

The retinoic acid signaling pathway regulates anterior/posterior patterning in the nerve cord and pharynx of amphioxus, a chordate lacking neural crest

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SUMMARY

Amphioxus, the closest living invertebrate relative of the vertebrates, has a notochord, segmental axial musculature, pharyngeal gill slits and dorsal hollow nerve cord, but lacks neural crest. In amphioxus, as in vertebrates, exogenous retinoic acid (RA) posteriorizes the embryo. The mouth and gill slits never form, *AmphiPax1*, which is normally downregulated where gill slits form, remains upregulated and *AmphiHox1* expression shifts anteriorly in the nerve cord. To dissect the role of RA signaling in patterning chordate embryos, we have cloned the single retinoic acid receptor (*AmphiRAR*), retinoid X receptor (*AmphiRXR*) and an orphan receptor (*AmphiTR2/4*) from amphioxus. *AmphiTR2/4* inhibits *AmphiRAR*-*AmphiRXR*-mediated transactivation in the presence of RA by competing for DR5 or IR7 retinoic acid response elements (RAREs). The 5' untranslated region of *AmphiTR2/4* contains an IR7 element, suggesting possible auto- and RA-regulation. The patterns of *AmphiTR2/4* and *AmphiRAR* expression during embryogenesis are largely complementary: *AmphiTR2/4* is strongly expressed in the cerebral vesicle (homologous to the diencephalon plus anterior midbrain), while *AmphiRAR* expression is high in the equivalent of the hindbrain and spinal cord. Similarly, while *AmphiTR2/4* is expressed most strongly in the anterior and posterior thirds of the endoderm, the highest *AmphiRAR* expression is in the

middle third. Expression of *AmphiRAR* is upregulated by exogenous RA and completely downregulated by the RA antagonist BMS009. Moreover, BMS009 expands the pharynx posteriorly; the first three gill slit primordia are elongated and shifted posteriorly, but do not penetrate, and additional, non-penetrating gill slit primordia are induced. Thus, in an organism without neural crest, initiation and penetration of gill slits appear to be separate events mediated by distinct levels of RA signaling in the pharyngeal endoderm. Although these compounds have little effect on levels of *AmphiTR2/4* expression, RA shifts pharyngeal expression of *AmphiTR2/4* anteriorly, while BMS009 extends it posteriorly. Collectively, our results suggest a model for anteroposterior patterning of the amphioxus nerve cord and pharynx, which is probably applicable to vertebrates as well, in which a low anterior level of *AmphiRAR* (caused, at least in part, by competitive inhibition by *AmphiTR2/4*) is necessary for patterning the forebrain and formation of gill slits, the posterior extent of both being set by a sharp increase in the level of *AmphiRAR*.

Supplemental data available on-line

Key words: Neural patterning, Pharynx, Lancelet, Amphioxus, RAR, RXR, TR2/4, RA

INTRODUCTION

Retinoic acid (RA), an endogenous vitamin A-derived morphogen, regulates cellular proliferation and differentiation in chordate embryos and adults. Too much or too little RA during embryogenesis causes malformations, particularly of the head and pharynx. RA functions via binding to retinoic acid receptors (RARs), which in turn bind preferentially as heterodimers with the retinoid X receptor (RXR) to retinoic acid response elements (RAREs) in the regulatory regions of target genes. Vertebrates have three retinoic acid receptors [RAR (NR1B)] and retinoid X receptors [RXR (NR2B)]

[α , $-\beta$, $-\gamma$ (1,2,3)], each with several alternatively spliced isoforms (see Laudet et al., 1999). The RAR:RXR heterodimer activates transcription by binding to direct repeats (DR) of AGGTCA separated by 1, 2 or 5 nucleotides (reviewed by Laudet and Gronemeyer, 2001). Direct targets described to date include genes for transcription factors [e.g. *Hox* genes (Manzanares et al., 2000), *HNF3 β* (Jacob et al., 1999), *caudal* (Houle et al., 2000), *shh* (Chang et al., 1997)], genes involved in retinoic acid metabolism [e.g. cellular retinoic acid binding protein (CRABP) (Di et al., 1998)], and genes for some secreted and structural proteins (Li et al., 1996; Cho et al., 1998; Yan et al., 2001). RARs and RXRs are expressed in many

developing tissues: RAR α -1 in the mouse spinal cord and hindbrain, pons and basal ganglia; RAR- β preferentially in the foregut endoderm; and RAR- γ in the presomitic mesoderm, tailbud, and caudal neural groove. Treatment of embryos with excess RA induces ectopic expression of RARs, and RAREs have been characterized upstream of each RAR gene (Leid et al., 1992).

The RA-signaling pathway is regulated by rates of synthesis and degradation of RA and by amounts of specific coactivators, co-repressors, RARs and related orphan receptors (e.g. TR2, TR4). TR2/4 genes modulate the effects of RAR binding to target genes in several ways. Both can repress transcription of RA metabolic genes (Chinpaisal et al., 1997) and competitively inhibit activation induced by RAR-RXR binding to RAREs (Chinpaisal et al., 1997; Lee et al., 1997). TR2 can also activate transcription of several genes, including RAR- β 2 (Lee et al., 1997; Wei et al., 2000; Young et al., 1997; Zhang and Dufau, 2000). The *in vivo* function of TR2/4 is unknown. However, *in vitro* experiments and embryonic expression suggest that TR4 may activate CNTFR α (ciliary neurotrophic factor receptor) in mouse nervous systems (Young et al., 1997), while TR2 may repress erythropoietin-induced activation (Lee et al., 1996). TR2 and TR4 are expressed in several developing tissues/neural structures, skeletal muscle, and the second pharyngeal pouch, kidney, liver and ovary (Lee et al., 1996; Young et al., 1997; van Schaick et al., 2000). Expression in early embryos has not been characterized.

To investigate the evolution of embryonic patterning by RA, we are using the invertebrate chordate amphioxus as a model for the ancestral vertebrate. Amphioxus is vertebrate like, but far simpler. It has pharyngeal gill slits, a dorsal hollow nerve cord and notochord, but lacks paired eyes, ears, limbs and neural crest. Moreover, amphioxus has little gene duplication. In the absence of neural crest, patterning of the pharynx appears to be mediated solely by the pharyngeal endoderm. In spite of such simplicity, in amphioxus, as in vertebrates, comparable concentrations of RA applied during the gastrula prevent formation of pharyngeal gill slits and posteriorize the nerve cord (Holland and Holland, 1996).

To further investigate the role of RA signaling in amphioxus development, we cloned the single amphioxus homologs of RAR, RXR and TR2/4, tested their functions *in vitro*, and determined their embryonic expression with and without RA, and in the presence of an RA-antagonist. We show that in the presence of RA, *AmphiTR2/4* competitively inhibits RAR-RXR-mediated transactivation. Moreover, the expression patterns of *AmphiRAR* and *AmphiTR2/4* in early embryos are largely complementary. *AmphiRAR*-expression is lowest in the anterior and posterior thirds of the neurula. It is particularly low where *AmphiTR2/4* is most strongly expressed (in the cerebral vesicle, the anterior endoderm and posterior mesoderm). Exogenous RA upregulates *AmphiRAR* and induces its ectopic expression in the cerebral vesicle and pharynx, while RA-antagonist BMS009 downregulates *AmphiRAR* and induces ectopic gill slit primordia that do not penetrate. These results suggest that levels of *AmphiRAR* mediate the effects of RA in patterning the early amphioxus embryo and that antagonism mediated by *AmphiTR2/4* is necessary for gill-slit formation and for restricting expression of anterior Hox genes to the homolog of the hindbrain and spinal cord.

MATERIALS AND METHODS

Obtaining and manipulating embryos, and cDNA library screening

Methods for collection and embryonic culture of amphioxus (*Branchiostoma floridae*) were according to Holland and Holland (Holland and Holland, 1993). The RA antagonist BSM009 or all-trans retinoic acid in DMSO was added to late blastulae at a final concentration of $1\text{--}1.5 \times 10^{-6}$ M. The RA was removed at hatching (early neurula) by several washes in sea water with no added RA. The RA antagonist was not removed.

The length of the pharynx was measured on fixed specimens with an Olympus SZX17 microscope with a numeric camera JVC KY-FZOBU using the soft Imaging System GmbH, AnaliSIS 3.1 software.

For cDNA library screening, probes of *AmphiRAR* (U93411; 149 bp) and *AmphiTR2/4* (U93412; 134 bp) were obtained by PCR of genomic DNA with degenerate primers (Escriva et al., 1997). The *AmphiRXR* probe (700 bp) was obtained by semi-nested RT-PCR with degenerate primers and first-strand cDNA synthesized from total RNA of 36 hour embryos. Approximately 1×10^6 clones of a cDNA library of 5-24 hour embryos were screened with each probe. The IR7 sequence in the 5' UTR of *AmphiTR2/3* was obtained with a forward IR7 primer (5'-GGTCACGAACTCTGAC-3') (Le Jossic and Michel, 1998) and a reverse primer specific for *AmphiTR2/4* (5'-TAGCTCCTGTGGTTTGGTGTGCG-3'). The IR7 sequence in the 5' UTR of *AmphiTR2/4* was confirmed by inverted PCR with forward primer 51 (5'-AATAAGCACGTCAGTCCAGCG-3'), and nested reverse primers 31 (5'-ATTTTTCACCCATTTTCTGCAG-3') and 32 (5'-CTTCTGCCCATTTTTCACCCAT-3').

Quantification of mRNA

Total RNA was extracted by the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) from ~2000, 15 hour embryos treated with either 2×10^{-6} M RA or BMS009 added as 1:500 dilutions of 1×10^{-3} M stock solutions in DMSO. Control embryos were treated with DMSO alone. A few embryos from each treatment were allowed to develop for 48 hours to ensure that all exhibited affected phenotypes. RT-PCR was performed with 7 μ g total RNA, 1 μ g oligo dT primer and M-MLV reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions. Primers to the 3' UTR of cytoplasmic actin were 5'-AAAGCTACAGGGAGCTT-GTCAGGAC-3' and 5'-CTAGAGCTATGATTCTACGAGAAGTG-3'. For *AmphiTR2/4*, primers were 5'-GCTGGCAAACATACAGCAAGGTGAC-3' and 5'-GCTCGATCACGGTGGTCTGGTCC-TG-3'; for *AmphiRAR*, 5'-TCCTACCCCGCTGGCACGTG-3' and 5'-ACCTGCAGAACTGGCATCTG-3'; and for *AmphiRXR*, 5'-AGAGTCCTCACGGAAGTGGTC-3' and 5'-CCTGTGTGCC-TGCTGCTGTG-3'. The PCR program was 93°C (2 minutes), 93°C (30 seconds), annealing temperature (30 seconds), 72°C (1 minute). For RAR, 1 μ l cDNA was used with 30 cycles at an annealing temperature of 52°C. For TR2/4 the same program was used with 2 μ l cDNA and for cytoplasmic actin, 2 μ l of 1:10 dilution of cDNA was used with 25 cycles at an annealing temperature of 54°C. Samples were visualized on a 1.5% agarose gel stained with Ethidium Bromide.

Measurements of transactivation potential

Ros 17.2/8 (rat osteosarcoma) and Cos-1 (monkey kidney) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). A total of 10^5 cells in six-well plates were transfected using 4 μ l ExGen 500 (Euromedex, Souffelweyersheim, France) with 1.0 μ g (ROS) or 0.5 μ g (Cos-1) total DNA including 0.1 μ g reporter plasmid. *AmphiRAR*, *AmphiRXR* and *AmphiTR2/4* were cloned into the pSG5 expression vector (Stratagene, La Jolla CA). Three tandem repeats of oligonucleotides encompassing consensus DR5 or IR7 sequences were cloned respectively into pBLCAT4 (Stratagene, La Jolla, CA) and the pGL2-

promoter vector (Promega, Madison, WI). The culture medium was changed 6 hours after transfection and, when appropriate, all-trans retinoic acid (RA) or the RA antagonist BMS009 in DMSO was added to final concentrations of 1×10^{-7} M and 1×10^{-5} - 10^{-8} M, respectively. Cells were lysed 48 hours after transfection and assayed for luciferase or CAT activity.

In situ hybridization

AmphiRAR, *AmphiRXR* and *AmphiTR2/4* and *AmphiPax1/9* cDNAs were used for synthesis of antisense riboprobes. Fixation, whole-mount in situ hybridization and histological sections were obtained as previously described (Holland et al., 1996). To obtain good results, two probes were combined for *AmphiRAR* and *AmphiTR2/4*—one synthesized to the 3' UTR plus, for *RAR*, a 735 bp probe to the 5' end of the cDNA and for *TR2/4* a 2 kb probe to the 5' 2/3 of the 2.7 kb cDNA.

RESULTS

AmphiRAR and AmphiTR2/4 are mutual antagonists

Phylogenetic and Southern blot analyses showed that amphioxus has only one *AmphiRAR* gene, one *AmphiTR2/4* gene and one *AmphiRXR* gene (see Supplemental Data – <http://dev.biologists.org/supplemental>). The ability of *AmphiTR2/4* and *AmphiRAR*-*AmphiRXR* to bind to a synthetic RARE, the DR5 element (direct repeat of the core RGGTCA element with a 5 bp spacer), and to an IR7 element (inverted repeat with a 7 bp spacer) was tested by electrophoretic mobility shift assays (EMSA) (data not shown). The specificity of binding was assessed by competition experiments with cold oligonucleotides containing wild-type or mutated DR5 or IR7 elements. Both *AmphiTR2/4* and *AmphiRAR*-*AmphiRXR*, like their vertebrate counterparts, bind the DR5 and IR7 elements. *AmphiTR2/4* binds the latter element with an apparently higher affinity than it does the DR5 element (data not shown).

Transcriptional regulation was assayed in two mammalian cell lines, Ros 17/2.8 and Cos-1. In both, the *AmphiRAR*-*AmphiRXR* heterodimer activated transcription in the presence of RA (Fig. 1A). The EC 50 (50% maximal activation) occurred at 10^{-8} M RA (data not shown), similar to results with a mammalian RAR-RXR heterodimer (Laudet and Gronemeyer, 2001). However, co-transfection with increasing quantities of *AmphiTR2/4* repressed transcription (Fig. 1A), suggesting that in amphioxus, as in vertebrates, *AmphiTR2/4* can inhibit RA signaling.

To examine the regulation of *AmphiTR2/4*, we used RT-PCR and inverse PCR to reveal an IR7 element (5'-GGGTCA-CGAACTCTGACCC-3') in the 5' UTR of *AmphiTR2/4*. This element is 100% identical to that in *TR2* genes from the sea urchin and many vertebrates (Le Jossic and Michel, 1998). In Ros 17/2.8 cells, transcription from the IR7 or DR5 elements is stimulated 7- to 10-fold by *AmphiRAR*-*AmphiRXR* in the presence of RA (Fig. 1A,B). No activation occurred in Cos cells. Evidently, either Ros 17/2.8 cells contribute additional factors not present in Cos-1 cells or Cos-1 cells contain inhibitors.

Co-transfection of increasing amounts of the *AmphiTR2/4* expression vector markedly decreased *AmphiRAR*-*AmphiRXR*-stimulated transcription on the IR7 element (Fig. 1B). At the lowest concentrations of *AmphiTR2/4*, transcription on the IR7 element was repressed more than on

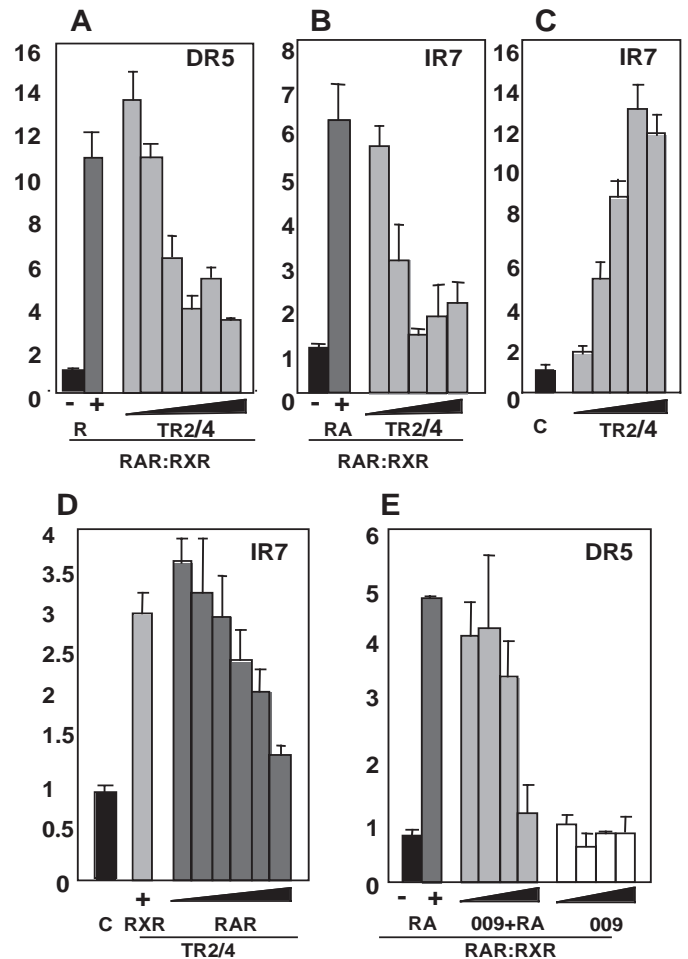


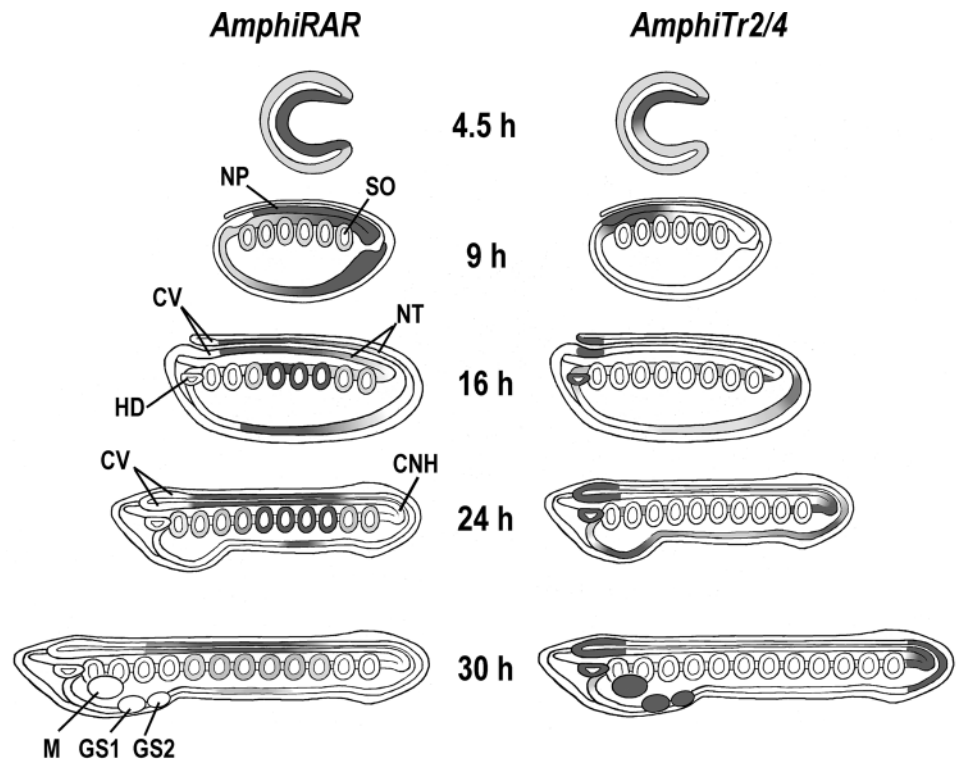
Fig. 1. Transcriptional activities of *AmphiRAR* and *AmphiTR2/4* on the consensus RARE (DR5) and IR7 elements expressed as the amount of chloramphenicol acetyl transferase (CAT) activity (A) or luciferase (Luc) activity (B-E) relative to control. Values are averages of three to five independent transfections in ROS17.2/8 cells. Error bars indicate \pm one s.d. (A) Dose-dependent repression by *AmphiTR2/4* (5-300 ng) of RA-induced transactivation and by *AmphiRAR*-*AmphiRXR* (10-500 ng each) on the DR5 element. (B) Dose-dependent repression by *AmphiTR2/4* of RA-induced transactivation on the IR7 element (as in A, except for co-transfection with the IR7-containing pGL2 plasmid instead of pBLCAT). (C) Transactivation induced by *AmphiTR2/4* on the IR7 element without RA or *AmphiRAR*-*AmphiRXR*. Conditions as in B. (D) Inhibition by *AmphiRAR*-*AmphiRXR* of transactivation induced by *TR2/4* (100 ng) on the IR7 element without RA. *AmphiRXR*, 300 ng. *AmphiRAR*, 10-500 ng. (E) Antagonism by BMS009 on transcription induced by RA. Dark bars on left represent activation by RAR-RXR±RA. Light gray bars represent activity with added BMS009. White bars represent activity with BMS009, no RA.

the DR5 element (compare Fig. 1B with 1A). However, even at the highest concentrations of *AmphiTR2/4*, transcription was higher than in the control without added RA (Fig. 1A,B). As transfection with *AmphiTR2/4* and the IR7 plasmid (no *AmphiRAR*, *AmphiRXR* or RA) activated transcription (Fig. 1C), it appears that *AmphiTR2/4* has a higher affinity than *AmphiRAR*-*AmphiRXR* for the IR7 element. Thus, *AmphiTR2/4* can competitively inhibit transcription mediated by *AmphiRAR*-*AmphiRXR*. However, because *AmphiTR2/4*

Fig. 2. *AmphiRAR* and *AmphiTR2/4*

expression in normal amphioxus embryos. Anterior is towards the left. At 4.5 hours (mid-gastrula), the expression of the two genes considerably overlaps. However, by 9 hours (early neurula), their expression patterns begin to become complementary. *AmphiRAR* is expressed posteriorly in the neural plate (NP), weakly in the somites (S) and throughout the mesendoderm, with expression strongest posteriorly, whereas *AmphiTR2/4* is most strongly expressed in the anterior neural plate and underlying mesendoderm. By 16 hours, complementarity is more pronounced. *AmphiRAR* is downregulated in posterior and anterior tissues. It is most strongly expressed in the middle third of the neural tube (NT), somites and endoderm but not in the cerebral vesicle (CV) or notochord (N). By contrast, *AmphiTR2/4* is most strongly expressed in the cerebral vesicle, Hatschek's anterior left diverticulum (HD) and the anterior and posterior endoderm. At 24 hours, endodermal expression of *AmphiRAR* is downregulated except in a small ventromedial area. Expression of *AmphiTR2/4* remains high in the cerebral vesicle, Hatschek's diverticulum, the chordoneural hinge (CNH) and anterior and posterior endoderm.

By 30 hours, the primordia of the mouth (M) and first two gill slits (GS1; GS2) have formed. Expression of *AmphiRAR* is restricted to the middle third of the nerve cord and weakly in the middle third of the somites and endoderm while that of *AmphiTR2/4* is strongest in the cerebral vesicle, Hatschek's diverticulum, the tailbud, mouth and gill slits. NP, neural plate; SO, somite; CV, cerebral vesicle; HD, Hatschek's left anterior diverticulum; NT, neural tube; CNH, chordoneural hinge; M, mouth; GS1 and GS2, gill slits.



itself is a transcriptional activator on an IR7 element, transcription is never reduced to zero. The competition model between RAR-RXR and TR2/4 for occupancy of their DNA-binding sites is supported by the repression of *AmphiTR2/4*-mediated activation by *AmphiRAR* in the absence of RA (Fig. 1D). In summary, functional antagonism between *AmphiTR2/4* and *AmphiRXR*-*AmphiRAR* probably reflects their competition for the IR7 element.

BMS009, which antagonizes RA-induced transactivation mediated by binding of RAR-RXR to DR5 elements (Benoit et al., 1999), inhibited activation induced by 1×10^{-7} M RA in Ros 17/2.8 cells transfected with *AmphiRAR*-*AmphiRXR* and the DR5 element (Fig. 1D). Inhibition was dose dependent. Without RA, BMS009 did not affect basal expression of the reporter plasmid (Fig. 1E). Similar results were obtained with a second RA antagonist BMS493 (data not shown).

Expression of *AmphiTR2/4* and *AmphiRAR* is largely complementary

Although expression patterns of *AmphiRAR* and *AmphiTR2/4*, initially overlap, as development proceeds the patterns become largely complementary (summarized in Fig. 2). Expression of both genes is undetectable in the blastula (Fig. 3A). However, by the mid-gastrula, the RA-sensitive period, *AmphiRAR* is strongly expressed throughout the mesendoderm and more weakly throughout the ectoderm (Fig. 3B; summarized in Fig. 2). At that stage, *AmphiTR2/4* is strongly expressed in the dorsal mesendoderm and more weakly expressed in the

ectoderm (Fig. 4A). By the early neurula (15 hours), *AmphiRAR* becomes downregulated anteriorly in the neural plate (arrow, Fig. 3C), in the anterior endoderm and in the non-neural ectoderm (Fig. 3C). Expression remains high in the posterior mesoderm, somites and the posterior three-quarters of the neural plate and endoderm (Fig. 3C-E). However, by the mid-neurula, expression is downregulated in the posterior third of the endoderm (Fig. 3F). By 20 hours, the only remaining strong expression of *AmphiRAR* is in the nerve cord, posterior to the cerebral vesicle, the somites in the middle third of the embryo and in a small region of the endoderm (Fig. 3I).

From 15-20 hours, expression of *AmphiTR2/4* becomes increasingly complementary to that of *AmphiRAR*. By the early neurula, *AmphiTR2/4* is strongly expressed in the anterior neuroectoderm, particularly in the future cerebral vesicle, and the dorsoanterior endoderm (Fig. 4B,C). It is never expressed in the somites. By 19 hours, expression is most intense posteriorly and anteriorly, especially in the posterior mesoderm, cerebral vesicle and Hatschek's anterior left gut diverticulum, which is homologous to part of the vertebrate pituitary (Fig. 4D). The pattern remains essentially the same as development progresses.

By 24 hours, expression of *AmphiRAR* is limited to the nerve cord posterior to the cerebral vesicle with some weak expression in the endoderm (Fig. 3J). By contrast, expression of *AmphiTR2/4* is strong in the cerebral vesicle, in Hatschek's diverticulum in (arrows, Fig. 4E,F), and in the primordia of the mouth and first gill slit (Fig. 4E, arrowhead). There is also

weak expression in the gut, strongest posteriorly (Fig. 4E,G). At 26 hours, expression persists in the tailbud, cerebral vesicle and in pharyngeal structures (particularly in the forming mouth), first gill slit and Hatschek's diverticulum (Fig. 4H,I).

The mouth and first gill slit penetrate by 30-36 hours. By 2-3 days, *AmphiRAR* is expressed only in the middle third of the nerve cord, somites and gut (Fig. 3K). In contrast, by 34 hours, expression of *AmphiTR2/4* in the mouth and the gill slit primordia has increased (Fig. 4J arrow). Strong expression persists in the cerebral vesicle, mouth and gill slits (Fig. 4K,L) and is downregulated elsewhere in the larva (Fig. 4M), except for the tailbud.

The expression of *AmphiRXR*, unlike that of *AmphiTR2/4* and *AmphiRAR*, is uniform and very weak, even when probes to the 3' and 5' halves of the cDNA were combined (data not shown). Moreover, attempts to quantify *AmphiRXR* by RT-PCR were unsuccessful, suggesting that levels of *AmphiRXR* expression may be fairly low (data not shown).

Excess RA and the RA antagonist BMS009 have opposite effects on anteroposterior patterning of the pharynx and on formation of the mouth, but both prevent gill slit penetration

Thirty to 36 hours after fertilization, the pharynx in normal embryos is clearly visible, as it bulges ventrally, its posterior limit being marked by a decrease of 20-25% in the height of the larva (Fig. 3K, Fig. 4J). The mouth, which has been considered to be a modified gill slit (van Wijhe, 1913), opens on the left side of the pharynx (Fig. 3K, Fig. 4K), while the first two gill slits form just posterior to the mouth on the ventral/right side of the larva (arrow Fig. 4J,L). At 30-36 hours, the posterior limit of the pharynx is just anterior to the level of the first pigment spot in the nerve cord (Fig. 4J). As the embryo adds more gill slits, the pharynx expands posteriorly. In normal embryos, *AmphiPax1/9* is a marker of the pharyngeal endoderm (Holland et al., 1995). It is initially expressed throughout the pharyngeal endoderm, subsequently becoming downregulated in the primordia of the mouth and gill slits (Fig. 3O) (Holland et al., 1995). Its posterior limit of expression, which coincides with the posterior limit of the pharynx, is just anterior to the level of the first pigment spot in the nerve cord, which is a good marker of anterior/posterior position (Fig. 3O).

Embryos treated with 1.5×10^{-6} M RA or BMS009 initially appear normal. However, in RA-treated embryos, the posterior limit of the pharynx has shifted anteriorly by 26 hours of development (compare Fig. 3K with 3N). In addition, the mouth and gill slits never form (Fig. 3N, Fig. 4O) (Holland and Holland, 1996). Concomitantly, the expression domain of *AmphiPax1/9* shifts anteriorly and remains upregulated where mouth and gill slits would normally penetrate (Holland and Holland, 1996). By contrast, by 26 hours, the pharynx of BMS009-treated larvae is expanded posteriorly (Fig. 3P, Fig. 4P). Not surprisingly, BMS009 broadens the expression of *AmphiPax1/9* posteriorly, extending it posterior to the first pigment spot (Fig. 3P). There is little or no downregulation of *AmphiPax1/9* where gill slits would be expected to penetrate (Fig. 3P).

By 36 hours, the length of the pharynx in BMS009-treated larvae has nearly doubled, from about 2.75 mm to about 4.8 mm (Fig. 4T, Fig. 5). This is mirrored by the domain of *AmphiPax1/9* expression, which extends well posterior to the first pigment spot (Fig. 3Q). The mouth, which penetrates at

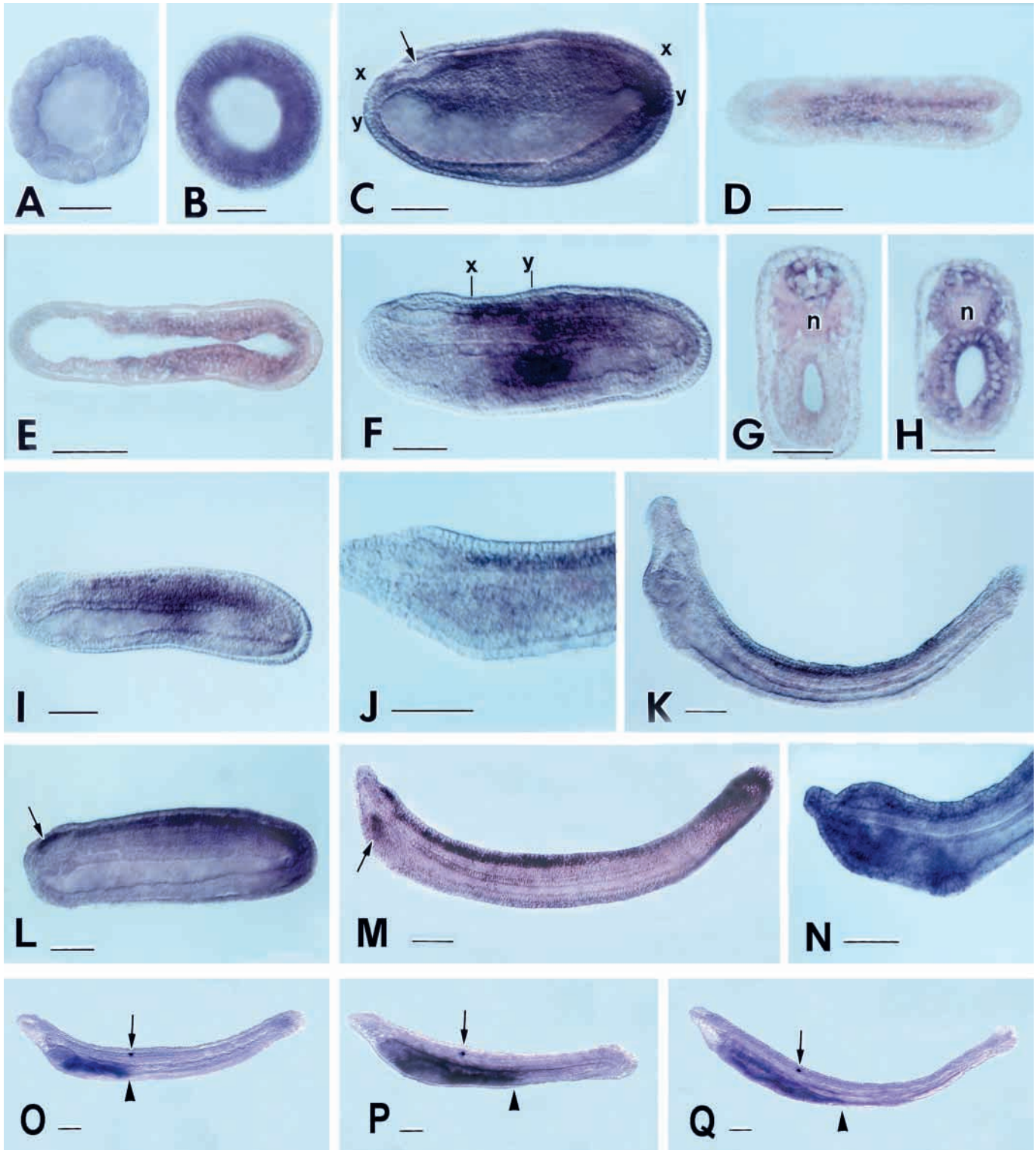
the normal time, is typically larger than normal (Fig. 4T). In embryos that are less severely affected by BMS009, gill slit primordia can be seen in living or fixed material as lines along the ventral surface of the pharynx. Rudiments of the first two gill slits form, but they are shifted posteriorly (Fig. 4T). Unlike the mouth, they fail to penetrate, and *AmphiPax1/9* remains upregulated throughout the pharynx posterior to the mouth (Fig. 3Q). That these are non-penetrating gill slit primordia is shown by their morphology in cross-section (compare Fig. 4L with 4U). In normal gill slit primordia, the pharyngeal endoderm is locally thickened with a medial cleft where each gill-slit will penetrate (Fig. 4L). By contrast, posterior to the pharynx, at the level of the first pigment spot, the endoderm is uniformly thin (Fig. 4M). However, in BMS009-treated larvae, the pharyngeal endoderm at the level of the first pigment spot is thickened ventrally with a median cleft (Fig. 4U), similar to normal gill slit primordia (Fig. 4L). In addition, in some BMS009-treated embryos, there are additional gill slit primordia extending to the posterior end of the expanded pharynx (Fig. 4T). Co-application of increasing amounts of RA together with BMS009 restores the normal length of the pharynx (Fig. 5), showing that the effect of BMS009 is due strictly to its antagonism of RA.

Excess RA and the RA-antagonist BMS009 have opposite effects on the expression of *AmphiRAR* and *AmphiTR2/4*

RA applied during the gastrula stage strongly upregulates expression of *AmphiRAR* (Fig. 6). Moreover, expression of *AmphiRAR* in the nerve cord is extended anteriorly into the cerebral vesicle (Fig. 3L-N, Fig. 7). In addition, it is upregulated in the posterior third of the endoderm (arrow, Fig. 3L,M). By 40 hours, endodermal expression is downregulated, except in the pharynx, where it is strongly upregulated, particularly where the mouth and first gill slit would have penetrated in untreated larvae (Fig. 3N). Expression remains strong throughout the nerve cord, including the cerebral vesicle (Fig. 3N).

RA slightly upregulates overall expression of *AmphiTR2/4* (Fig. 6) at 15 hours, and alters the pattern of expression in the endoderm. Anteriorly, the region of strongest endodermal expression is more restricted, while the region of strong posterior expression is expanded anteriorly (Fig. 4N). Although expression in the cerebral vesicle is not immediately affected (Fig. 4N), by 36 hours it is downregulated (Fig. 4O), as is expression in the pharynx and tailbud. Longer staining of embryos at this stage hybridized with the *AmphiTR2/4* riboprobe did not result in increased signal.

Not surprisingly, while gastrulae treated with BMS009 still express *AmphiRAR* throughout the mesendoderm, expression is no longer detectable by the late gastrula by in situ hybridization or by RT-PCR (Fig. 6). Normal embryos hybridized in parallel with the RAR probes labeled strongly (data not shown). In BMS009-treated embryos, although the overall level of *AmphiTR2/4* expression is scarcely affected, expression appears somewhat upregulated throughout the endoderm, particularly in Hatschek's diverticulum (Fig. 4P-S). Expression in the cerebral vesicle appears unaffected, and as in normal embryos, there is no expression in the notochord (Fig. 4Q-S). By 48 hours, expression is downregulated except for Hatschek's diverticulum and the expanded pharyngeal endoderm (Fig. 4T,U).



DISCUSSION

Conservation of the anterior/posterior distribution of RAR transcripts in amphioxus and vertebrates

A role for endogenous RA in patterning early embryos has been clearly demonstrated only for chordates – tunicates, amphioxus and vertebrates. RA-treated tunicate embryos, like

amphioxus and vertebrate embryos, are fore-shortened and lack pharyngeal gill slits (Katsuyama et al., 1995; Hinman and Degnan, 1998). Endogenous retinoids have been identified in tunicates (Kawamura et al., 1993), and *RAR* and *RXR* have been cloned (Hisata et al., 1998; Kamimura et al., 2000).

Regional differences of endogenous RA in vertebrate

Fig. 3. Expression of *AmphiRAR* (A-N) in amphioxus embryos in the absence (A-K) and presence (L-N) of 1×10^{-6} M RA and of *AmphiPax1* in the presence of 1.5×10^{-6} M BMS009 (O-Q). Whole mounts and frontal sections (counterstained pink) with anterior towards the left. Cross sections viewed from posterior end. (A) No expression in the blastula. (B) Gastrula with ubiquitous expression. (C) Early neurula (15 hours). Expression downregulated in the cerebral vesicle (arrow), anterior endoderm and non-neural ectoderm. (D) Frontal section through x-x in C. Transcripts abundant in posterior mesoderm, somites and neural plate posterior to the cerebral vesicle. (E) Frontal section through y-y in C. Transcripts most abundant in the posterior three quarters of the endoderm. (F) Eighteen hour neurula. Expression is downregulated in the anterior third of the nerve cord and upregulated in the middle third. (G) Cross section through x in F. Expression throughout the nerve cord and very weakly in the somites adjacent the notochord. (H) Cross section through y in F. Expression strong in the nerve cord, somites and endoderm. (I) Twenty hour neurula. Expression downregulated in the pharyngeal endoderm. (J) Twenty-four hour embryo. Expression strong in the nerve cord posterior to the cerebral vesicle and a small region of endoderm, but largely downregulated elsewhere. (K) Two day larva. Expression most pronounced in middle of nerve cord. No expression in posterior quarter of the embryo or in forming gill slits. (L) Twenty-two hour embryo (RA treated). Expression generally upregulated extending into the dorsal part of the cerebral vesicle (arrow). (M) Twenty-six hour embryo (RA treated). Expression in the cerebral vesicle and upregulated in the pharynx. (N) Forty hour larva (RA treated). Gill slits and mouth absent. Expression anteriorized and upregulated in the pharynx. (O) Expression of *AmphiPax1/9* in the pharynx in a normal 26 hour embryo (arrow indicates first pigment spot in nerve cord; arrowhead indicates posterior limit of pharynx marked by the posterior limit of *Pax1/9* expression). (P) *AmphiPax1/9* expression is expanded posteriorly in a 24 hour embryo treated with BMS009 (arrow and arrowhead as in O). (Q) *AmphiPax1/9* expression remains posteriorly expanded in a 36 hour larva treated with BMS009 (arrow and arrowhead as in O). Scale bars: 50 μ m for whole mounts; 25 μ m for sections. n, notochord.

embryos have been measured directly (Maden et al., 1998) and indirectly by expression of genes involved in RA synthesis and metabolism or containing RAREs (Rossant et al., 1991; Båvik et al., 1997; Berggren et al., 1999; Perz-Edwards et al., 2001). In general, levels of RA or enzymes of RA-metabolism are lowest in all three germ layers in the anterior third of vertebrate embryos, highest in the middle third and intermediate in the posterior third (Creech Kraft et al., 1994; Båvik et al., 1997; Hollemann et al., 1998; Morriss-Kay and Ward, 1999; Chen et al., 1994; Chen et al., 2001). For example, in the nerve cord, the highest levels are in the posterior hindbrain and spinal cord (Wagner et al., 1992).

In general, patterns of RAR expression parallel levels of endogenous RA. RAREs occur in the regulatory regions of some RAR genes (Leid et al., 1992; Perz-Edwards et al., 2001), suggesting regulation by RA. Moreover, excess RA induces ectopic expression of RARs (Leid et al., 1992). Thus, although most tissues express RARs (Joore et al., 1994; Mollard et al., 2000), levels are highest in the posterior hindbrain/anterior spinal cord and in somites and gut in the middle third of vertebrate embryos.

Amphioxus embryos are too small for direct measurements of regional differences in RA. However, our results show that, as in vertebrates, *AmphiRAR* expression is sensitive to RA

levels, being strongly upregulated by excess RA and completely downregulated by an RA antagonist. Therefore, *AmphiRAR*, the only RAR in amphioxus, may contain an RARE. Consequently, its expression probably reflects levels of endogenous RA. As in vertebrates, expression of *AmphiRAR* in the mid-neurula is lowest in the anterior third, highest in the middle third and intermediate in the posterior third of the embryo.

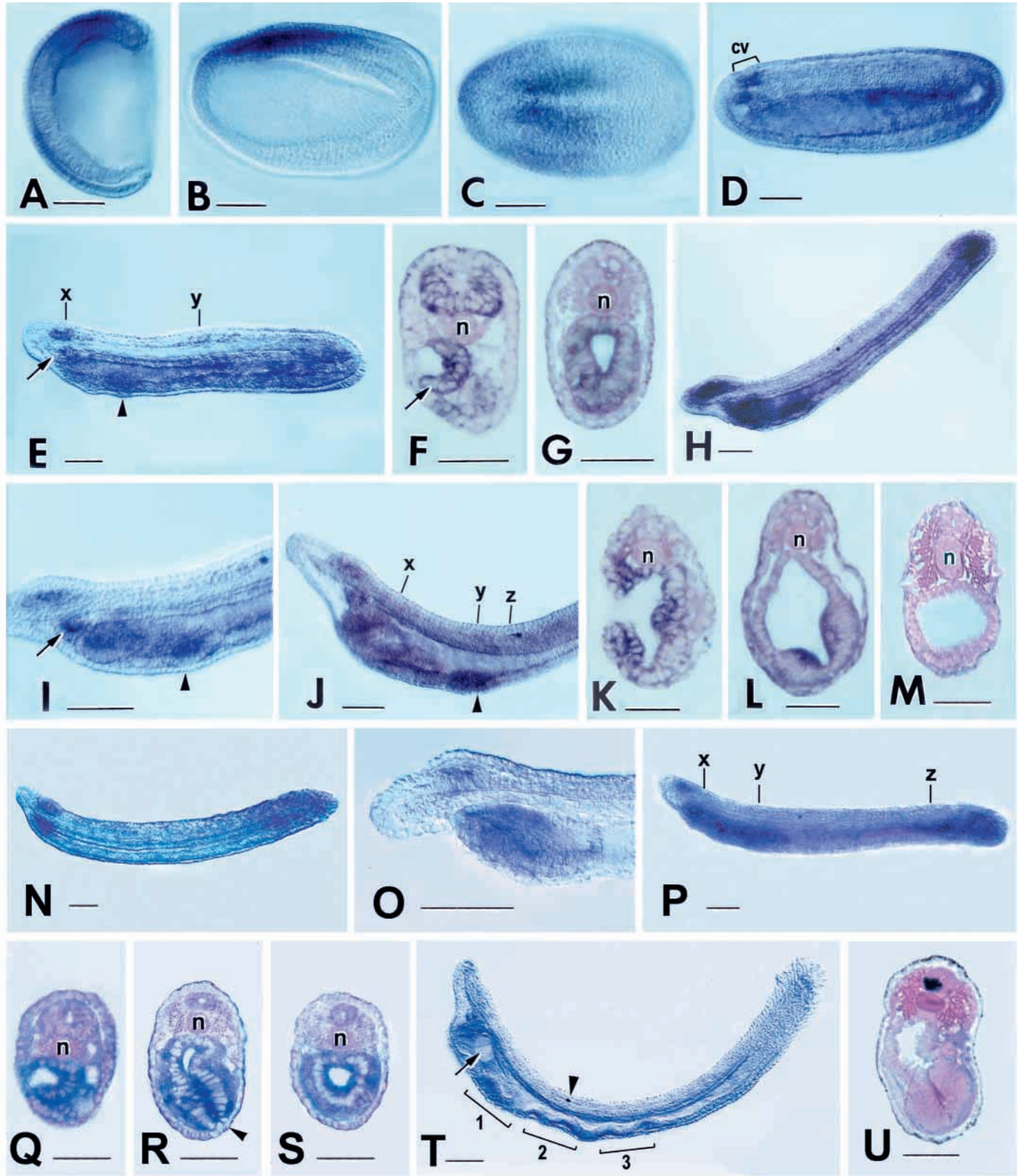
The amphioxus nerve cord includes an anterior swelling, the cerebral vesicle, which is homologous to the diencephalon plus the anterior part of the midbrain (Holland and Chen, 2001). *AmphiRAR* is expressed posterior to the cerebral vesicle, with the highest expression in the hindbrain homolog. Similarly, in the endoderm, expression declines abruptly just behind the primordium of the third gill slit, while in the somites, the peak level of expression is posterior to that in the nerve cord and endoderm. Just as RA is undetectable in the notochord and ectoderm of chick embryos (Maden et al., 1998), expression of *AmphiRAR* in the notochord and ectoderm of amphioxus neurulae is not detectable. Thus, the pattern of endogenous RA in amphioxus embryos is probably very like that in vertebrates, suggesting an evolutionarily conserved role for RA signaling in patterning along the anterior/posterior axis in amphioxus and vertebrates.

TR2/4 probably functions in vivo to downregulate RA signaling

Our results show that *AmphiTR2/4*, like its vertebrate homologs (Harada et al., 1998), can competitively inhibit *AmphiRAR*-*AmphiRXR* activated transcription. Conversely, in the absence of RA, *AmphiRAR*-*AmphiRXR* can inhibit activation induced by *AmphiTR2/4*. The IR7 element in the 5' UTR of *AmphiTR2/4* suggests that *AmphiTR2/4* is both transcriptionally autoregulated and crossregulated by *AmphiRAR*-*AmphiRXR*. The mutual antagonism between *AmphiTR2/4* and *AmphiRXR*-*AmphiRAR* suggests a complex regulation. The relatively small effect on *AmphiTR2/4* mRNA levels by RA and BMS009 supports this conclusion. Thus, autoregulation may predominate in anterior regions of amphioxus embryos where RA concentrations are probably low, whereas more posteriorly, where RA levels are higher, regulation by *AmphiRAR*-*AmphiRXR* may predominate. The conservation of the IR7 element in *TR2/4* homologs in amphioxus, sea urchins and vertebrate *TR2* (but not *TR4*) genes (Le Jossic and Michel, 1998) suggests that this complex regulation is an ancient property of *TR2/4* genes, which was lost in vertebrate *TR4*. Interestingly, sea urchin *TR2/4* does not appear to be regionally localized (Kontrogianni-Konstantopoulos et al., 1998). As RA has little effect on sea urchin development, *TR2/4* may function differently in sea urchins. The largely complementary expression patterns of *AmphiTR2/4* and *AmphiRAR* suggest that in vivo, as in vitro, *AmphiTR2/4* inhibits *AmphiRAR*. A similar relationship may occur in vertebrates.

Evolutionarily conserved role of RA in patterning the nerve cord

In vertebrates and amphioxus, RA signaling appears to function similarly in anteroposterior patterning of the hindbrain. In both, excess RA posteriorizes the nerve cord as shown by an anterior shift in expression of *Hox1* and *Hox3*



genes (Holland and Holland, 1996). Moreover, in zebrafish, as in amphioxus, excess RA induces ectopic expression of RARs in anterior brain structures (Joore et al., 1994). Whether this function in hindbrain patterning evolved only with amphioxus or whether it evolved earlier is unclear. Although RA treatment

of ascidian tunicates fore-shortens the trunk, effects on *Hox1* expression differ from those in other chordates. *Hox1* expression is ectopically induced in non-neural ectoderm and at the anterior tip of the nerve cord, but expression more posteriorly is unchanged (Katsuyama et al., 1995). Perhaps the

Fig. 4. Expression of *AmphiTr2/4* in untreated (A-M) amphioxus embryos and in embryos treated with 1×10^{-6} RA (N,O) or BMS009 (P-U). For whole mounts, anterior is towards the left. Cross-sections (counterstained pink) viewed from posterior end. (A) Mid-gastrula. Expression in dorsal hypoblast and epiblast. (B) Thirteen hour neurula. Intense expression in anterior neural plate and endoderm. (C) Dorsal view of embryo in B. (D) Nineteen hour embryo. Transcripts present in the cerebral vesicle (cv) and the endoderm, most strongly in the posterior third and in Hatschek's anterior left diverticulum (just below the cerebral vesicle). (E) Twenty-four hour neurula. Strong expression in cerebral vesicle and throughout the endoderm, in Hatschek's anterior left diverticulum (arrow) and primordium of first gill slit (arrowhead). (F) Cross-section through x in E. Strong expression throughout the cerebral vesicle and in Hatschek's anterior left diverticulum (arrow). (G) Cross-section through y in E. Weak expression throughout pharyngeal endoderm. (H) Twenty-six hour embryo, overstained. Expression in tailbud, cerebral vesicle and pharyngeal endoderm. (I) Higher magnification of an embryo at the same stage as in H. (arrow indicates Hatschek's diverticulum; arrowhead indicates primordium of first gill slit). (J) Thirty-four hour larva. Expression upregulated in endoderm of first gill slit (arrowhead). (K) Cross-section through x in J. Strong expression in pharyngeal endoderm and ectoderm around the open mouth. (L) Cross-section through y in J. Strong expression in gill slit primordium. (M) Cross-section through z in J. Very weak expression in non-pharyngeal endoderm. (N) Twenty-four hour embryo, RA treated. *AmphiTR2/4* expression anteriorized in the pharynx, but unaffected in cerebral vesicle. (O) Thirty-six hour larva, RA treated. Compare with control in J. Expression anteriorized in pharynx and largely downregulated in cerebral vesicle. (P) Twenty-six hour embryo, BMS009 treated, with slightly expanded pharynx. Expression of *AmphiTR2/4* is strong in the endoderm (particularly in the anterior and posterior thirds) and posterior mesoderm and moderate in the cerebral vesicle. x, y, z indicate levels of cross sections in Q-S. (Q) Cross-section through level x in P. Expression is strong in endoderm, particularly in Hatschek's diverticulum, and weaker in the cerebral vesicle. (R) Cross-section through level y in P. Expression is strong in gill slit primordium (arrowhead). (S) Cross-section through level z in P. (T) Forty-eight hour embryo (arrow indicates the mouth; arrowheads indicate the first pigment spot in the nerve cord; 1, 2, 3 indicate expanded non-penetrating gill slit primordia). (U) Cross-section through a 48 hour larva at the level of first pigment spot in the nerve cord. Expression is weak in the expanded pharyngeal endoderm. Scale bar: 50 μ m in whole mounts; 25 μ m in sections.

role of RA in nerve cord patterning became modified in ascidians when nerve cell bodies in the tail nerve cord were lost. Nerve cell bodies are present in the tail nerve cord of appendicularian tunicates, which are basal within the tunicates, and the expression of *Hox1* in these tunicates could be informative.

The expression of RARs suggests that in both amphioxus and vertebrates, the intensity of RA signaling is very low anterior to a boundary just rostral to or within the hindbrain and much higher posterior to this boundary. In amphioxus, this boundary is between the cerebral vesicle and the hindbrain homolog. Among different vertebrates, it usually lies somewhere in the hindbrain (Ruberte et al., 1991; Joore et al., 1994; Smith, 1994; Mollard et al., 2000). Not surprisingly, an increase of either RA or RAR levels posteriorizes the hindbrain, while a decrease anteriorizes (Holleman et al., 1998; Dupé et al., 1999). Since RA regulates patterning in the hindbrain, at least in part, through RAREs (DR5) in the 5'

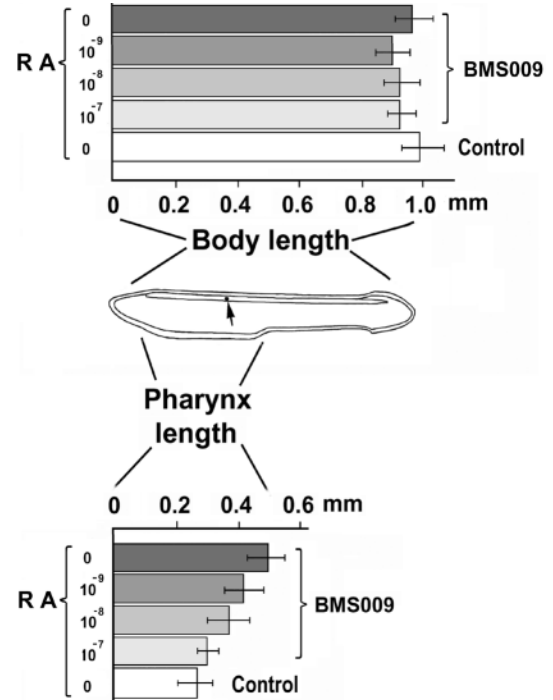


Fig. 5. RA treatment inhibits expansion of the pharynx in BMS009-treated larvae. (Top) Body length in larvae treated with 1×10^{-6} M BMS009 and $0-1 \times 10^{-6}$ M RA is same as in untreated controls. (Bottom) The length of the pharynx is approximately doubled in larvae treated with 1×10^{-6} M BMS009. Pharyngeal length is progressively restored to normal by increasing amounts of RA. Each bar represents the average of 10 larvae. Error bars represent \pm one sd.

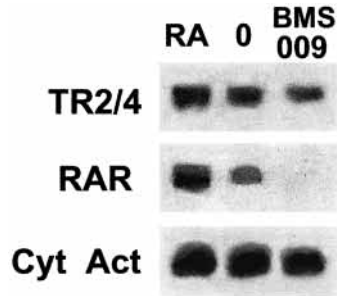
regulatory regions of *Hoxa1* and *Hoxb1* (Frasch et al., 1995), overexpression of *Hoxa1*, *Hoxb1* or *Hoxb2*, like excess RA or overexpression of RAR, posteriorizes, giving rhombomere 2 a rhombomere 4 identity (Zhang et al., 1994; Alexandre et al., 1996). Similarly, low levels of RA upregulate expression of *Hoxb1* and *Hoxb2* and shift the pattern of expression anteriorly (Marshall et al., 1992).

The role of RA signaling in patterning the hindbrain appears to be similar in amphioxus and vertebrates. Excess RA shifts *AmphiHox1* and *AmphiRAR* expression anteriorly in the nerve cord (Holland and Holland, 1996). Not surprisingly, *AmphiHox-1*, like its vertebrate homologs, contains an RARE (Manzanares et al., 2000). In *Xenopus*, RAR-RXR levels directly control the number of primary neurons in the hindbrain (which express *Isl1*) (Sharpe and Goldstone, 2000). In amphioxus, as in vertebrates, *islet*-expressing motoneurons occur on either side of the floor plate (Lacalli and Kelly, 1999; Jackman et al., 2000), and it seems likely that they may also be controlled by RA signaling.

Segmentation of the pharynx is mediated by RA signaling in the pharyngeal endoderm in amphioxus and other chordates

Until recently, pharyngeal defects in vertebrates caused by excess RA were thought due to abnormalities in neural crest. The chief evidence is that neural crest-derived structures such as the branchial cartilages are abnormal and fused in RA-treated

Fig. 6. Quantification of mRNA of *AmphTR2/4* and *AmphiRAR* by RT-PCR normalized to that of cytoplasmic actin in 15 hour embryos treated with 2×10^{-6} M RA (RA), in control embryos treated with DMSO alone (0) and with 2×10^{-6} M BMS009 (BMS009). Even when additional sample was loaded, no amplification of *AmphiRAR* was detectable in BMS009-treated embryos.



embryos. Moreover, these embryos resemble those in which *Hox1* and *Hox2* genes are mis-expressed in neural crest migrating into the pharyngeal arches (Alexandre et al., 1996). However, recent experiments show that patterning of the pharynx by neural crest is secondary to patterning by the pharyngeal endoderm. Thus, when neural crest is ablated or prevented from forming, the pharyngeal arches form and are patterned correctly (Gavalas et al., 2001; Graham and Smith, 2001). Conversely, in a mutant zebrafish lacking pharyngeal arch segmentation, gill slits do not form, although early migration of neural crest cells is normal (Piotrowski and Nüsslein-Vollhard, 2000).

The role of pharyngeal endoderm in patterning the pharynx evidently preceded the evolution of neural crest. In hemichordates, tunicates and amphioxus, which lack neural crest, the pharyngeal endoderm expresses similar suites of genes during gill slit formation (e.g. *Pax1/9*) as in vertebrates (Holland et al., 1995; Müller et al., 1996; Peters et al., 1998; Ogasawara et al., 1999; Ogasawara et al., 2000). Furthermore, RA treatment eliminates pharyngeal gill slits in at least tunicates (Hinman and Degnan, 1998), amphioxus (Holland and Holland, 1996) and lampreys (Kuratani et al., 1998), as well as vertebrates (Helms et al., 1997). In both amphioxus and vertebrates, expression of *Pax1/9* in pharyngeal endoderm is crucial for gill slit formation and is regulated by RA (Holland and Holland, 1996; Müller et al., 1996; Wallin, 1996; Peters et

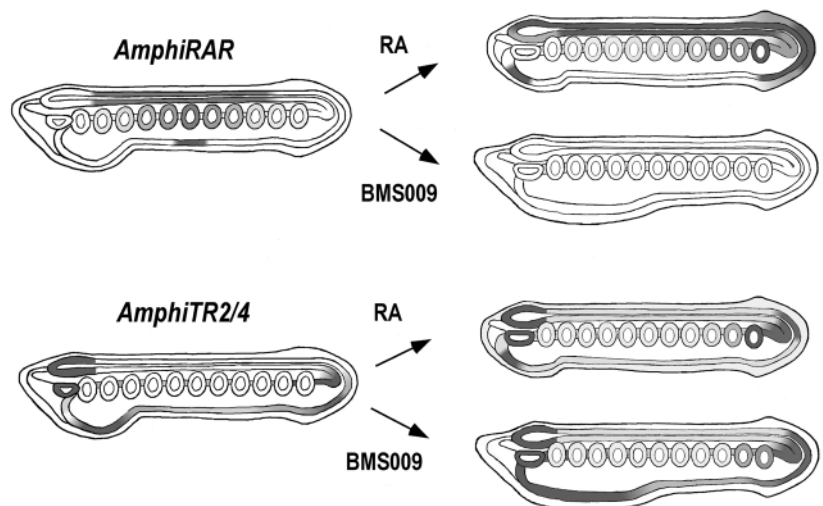
al., 1998; Wendling et al., 2000). As the present results show, in the presence of an RA antagonist, the pharynx expands posteriorly. The gill slit primordia are shifted posteriorly and additional ectopic primordia induced. Thus, segmental patterning of the pharynx is evidently an ancient chordate characteristic, mediated by RA signaling and expression of *Pax1/9* in the pharyngeal endoderm. Whether *Pax1/9* is a direct or indirect target of RA signaling is unknown.

Several other genes are expressed in pharyngeal endoderm and may act downstream of RA signaling. In amphioxus, *AmphiPax2/5/8* is expressed in the endostyle, which is homologous to the thyroid, and in the gill slit primordia in a pattern complementary to that of *AmphiPax1/9* (Kozmik et al., 1999). Its expression is abolished by exogenous RA (H. E., N. D. H., H. G., V. L. and L. Z. H., unpublished). Similarly, in vertebrates, *Pax2/5/8* homologs are expressed in the visceral arches and thyroid (Heller and Brändli, 1999). The pharyngeal endoderm in amphioxus also expresses *Ptx* (Yasui et al., 2000), *Shh* (Shimeld, 1999) and *HNF3 β* (Shimeld, 1997). In vertebrates, *Shh* is expressed in the pharyngeal endoderm (Helms et al., 1997; Piotrowski and Nüsslein-Vollhard, 2000), its regulatory region contains an RARE (Chang et al., 1997), and both the intensity and the extent of expression are reduced by exogenous RA (Helms et al., 1997). Expression of vertebrate *Pitx2* and *Hnf3b* in pharyngeal endoderm has not been described, although they are expressed elsewhere in the anterior endoderm (Dufort et al., 1998).

Initiation of gill slit formation and penetration of gill slits are separate events mediated by different levels of RA signaling

Gill slit formation evidently requires a low level of RAR signaling. In both amphioxus and vertebrates RAR-expression is low in the anterior gill slits or pouches (Smith et al., 1994). Moreover, in *Xenopus*, an RA hydroxylase, which reduces RA levels, is prominently expressed in first three pharyngeal arches (Holleman et al., 1998). Paradoxically, however, blocking RA signaling has similar effects as excess RA – gill slits do not penetrate in amphioxus and pharyngeal arches are reduced or fused in vertebrates (Dupé et al., 1999; Maden et al., 1996;

Fig. 7. Diagram of the effects RA and the RA-antagonist BMS009 on pharyngeal morphology and expression of *AmphiRAR* and *AmphiTR2/4* at the late neurula/early larva stage. (Left) In normal embryos, *AmphiRAR* and *AmphiTR2/4* are expressed in approximately complementary patterns: *AmphiTR2/4* expression being high in the cerebral vesicle, pharynx and tailbud where *AmphiRAR* expression is low. (Right) Application of RA shortens the pharynx and shifts it anteriorly (mouth and gill slits never form), while the RA antagonist BMS009 expands the pharynx posteriorly (subsequently an enlarged mouth forms; gill slit primordia shift posteriorly; extra ones are initiated, but none penetrates). *AmphiRAR* expression is upregulated and shifted anteriorly by RA and completely downregulated by BMS009. By contrast, although the level of *AmphiTR2/4* expression is only slightly affected by RA and BMS009, RA shifts pharyngeal expression anteriorly, while BMS-009 expands it posteriorly. Together, these results suggest that levels of RA signaling, mediated in part by competitive inhibition of *AmphiRAR* by *AmphiTR2/4*, regulate anterior/posterior patterning in the nerve cord and endoderm.



Mulder et al., 1998; Wendling et al., 2000). However, the pharyngeal phenotypes are not identical. In RA-treated embryos, the pharyngeal region is reduced, there is not even a rudiment of mouth or gill slits, and *AmphiRAR* expression is upregulated throughout the pharynx. By contrast, the RA-antagonist expands the pharynx posteriorly, enlarges the mouth and initiates gill slit formation (although gill slits do not penetrate), and downregulates *AmphiRAR* expression. Thus, a low level of RA signaling both initiates gill slit formation and allows them to penetrate. If RA signaling is completely blocked, gill slits can initiate, but not penetrate. The high level of RA signaling in the middle third of normal embryos apparently sets a posterior limit for gill slits by preventing their initiation.

In summary, amphioxus is proving to be an excellent model for understanding the role of the RA signaling pathway in patterning along the anterior/posterior axis in chordates both in the nerve cord and pharynx. Moreover, the presence of single genes for *RAR*, *RXR* and *TR2/4* in amphioxus, together with the absence of neural crest greatly facilitates an understanding of the function of these genes in patterning of the pharynx by the pharyngeal endoderm.

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