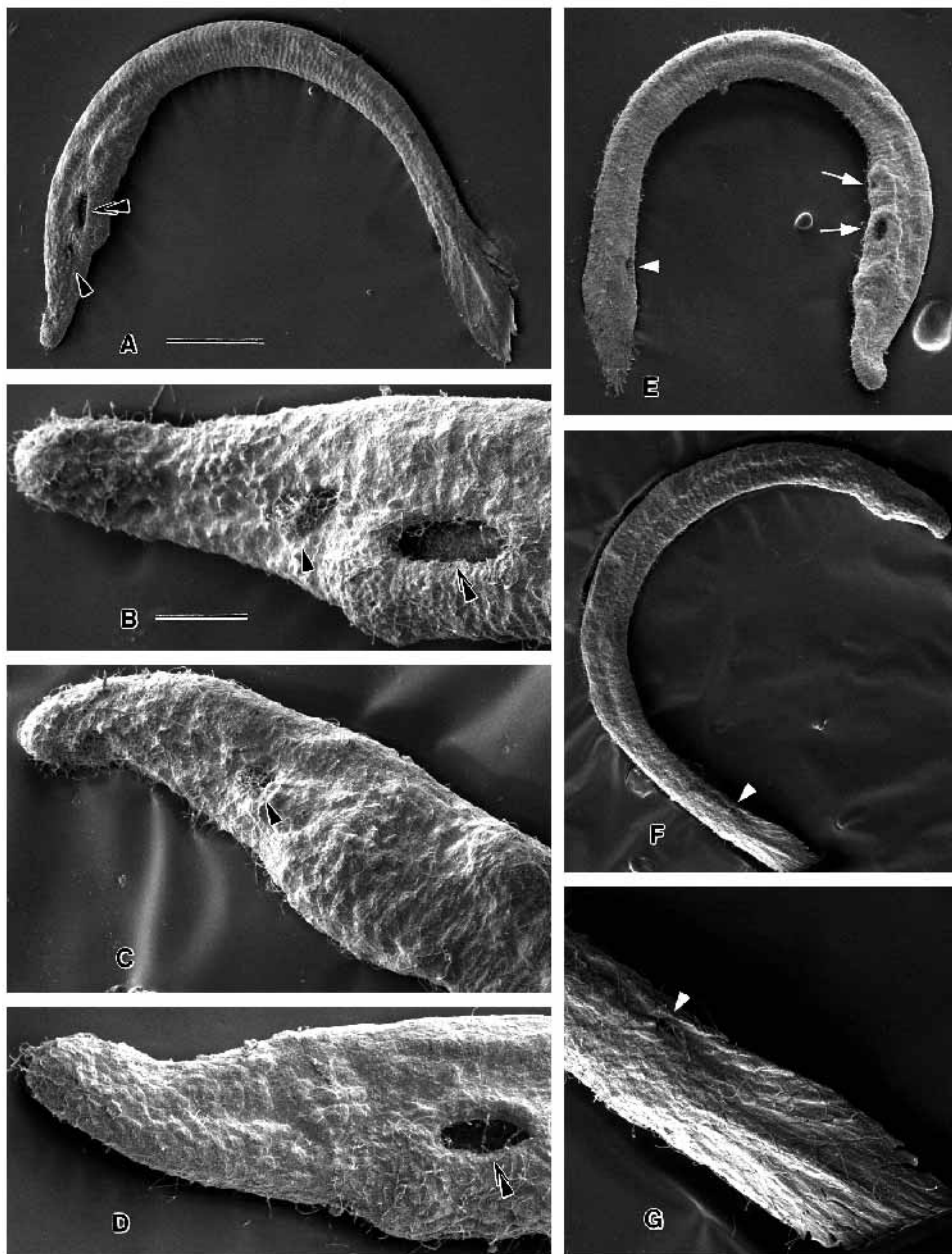


Fig. 1. SEM of 2.5 day larvae of *Branchiostoma floridae*. DMSO applied at 2.5 hours for controls; RA (1×10^{-6} M) at 4.5 hours (early gastrula). (A) Control. Left side showing ciliated pit (single arrowhead) and mouth (tandem arrowheads). (B) Control. Enlargement of anterior end of larva in A showing ciliated pit (single arrowhead) and mouth (tandem arrowheads). (C) RA treatment. Anterior left side showing small ciliated pit (single arrowhead), but no mouth. (D) RA treatment. Anterior left side showing mouth (tandem arrowhead) but no ciliated pit. (E) Control. Right side showing two gill slits (arrows) and an anus (arrowhead). (F) RA treatment. Right side showing absence of gill slits and small anus (arrowhead). The left sides of larvae with these abnormalities are as in C and D. (G) Enlargement of posterior end of F showing small anus (arrowhead). Bar A,E,F, 100 μ m; B-D,G, 30 μ m.

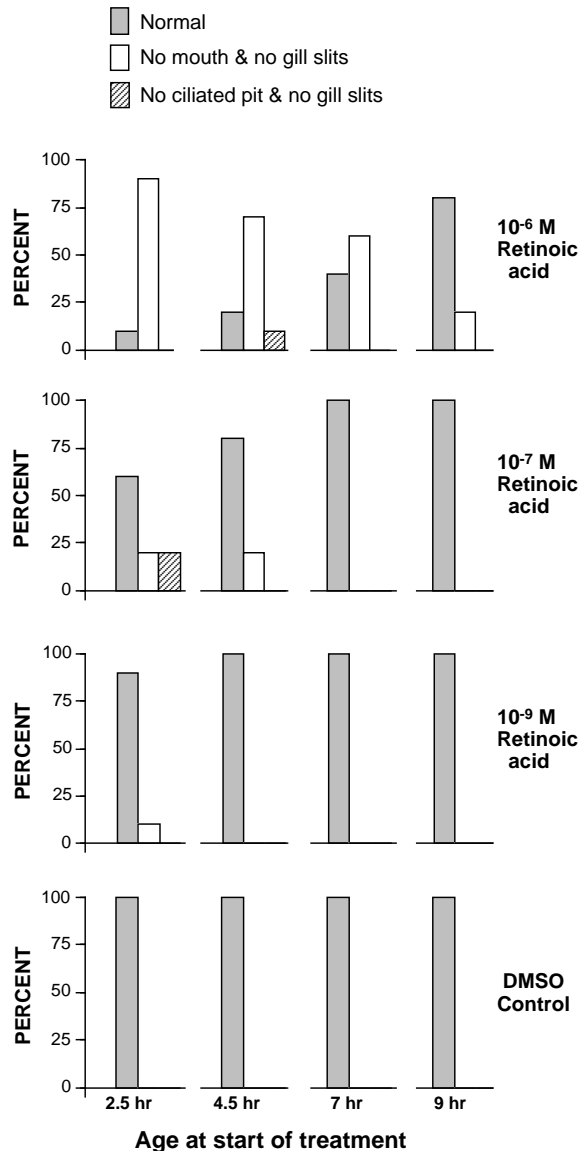


Fig. 2. Time- and concentration-dependence of excess RA on development of *Branchiostoma floridae*. Each point includes 40 embryos assayed by SEM at 2.5 days when mouth and gill slits are normally open. 2.5 hours = early blastula; 4.5 hours = early gastrula; 7.5 hours = late gastrula; 9 hours = early neurula.

The effect of RA on expression of *AmphiPax-1*

AmphiPax-1 is normally expressed in the pharyngeal endoderm before and during the formation of the mouth and first 2 gill slits (Holland et al., 1995b). The mouth penetrates on the left just anterior to the zone of *AmphiPax-1* expression. In 18-hour controls (Fig. 3N), expression ceases in the ventral pharyngeal region where the first gill slit will penetrate. By 24 hours expression is also down-regulated where the second gill slit will penetrate (Fig. 4A). At 28 hours, *AmphiPax-1* expression remains high in the pharyngeal endoderm except immediately bordering the newly formed gill slits (Fig. 4D).

RA treatment causes the expression domain of *AmphiPax-1* to compress and shift anteriorly (Fig. 3O). At 18 hours, there is little, if any, down-regulation in the ventral part of

the pharynx. By 24 hours, there are two patterns of *AmphiPax-1* expression. In one, there is a single down-regulated zone (instead of the normal two) (Fig. 4B). In the other, there is no down-regulation of *AmphiPax-1* expression (Fig. 4C). By 28 hours the domain of *AmphiPax-1* expression is greatly reduced (Fig. 4E, F). Expression may include the cells where the first gill slit would normally perforate (Fig. 4F) or may be down-regulated in one small ventrolateral area (Fig. 4E).

Effects of RA on expression of *AmphiMLC-alk*

AmphiMLC-alk is expressed from 13 hours of development through the adult in the somites and differentiating and adult axial musculature (Holland et al., 1995a). *AmphiMLC-alk* is, therefore, a good marker for somite differentiation. RA, even applied during the blastula, does not affect expression of *AmphiMLC-alk*. At 15 hours expression as in controls is strong throughout all somites, though somewhat weaker in somite 1 (Fig. 4G). By 20 hours expression has extended into the anterior projection from somite 1 (Fig. 4H) and, by 24 hours, expression is strong throughout all the somites (Fig. 4I). Together with the observation that RA-treated larvae swim by muscular undulations, these results show that RA does not inhibit development of axial muscles.

DISCUSSION

The origin and early evolution of the vertebrate body plan is controversial, and has been explained by highly diverse scenarios (e.g. Jefferies, 1986 as compared to Gans, 1989). In the present research, we have addressed this question through the use of a teratogen, retinoic acid (RA), to perturb morphogenesis and the expression patterns of developmental genes in amphioxus, the closest living invertebrate relative of the vertebrates. A comparison with the effects of RA on the morphology and expression patterns of homologous genes in vertebrate embryos yields insights into the evolution and mechanism of patterning of the chordate nerve cord and pharynx.

Hox gene expression and evolution of the chordate hindbrain

Previous studies on *Hox* expression in the embryonic nerve cords of amphioxus and vertebrates have shown that, although the amphioxus nerve cord has no obvious divisions into rhombomeres or forebrain or midbrain, it does have a counterpart of at least the vertebrate postotic hindbrain (Holland et al., 1992; Holland and Garcia-Fernández, 1996). The expression of both *AmphiHox-3* in amphioxus and its homolog *Hoxb-3* in the mouse extends from the posterior end of the nerve cord to a discrete anterior limit – at the level of the somite 4/5 boundary in amphioxus (Holland et al., 1992) and at the rhombomere 4/5 boundary in the mouse (Morriss-Kay et al., 1991). In addition, the domain of *Hoxb-1* is restricted to rhombomere 4 in the mouse hindbrain (about the level of the otic vesicle) and that of *AmphiHox-1* to a stripe in the amphioxus nerve cord from the level of the posterior half of somite 3 through somite 5 (Holland and Garcia-Fernández, 1996). Thus, the amphioxus nerve cord about the level of somites 3.5 and 4 appears to correspond approximately to rhombomere 4 in the vertebrate

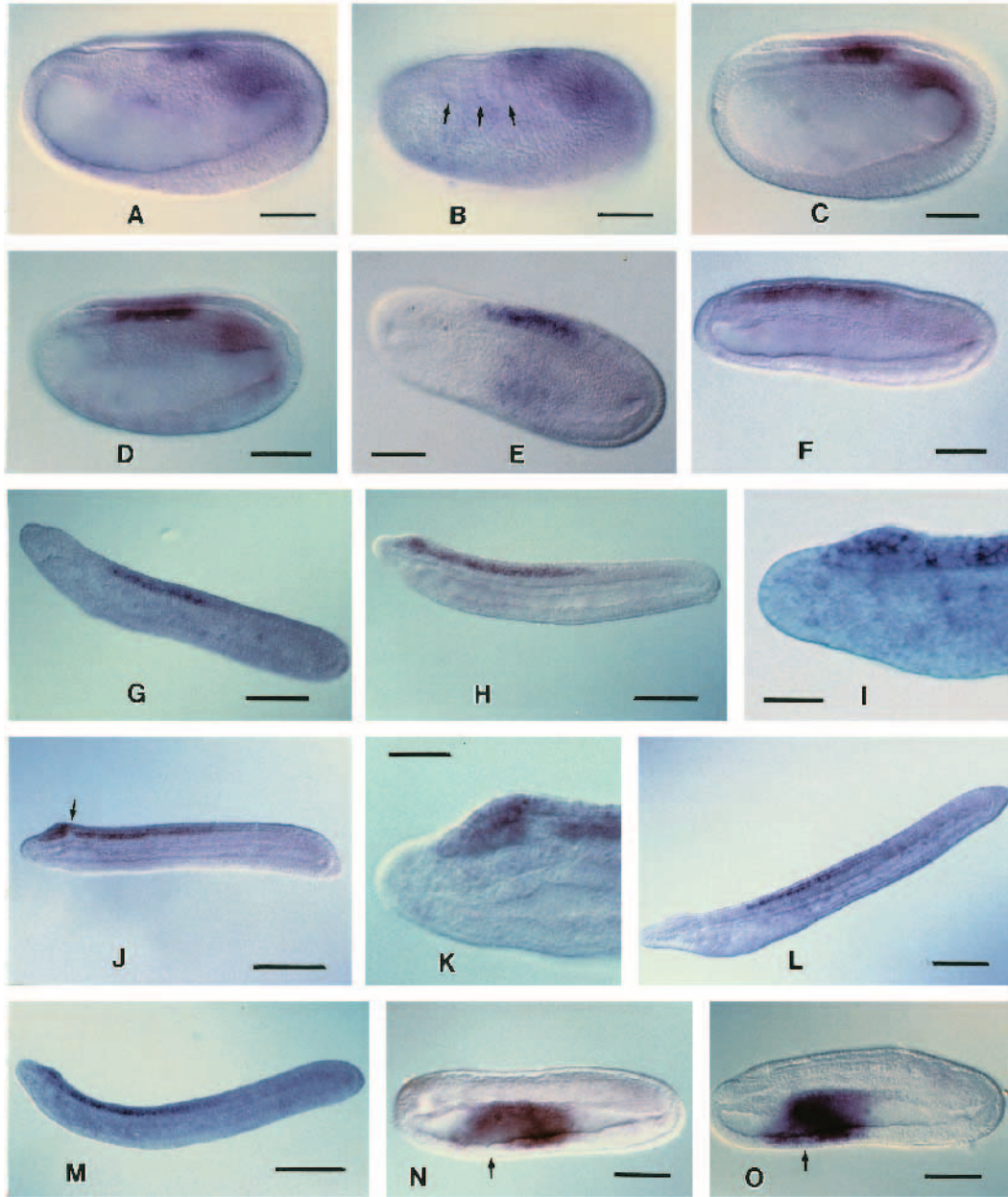


Fig. 3. Effects of excess RA on expression of *AmphiHox-1* (A-L) and *AmphiPax-1* (M-O) in *Branchiostoma floridae*. Anterior at left. Medial views except as noted. DMSO added at 2.5 hours; RA at 1×10^{-6} M or 1×10^{-7} M at 4.5 hours (early gastrula) or 7.5 hours (late gastrula). (A) DMSO-control, 15-hour neurula. Neurenteric canal at top; neuropore at top left. *AmphiHox-1* is expressed in a band in the nerve cord and in posterior mesoderm. (B) Same neurula as in A. View through somites; somites 1-3 arrowed. The anterior limit of *AmphiHox-1* expression in the nerve cord is adjacent to the posterior half of somite 3. (C) 15-hour neurula, 1×10^{-6} M RA at 7.5 hours, showing the least severe effect of RA. Anterior limit of *AmphiHox-1* has extended to the level of somite 2. (D) 15-hour neurula, 1×10^{-6} M RA at 4.5 hours showing the most severe effect of RA. Anterior limit of *AmphiHox-1* is at level of somite 1. (E) DMSO-control 15-hour neurula. *AmphiHox-1* expression has extended posteriorly, but the anterior limit remains at the level of somite 3.5-4. The posterior mesoderm no longer expresses *AmphiHox-1*. (F) 20-hour embryo, 1×10^{-7} M RA at 7.5 hours, showing a relatively severe effect of RA. Anterior limit of *AmphiHox-1* expression has extended to the level of somite 1. (G) 24-hour

DMSO-control. Anterior limit of *AmphiHox-1* expression remains at the level of somite 3.5/4. (H) 24-hour embryo, 1×10^{-7} M RA at 4.5 hours; continuous pattern of *AmphiHox-1* expression. Anterior limit of *AmphiHox-1* is at the anterior tip of the cerebral vesicle, which is not drastically shortened. (I) Higher magnification of the anterior end of the embryo in H. All cells of the cerebral vesicle express *AmphiHox-1*. (J) 24-hour embryo, 1×10^{-7} M RA at 4.5 hours; discontinuous pattern. The anterior limit of *AmphiHox-1* expression is at anterior tip of the cerebral vesicle, but cells at the posterior end of the cerebral vesicle (arrow) do not express *AmphiHox-1*. (K) Higher magnification of the embryo in K. (L) 28-hour DMSO-control. The domain of *AmphiHox-1* expression in the nerve cord has broadened posteriorly, but the anterior limit remains at the level of somite 3.5/4. (M) 28-hour larva, 1×10^{-6} M RA at 4.5 hours; continuous pattern. The anterior limit of *AmphiHox-1* expression is at the anterior tip of the cerebral vesicle. The curve in the larva is due to in situ hybridization, not to RA treatment. (N) DMSO-control, 20-hour embryo. *AmphiPax-1* is expressed in ventral endoderm except where the first gill slit will perforate (arrow). (O) 20-hour late neurula, 1×10^{-6} M RA at 4.5 hours. *AmphiPax-1* expression is shifted slightly anteriorly and is continuous throughout the pharyngeal endoderm. Arrow = continued expression of *AmphiPax-1* in cells where first gill slit normally penetrates. Bar A-F,N,O = 50 μ m; G,H,I,K = 30 μ m.

hindbrain. These studies, however, did not show whether amphioxus has a homolog of the preotic hindbrain.

In all vertebrates tested, RA shifts the expression of anterior *Hox* genes rostrally into the preotic hindbrain (Sundin and Eichele, 1992; Wood et al., 1994; Kolm and Sive, 1995). In

the mouse, there are two altered expression patterns, continuous and discontinuous (Fig. 5). In the former, rhombomeric segmentation is repressed, and the preotic hindbrain is shortened, expressing *Hoxb-1* throughout; in the latter, the hindbrain is not shortened and there is a gap in *Hoxb-1*

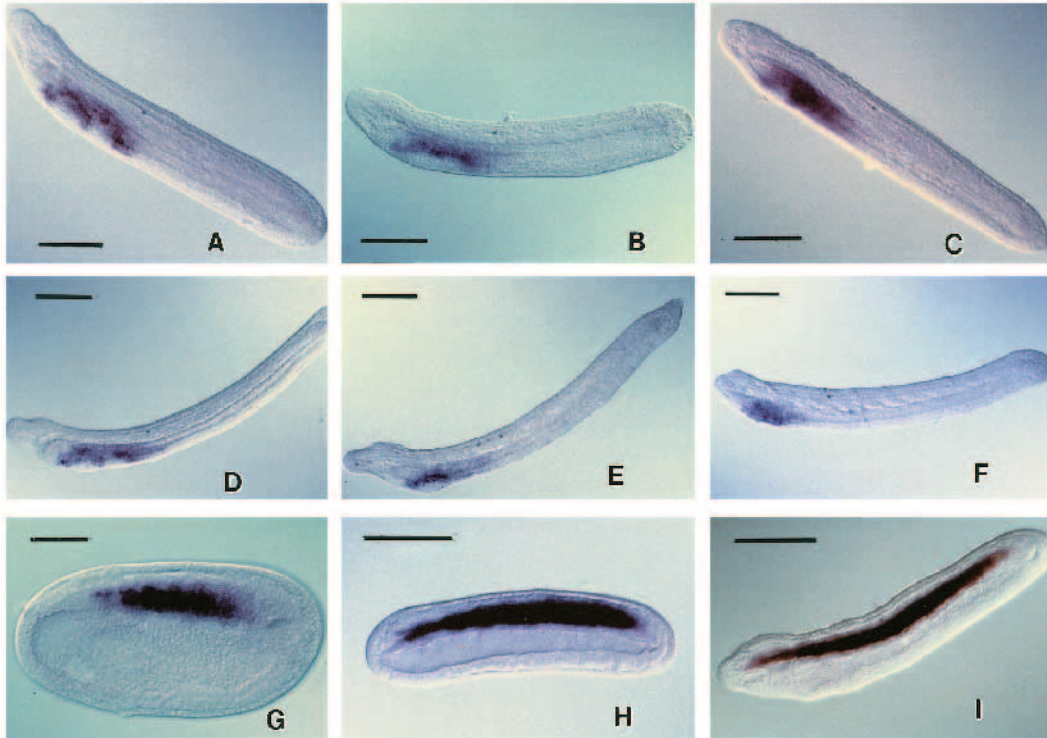


Fig. 4. Effects of excess RA on expression of *AmphiPax-1* (A-F) and *AmphiMLC-Alk* (G-I) in *Branchiostoma floridae*. Anterior at left. Plane of focus is on right side in A-F and on somites G-I. RA treatments at either 1×10^{-7} M or 1×10^{-6} M RA starting at 2.5 hours (early blastula) or 4.5 hours (early gastrula). (A) 24-hour embryo, DMSO-control. Endodermal cells where the first 2 gill slits will perforate no longer express *AmphiPax-1*. (B) 24-hour embryo, 1×10^{-7} M RA at 4.5 hours. *AmphiPax-1* expression is shifted slightly anteriorly; the 2 ventral regions not expressing *AmphiPax-1* appear to have fused. (C) 24-hour embryo, 1×10^{-7} M RA at 2.5 hours. *AmphiPax-1* expression is shifted anteriorly and includes cells where the first two gill slits and mouth

would normally perforate. (D) DMSO-control 28-hour larva. *AmphiPax-1* is down-regulated where the first 2 gill slits will perforate. (E) 28-hour larva, 1×10^{-6} M at 4.5 hours. The domain of *AmphiPax-1* expression is narrowed. (F) 28-hour larva, 1×10^{-6} M RA at 2.5 hours. The anterior end of the larva is shortened. *AmphiPax-1* expression is compressed and occurs in cells where gill slits should have perforated. (G) 15-hour neurula, 1×10^{-6} M RA at 2.5 hours. *AmphiMLC-alk* expression in all somites. (H) 20-hour neurula, 1×10^{-6} M RA at 2.5 hours. *AmphiMLC-alk* expression in all somites. (I) 24-hour embryo, 1×10^{-6} M RA at 2.5 hours. *AmphiMLC-alk* expressed in all somites. Bar A-F, H-I, 100 μ m; G, 50 μ m.

expression in rhombomere 3 (Fig. 5). RA effects on *AmphiHox-1* expression in the amphioxus nerve cord are strikingly similar to those on expression of *Hoxb-1* in the mouse. The domain of *AmphiHox-1* expression is shifted far anteriorly and there is both a continuous pattern and a discontinuous one with a gap in expression of *AmphiHox-1* about the level of somite 2. This similarity suggests that amphioxus has a homologue not only of the postotic hindbrain but also of the preotic hindbrain.

To confirm this suggestion, the expression patterns of more genes in both normal and RA-treated embryos would be desirable. For example, the homolog of *Krox20* should be expressed in the amphioxus equivalents of rhombomeres 3 and 5, while the anterior limit of *AmphiHox-2* should be at the anterior boundary of either the rhombomere 2 or 3 homolog. Furthermore, to define the anterior limit of the putative amphioxus hindbrain homolog, it would be useful to know the expression patterns of amphioxus homologs of genes expressed in the vertebrate forebrain and midbrain (e.g. *Emx* and *Otx*) and the midbrain/hindbrain boundary (*En*) (Davis, 1991; Fjose et al., 1992; Holland et al., 1993). In preliminary work, we observed expression of the amphioxus homolog, *AmphiEn*, in a few cells within the cerebral vesicle in normal 20-hour embryos (Holland et al., 1994). We could not test whether RA eliminates *AmphiEn* expression as it does at the midbrain/hindbrain junction in vertebrates (Holder and Hill, 1991), because available *En* probes (a 3' *AmphiEn* antisense

riboprobe and antibody INV 4D9) label normal embryos weakly at best and often not at all.

To help interpret RA effects on the amphioxus nerve cord, corresponding anatomical studies would be useful. Unfortunately, such studies cannot be done on RA-treated larvae, since they die from starvation before cerebral vesicle cells have fully differentiated into neurons with axonal processes. Even in normal amphioxus it would be difficult to correlate cells in the embryonic nerve cord expressing genes such as *AmphiHox* and *AmphiEn* with late larval and adult structures, since expression of such genes ceases in the nerve cord long before structures have differentiated in the cerebral vesicle. Furthermore, it is not known whether portions of the cerebral vesicle grow disproportionately during development. Thus, solely on the basis of their relative positions, cells in the embryonic cerebral vesicle cannot be correlated with late larval structures, such as the cells in the anterior half of the cerebral vesicle which resemble infundibular cells of the vertebrate diencephalon (Olsson, 1986; Olsson et al., 1994; Lacalli et al., 1994). Cell lineage tracings would be very valuable in this regard.

Our evidence for amphioxus homologs of both the preotic and postotic hindbrain suggests that none of the older anatomy-based theories concerning homologies of the amphioxus nerve cord are entirely correct. Rathke (1841) certainly erred in claiming that amphioxus is completely brainless, but it is also clear that the amphioxus cerebral vesicle is not homologous to the entire vertebrate brain as proposed, for example, by Stieda

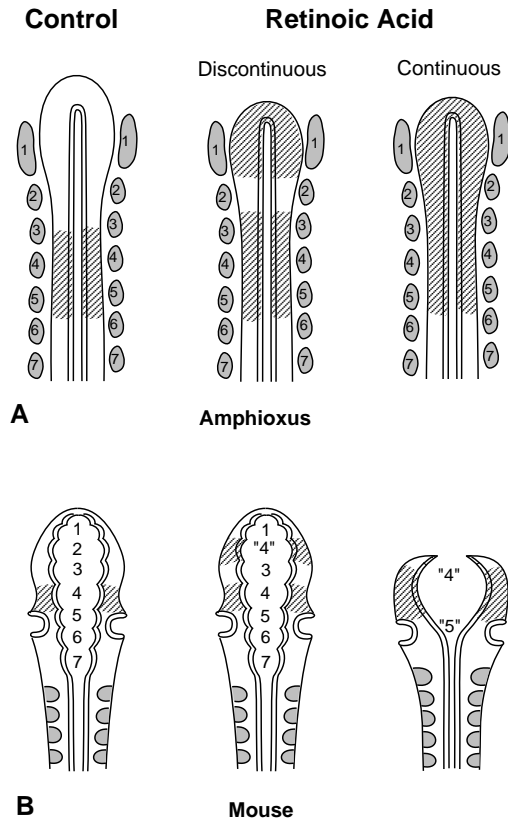


Fig. 5. Schematic dorsal views (anterior at top) of the embryonic (A) amphioxus nerve cord; somites shaded and numbered, and (B) mouse hindbrain; somites shaded, rhombomeres numbered, showing effects of RA on expression patterns of *AmphiHox-1* and mouse *Hoxb-1*. In both RA causes an anterior expansion of *Hox* expression (hatching); in both there are two patterns: (1) discontinuous with a gap in expression at the level of somite 2 in amphioxus and rhombomere 3 in the mouse and (2) continuous with an unbroken expression domain. The continuous and discontinuous patterns in the mouse result from RA application before and after somitic segmentation respectively. In amphioxus, both patterns occur in samples treated identically. RA is water-insoluble and must be administered to amphioxus in DMSO; thus the amount of RA taken up by individual embryos may vary. Mouse figure after Wood et al. (1994).

(1873) and Olsson (1986). Other theories homologizing anterior parts of the amphioxus nerve cord with only a part of the vertebrate brain are closer to the truth. Thus, Owsjannikow (1868) may have been partly right in homologizing the cerebral vesicle with the hindbrain, although our results show that the amphioxus hindbrain homolog extends far posterior to the cerebral vesicle. Gans and Northcutt (1983) appear to have been even closer to the truth in suggesting that the amphioxus nerve cord is homologous to the postotic hindbrain and spinal cord, although our results indicate that there is at least a homolog of the preotic hindbrain as well.

An ascidian homolog of *AmphiHox-1*, *HrHox-1*, and its upregulation by exogenous RA in the anterior region of the central nervous system has recently been described (Katsuyama et al., 1995). Development of sea urchin larvae is apparently normal, though delayed (Sciarrino and Matranga, 1995). In *Drosophila*, excess RA apparently has no effect on

the nervous system (Harmon et al., 1995). Thus, the involvement of RA in patterning of the central nervous system may have evolved after the split of echinoderms and hemichordates plus chordates.

Pattern formation of the chordate hindbrain

RA has been extensively used as a tool to investigate the mechanism of pattern formation in vertebrate embryos. RA applied to early embryos causes abnormalities mainly in the head and pharynx, which have been attributed to a disruption of a gradient of endogenous RA. Endogenous RA has been detected in mouse and chick embryos (Chen et al., 1992; Hogan et al., 1992; Wagner et al., 1992). In *Xenopus* embryos, a gradient low anteriorly and high posteriorly been shown (Chen et al., 1994). Excess RA can result in: loss of forebrain and midbrain in *Xenopus* (Durstun et al., 1989), defects in the midbrain and hindbrain in the chick (Sundin and Eichele, 1992) and shortening of the hindbrain and loss of obvious rhombomeres in rodents (Wood et al., 1994). To what extent RA acts directly on the nervous system or indirectly via effects on the mesoderm is controversial, because RA affects both mesoderm and neuroectoderm in vertebrates. In vitro experiments in *Xenopus* suggested that loss of anterior neuroectoderm was at least partly due to RA effects on the embryonic mesoderm (Ruiz i Altaba and Jessell, 1991). However, RA may also directly influence patterning of the neuroectoderm (e.g. Durstun et al., 1989) and can activate *Hox* gene expression in tissue-cultured embryonal carcinoma cells in the absence of mesodermal tissue (Stornaiuolo et al., 1990). In addition, binding sites for retinoic acid receptors (RAR) occur in the promoter of *Hoxb-1* (Ogura and Evans, 1995) and in the downstream enhancer of both *Hoxa-1* and *Hoxb-1* (Langston and Gudas, 1992; Marshall et al., 1994). However, RA treatment can induce additional somites adjacent the hindbrain in the mouse (Morriss-Kay et al., 1991) and cause abnormal somites and notochord in *Xenopus* and the rat (Ruiz i Altaba and Jessell, 1991; Kraft et al., 1994). Thus, a mesodermally mediated effect of RA on patterning of the vertebrate hindbrain remains possible.

Although our result that RA affects patterning of the amphioxus nerve cord but not development of somites or notochord is consistent with patterning of the nerve cord being independent of mesodermal influences, a role for the mesoderm in neuroectodermal patterning cannot be ruled out. First, even though development of the mesoderm appears normal, genes involved in anteroposterior patterning of the mesoderm have not been identified. Thus, it is not known if RA affects anteroposterior patterning of the mesoderm in amphioxus embryos. Furthermore, even if endogenous RA acts directly on *AmphiHox* gene expression to pattern the nerve cord, the levels of RA in the nerve cord could be controlled by mesodermal signals, which would then be bypassed by exogenous RA. In this regard, an investigation of the mechanism of RA effects in amphioxus would be valuable. An amphioxus retinoic acid receptor (RAR) has been cloned (H. Escriva and V. Laudet, personal communication), although its expression is not yet known. Given the similar effects of RA on *Hox* gene expression in the amphioxus and vertebrate nerve cords and the finding that RA alters *Hox* expression in the nerve cord of ascidian tunicates (Katsuyama et al., 1995), it is tempting to speculate that RA acts via the same mechanism in

all chordates. The presence of RARs in tunicates, however, remains to be investigated.

Patterning of the chordate pharynx

Just as amphioxus has a homolog of the vertebrate hindbrain, it has branchial structures – gill slits and a mouth – similar to the branchial gill slits in vertebrates. However, morphological similarity does not necessarily mean homology. A major difference between amphioxus and vertebrates is that amphioxus apparently lacks migrating neural crest, which in vertebrates contributes to the pharyngeal mesoderm. The neural crest cells express the *Hox* code of the region of the brain from which they emanate and induce *Hox* expression in the branchial ectoderm (Hunt et al., 1991), which is thought to be involved in patterning of the pharyngeal arches (Wilkinson, 1993).

In vertebrates RA can cause loss or fusion of pharyngeal arches (Kraft et al., 1994; Lee et al., 1995), which has been attributed to abnormal patterns of neural crest migration into the arches (Lee et al., 1995). The neural crest takes a more anterior path and, since the *Hox* code of the preotic hindbrain is altered, cells migrating into the first branchial arch express the *Hox* code characteristic of more posterior arches. Thus, while *Hoxb-1* expression in neural crest cells normally occurs only in those migrating from rhombomere 4 into the VII/VIII cranial ganglion, in RA-treated embryos, neural-crest-expressing *Hoxb-1* also migrates into the maxillary region (Morriss-Kay et al., 1993).

RA also affects the amphioxus mouth and gill slits, but there is no evidence for migrating neural crest cells or for expression of either *AmphiHox-3* or *AmphiHox-1* in pharyngeal tissues (the expression pattern of *AmphiHox-2* is not yet known). Thus, effects of RA on pharyngeal patterning in amphioxus must be due to something other than altered *Hox* expression in neural crest. A good candidate is *AmphiPax-1*. The expression pattern of *AmphiPax-1* in the pharyngeal endoderm of amphioxus suggests that it has a role in pharyngeal patterning (Holland et al., 1995b). In RA-treated embryos, *AmphiPax-1* often fails to turn off as it normally does in cells where the gill slits will penetrate.

In vertebrates, the major expression domain of *Pax-1* and the closely related *Pax-9* is in the axial mesoderm (Neubüser et al., 1995). However, both genes are also expressed in the endoderm of the pharyngeal pouches (Neubüser et al., 1995; Peters et al., 1995). Discussions concerning their roles in development have been limited to their roles in formation of the axial skeleton and RA effects on their expression have not been studied, in the pharynx or elsewhere. However, the role of *AmphiPax-1* in patterning the amphioxus pharynx suggests a similar role for *Pax-1* and *Pax-9* in the vertebrate pharynx, a role that has been overshadowed by a focus on the role of neural crest in pharyngeal patterning and by the roles of *Pax-1* and *Pax-9* in the axial mesoderm. Furthermore, the similarity of *Pax* gene expression in the amphioxus and vertebrate pharynx suggests that the amphioxus and vertebrate branchial structures are homologous. Thus in the vertebrate lineage, the evolution of neural crest cells may have been important for the elaboration of the relatively simple branchial arches present in the common ancestor of amphioxus and the vertebrates.

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