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An amphioxus *snail* gene: Expression in paraxial mesoderm and neural plate suggests a conserved role in patterning the chordate embryo

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Abstract Homologs of the *Drosophila snail* gene have been characterized in several vertebrates. In addition to being expressed in mesoderm during gastrulation, vertebrate *snail* genes are also expressed in presumptive neural crest and/or its derivatives. Given that neural crest is unique to vertebrates and is considered to be of fundamental importance in their evolution, we have cloned and characterized the expression of a *snail* gene from amphioxus, a cephalochordate widely accepted as the sister group of the vertebrates. We show that, at the amino acid sequence level, the amphioxus *snail* gene is a clear phylogenetic outgroup to all the characterized vertebrate *snail* genes. During embryogenesis *snail* expression initially becomes restricted to the paraxial or presomitic mesoderm of amphioxus. Later, *snail* is expressed at high levels in the lateral neural plate, where it persists during neurulation. Our results indicate that an ancestral function of *snail* genes in the lineage leading to vertebrates is to define the paraxial mesoderm. Furthermore, our results indicate that a cell population homologous to the vertebrate neural crest may be present in amphioxus, thus providing an important link in the evolution of this key vertebrate tissue.

Key words Amphioxus · Snail · Neural crest · Evolution · Chordate

Introduction

Embryologists and evolutionists have long understood that their fields are linked and that insights into the modification of animal form during evolution can be gained by comparing the development of organisms in closely related taxa. The advances of developmental genetics over the last two decades are now allowing such comparisons to be undertaken at the level of individual genes. As the closest living sister group to the vertebrates (Wada and Satoh 1994), the cephalochordate amphioxus has become an important reference organism for understanding the origin of the vertebrate body plan. Amphioxus embryology is well understood from classical approaches (Hatschek 1893; Conklin 1932) and its gastrular and neurular development have been characterized as representing the common, generalized embryonic form of a vertebrate ancestor (Nelson 1953; see Fig. 1). More recently, expression studies of several amphioxus genes such as *Hox* genes (Holland et al. 1992), *Otx* (Williams and Holland 1996), *HNF-3* (Shimeld 1997), *Dll* (Holland, LZ et al. 1996), and *Brachyury* (Holland et al. 1995) clearly demonstrate that homologies can be assigned between amphioxus and vertebrate embryos at the molecular level. These studies have been crucial in furthering our understanding of vertebrate origins.

Gans and Northcutt (1983) have put forth a provocative and influential theory for the origin of the vertebrate body plan based upon a careful examination of the morphological differences between vertebrates and amphioxus. They proposed that the shared-derived characters of vertebrates consist of specialized head structures necessary for a lifestyle of active predation. Furthermore, they noted that these new structures arise from a very limited set of embryonic tissues, primarily the neural crest and the epidermal placodes. From their initial specification along the dorso-lateral edge of the neural tube, neural crest cells migrate ventrally along specific pathways and differentiate into a variety of sensory, pigment, and connective tissue cell types (reviewed in Hall and Hörstadius 1988; Bronner-Fraser 1995). If the Gans and Northcutt theory is correct, it suggests that the transition to the vertebrate body plan was dependent upon either the acquisition of completely novel genetic programs specifying neural crest, or else the developmental modification of pre-existing tissues. Non-vertebrate chordates such as

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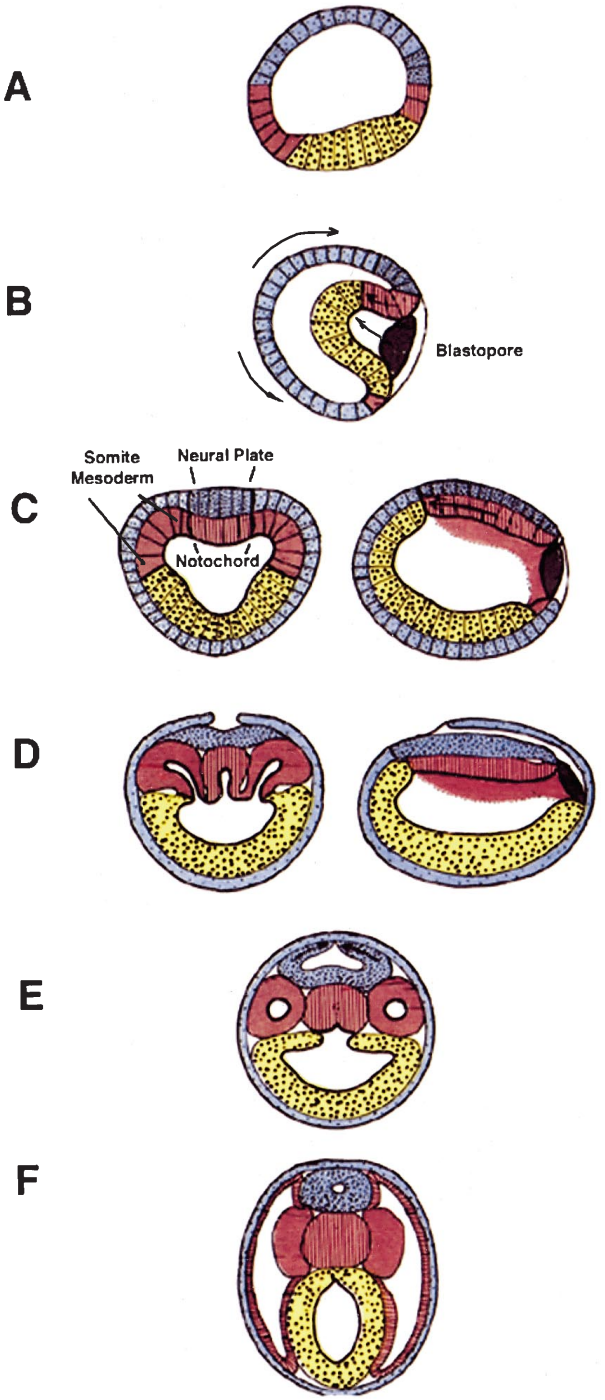


Fig. 1A–E An overview of morphodynamic movements during amphioxus gastrulation and neurulation. Early gastrulation (**A, B**) is characterized by involution of the chordamesoderm and somite mesoderm to the interior. During mid to late gastrulation (**C, D**), the mesoderm extends along the antero-posterior axis and converges dorsally. The mesoderm separates by constriction laterally to form the somites and middorsally to form the notochord. These stages are also marked by the formation of the neural plate, and the spreading of the epidermal ectoderm over the neural plate. During neurulation (**E,F**), the neural tube forms by the dorsal folding of the lateral edges of the neural plate until they fuse at the midline. The lateral walls of the somites spread laterally and ventrally forming the general body coelom. Adapted from Lehman (1987)

amphioxus and ascidians have no cells resembling neural crest, either in their migratory behavior or derivatives, and thus it is currently unknown what the evolutionary precursors to neural crest might have been.

The *snail* gene was first identified and characterized in *Drosophila* (Simpson 1983; Alberga et al. 1991), and one or more homologs have been cloned and characterized from a number of vertebrates, including mouse (Neito et al. 1992; Smith et al. 1992), chick (Neito et al. 1994; Isaac et al. 1997), frog (Sargent and Bennett 1990), and zebrafish (Hammerschmidt and Nüsslein-Volhard 1993; Thisse et al. 1993, 1995). These genes encode zinc-finger type DNA-binding proteins and are presumed to act as tissue-specific transcription factors. In *Drosophila*, *snail* is expressed in the invaginating ventral furrow during gastrulation and is required for mesoderm formation. Similarly, in each of the above vertebrate species, *snail* expression is first detected during early gastrulation. *Snail* expression typically becomes restricted to presomitic mesoderm, and in some cases persists as the definitive somites form. In addition to this early mesodermal expression, another common feature of *snail* gene expression in vertebrates is in premigratory neural crest and its pharyngeal arch derivatives.

Snail genes are one of the few molecular markers known for the initial specification of neural crest in vertebrates; they thus present an excellent tool with which to trace the origin of this key tissue in the closest relatives of the vertebrates. In order to pursue this question, we have cloned a *snail* homolog from amphioxus and examined its embryonic expression. We report that amphioxus possesses a *snail* gene that is a clear phylogenetic outgroup to all the characterized vertebrate *snail* genes and appears to be the sole *snail* homolog in the amphioxus genome. *Snail* expression initially becomes restricted to the paraxial or presomitic mesoderm, indicating that this is an ancestral function of *snail* genes in the lineage leading to vertebrates. Later, *snail* is expressed at high levels in the lateral neural plate and persists during neurulation, while somitic expression is extinguished. This finding indicates that a cell population is present in amphioxus which may be homologous to the premigratory vertebrate neural crest, thus providing an important link in the origin and evolution of a tissue thought to be of critical importance in vertebrate origins.

Materials and methods

Library construction

Spawning adult amphioxus (*Branchiostoma floridae*) were collected from Old Tampa Bay, Florida, in the summer of 1995, and eggs were fertilized in vitro. Total RNA was prepared from staged embryos ranging from 6 to 20 h post fertilization. cDNA was made from polyA+ selected mRNA using the SuperScript Choice System (Gibco), and size-selected cDNA was ligated into the Lambda Zap II vector (Stratagene) and packaged using Gigapack III Gold packaging extract (Stratagene).

Cloning of *snail*

Approximately 5×10^5 pfu were plated and screened at low stringency using a labelled zebrafish *snail1* cDNA (Thisse et al. 1993) as a probe. Hybridization was carried out in 40% formamide, 5×SSPE [0.9 M NaCl, 0.05 M NaH_2PO_4, 0.005 M ethylene diamine tetraacetic acid (EDTA), pH 7.5], 1% SDS (sodium dodecyl sulfate) and 100 µg/ml herring sperm DNA at 37°C overnight. Stringent washes were carried out in 2×SSPE at 40°C.

Of ten positive plaques, six were eventually purified and insert sizes determined. The clone with the largest insert was sequenced on both strands by primer walking using an ABI 377 automated sequencer. The sequence was compared to known *snail* genes and has been submitted to GenBank under accession number AF081809.

Genomic southern blot analysis

Total amphioxus and zebrafish genomic DNA was restricted with EcoR1, electorphoresed and blotted onto nylon membrane. This blot was then hybridized at low stringency with alternately, a labelled zebrafish *snail1* cDNA, zebrafish *snail2* cDNA and a portion of the amphioxus *snail* cDNA. Hybridization and wash conditions were the same as those for cDNA cloning.

Sequence analysis

Sequences were aligned using the Clustal X algorithm (Thompson et al. 1994) and phylogenetic trees were constructed using the neighbor-joining method of Saitou and Nei (1987).

In situ hybridizations

Staged embryos were collected in the summers of 1995 and 1996 (see above). Embryos were fixed for 30 min in 4% formaldehyde, 0.1 M MOPS 3-[N-Morpholino] propanesulfonic acid, 2 mM $MgSO_4$, 1 mM ethylene glycol-bis-(β-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA), 0.5 M NaCl pH 7.5, and then dehydrated and stored in 100% ethanol at –20°C.

Digoxigenin-labelled antisense RNA was generated from the full-length amphioxus *snail* cDNA, as well as from an 840-bp EcoR1-BamHI fragment containing only 3' untranslated sequence from the full length clone using a Genius kit (Boehringer-Mannheim). These were used as probes on whole, fixed embryos as follows. Embryos were rehydrated into phosphate-buffered saline (PBS), and hybridized overnight at 65°C in the following hybridization solution: 50% formamide, 5×sodium sodium cibrate (SSC), 0.1% Tween, 5 mM EDTA, 1 mg/ml tRNA, 100 µg/ml heparin, 1×Denhardt's. Stringent washes were performed in 0.2×SSC at 65°C. Embryos were developed using the antibody and coloration components of the Genius kit (Boehringer-Mannheim).

Sections

Heavily stained specimens were dehydrated quickly through a graded series of methanol, cleared in two changes of propylene oxide (PO), infiltrated with a 1:1 mixture of PO and Epon for 45 min, followed by 3:1 Epon and PO for 6 h, and finally pure Epon for 2 h. The tissue was embedded in fresh Epon and the blocks polymerized overnight at 60°C. Ten micron sections were cut on a glass knife and dried on subbed slides. All slides were cover-slipped in Epon and polymerized overnight at 60°C.

Results

Genomic southern analysis of amphioxus *snail* genes

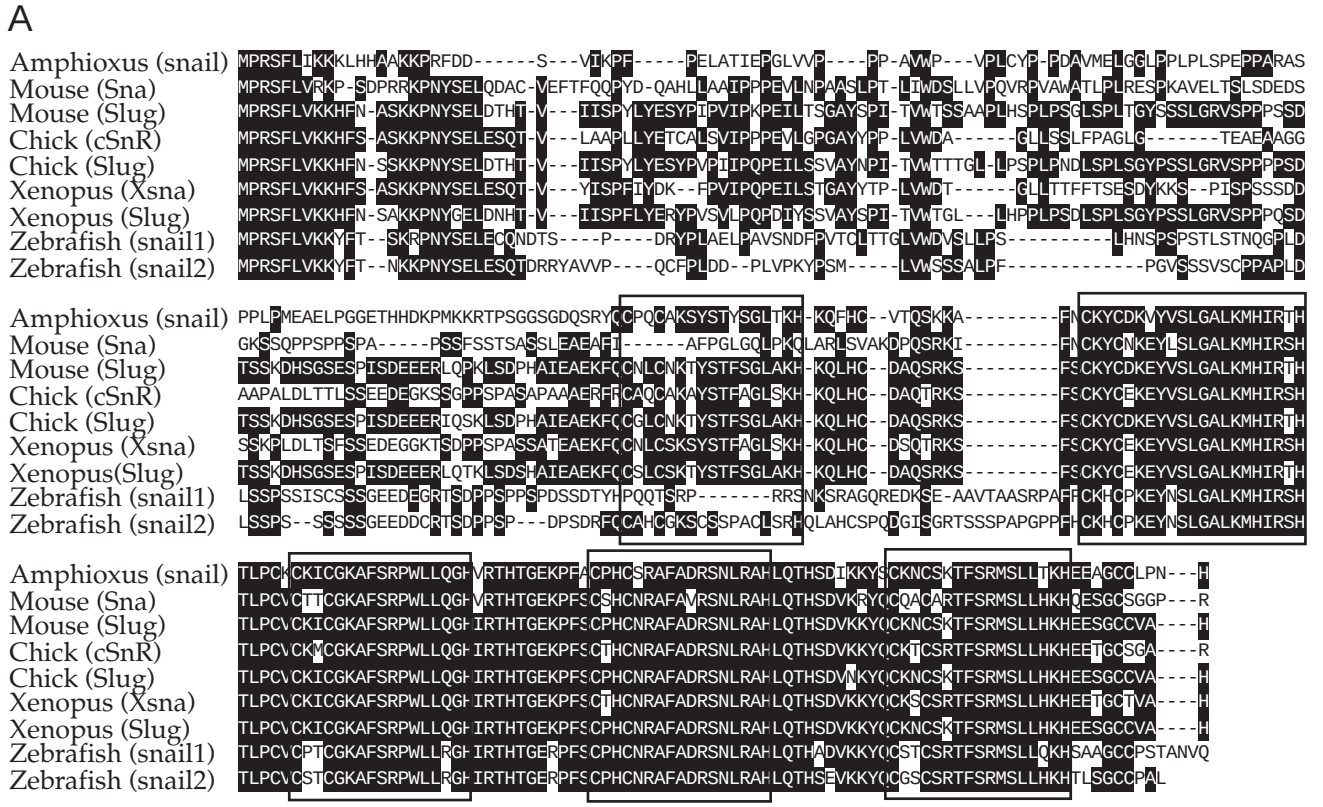
In order to assess the complexity of the amphioxus genome with respect to *snail* gene homologs, we probed digests of total amphioxus genomic DNA with a labelled zebrafish *snail1* cDNA (Thisse et al. 1993), and a labelled zebrafish *snail2* cDNA (Thisse et al. 1995) at low stringency. We included total zebrafish genomic DNA as a control. The resulting autoradiogram (not shown) indicates multiple hybridizing bands within the zebrafish genome, consistent with their being multiple described *snail* homologs in zebrafish (including *snail1*; Hammerschmidt and Nüsslein-Volhard 1993; Thisse et al. 1993; and *snail2*; Thisse et al. 1995). In contrast, we detect only a single 2.5-kb hybridizing band in the amphioxus genome, which indicates that amphioxus has only a single *snail* homolog.

Sequence analysis of amphioxus *snail*

We constructed a gastrula stage cDNA library and screened it by low-stringency hybridization with the zebrafish *snail1* gene. Of several positive clones, the one with the largest insert (1.9 kb) was completely sequenced and determined to be a full-length cDNA encoding a 251-amino acid residue protein containing five zinc fingers . Three other clones were determined to be partial cDNAs corresponding to the same gene.

This amino acid sequence of this amphioxus *snail* homolog was used in a comparative sequence alignment of known vertebrate *snail* homologs (Fig. 2B). Although two of the vertebrate *snail* genes (mouse *Sna* and zebrafish *snail1*) contain four rather than five zinc fingers, this alignment reveals a high degree of sequence conservation throughout the zinc finger region, indicating that the amphioxus gene is a bona fide *snail* homolog. It is also interesting to note that the amino terminus of amphioxus *snail* matches the vertebrate *snail* genes exactly (Fig. 2B) while *Drosophila* and ascidian *snail* genes do not (data not shown).

Figure 2B illustrates the results of a phylogenetic analysis of the zinc finger regions of multiple *snail* genes using the neighbor-joining method. The tree indicates that the amphioxus *snail* gene is an outgroup to the vertebrate *snail* genes (bootstrap value 88.8%), while the homologs from *Drosophila* and the ascidian *Ciona intestinalis* (*Ci-snail*; Corbo et al. 1997) are outgroups to the amphioxus+vertebrate clade. Phylogenetic groupings among the vertebrate *snail* genes suggest that multiple independent *snail;*gene duplication events have occurred since the cephalochordate-vertebrate divergence.



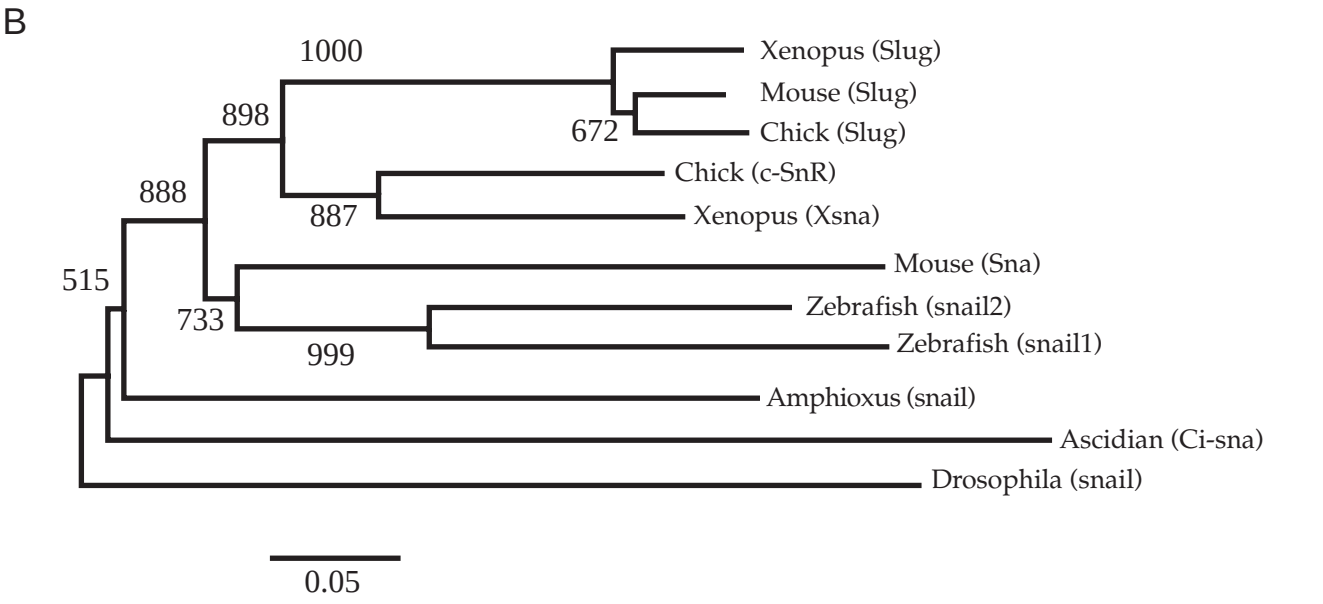


Fig. 2A, B Sequence analysis of amphioxus *snail*. **A** Comparative sequence alignment of *snail* homologs from amphioxus (*Branchiostoma floridae, snail*) and representative vertebrate classes including zebrafish (*Danio rerio, snail1*; Hammerschmidt and Nüsslein-Volhard 1993; Thisse et al. 1993; and *snail2*; Thisse et al. 1995), Xenopus (*X. laevis, Xsna* ; Sargent and Bennett 1990; *Slug*; Mayor et al. 1995), chick (*Gallus gallus, Slug*; Neito et al. 1994; and *cSnR*; Isaac et al. 1997), and mouse (*Mus musculus, Sna*; Neito et al. 1992; Smith et al. 1992; *Slu*; Sefton et al. 1998). Residues that match the consensus are *shaded* and the zinc fingers are *boxed*. There is extensive homology in the zinc finger regions (note that mouse *Sna* and zebrafish *snail1* have only four zinc fingers while the rest have five fingers). Clustal algorithm (Thompson et al. 1994) used for generating alignment. **B** Phylogenetic tree of *snail* proteins based on the zinc finger regions of an alignment similar to that shown in **A**, but including snail homologs from an ascidian (*Ciona intestinalis, Ci-Sna*; Corbo et al. 1997), as well as from *Drosophila* (*snail*; Boulay et al. 1987). This tree was generated using the neighbor-joining method (Satoh and Nei 1987). *Numbers* at internal branches indicate the bootstrap value (%) for each group. As expected, amphioxus snail is an outgroup to the vertebrate *snail* genes and the ascidian and *Drosophila* homologs are outgroups to the amphioxus+vertebrate clade. However, the grouping within the vertebrates suggests that multiple independent gene duplication events have occurred since the cephalochordate-vertebrate divergence

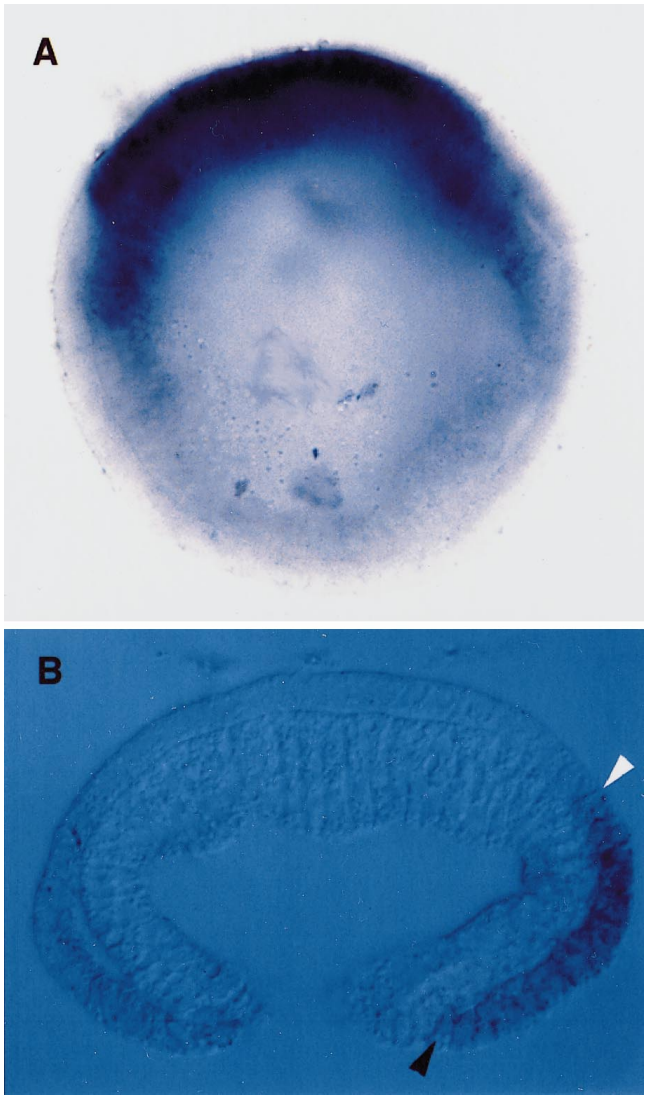


Fig. 3A, B *Snail* expression is initially restricted to the dorsal, ectodermal cell layer of the amphioxus early gastrula. **A** Whole-mount posterior view of early gastrula, approximately 7 h post fertilization labeled with *snail* antisense RNA. *Snail* transcripts are detected at high levels in roughly the dorsal third of the gastrula. **B** Sagittal section of similarly staged embryo. *Snail* expression is restricted to the outer, ectodermal cell layer, and extends from the blastopore lip (*black arrow*) to roughly one half the distance to the anterior pole (*white arrow*)

Early mesodermal expression of amphioxus *snail*

The patterns of *snail* expression during amphioxus embryogenesis were determined by in situ hybridization using digoxigenin labelled antisense RNA generated from the full-length *snail* cDNA as well as from a clone representing only the 3' untranslated region of the amphioxus *snail* cDNA. Since the expression patterns obtained with these two probes are identical (data not shown), the full length probe was used for subsequent figures as it produces a more robust signal.

Snail transcripts are first detected during early gastrulation, approximately 7 h after fertilization (Fig. 3). At

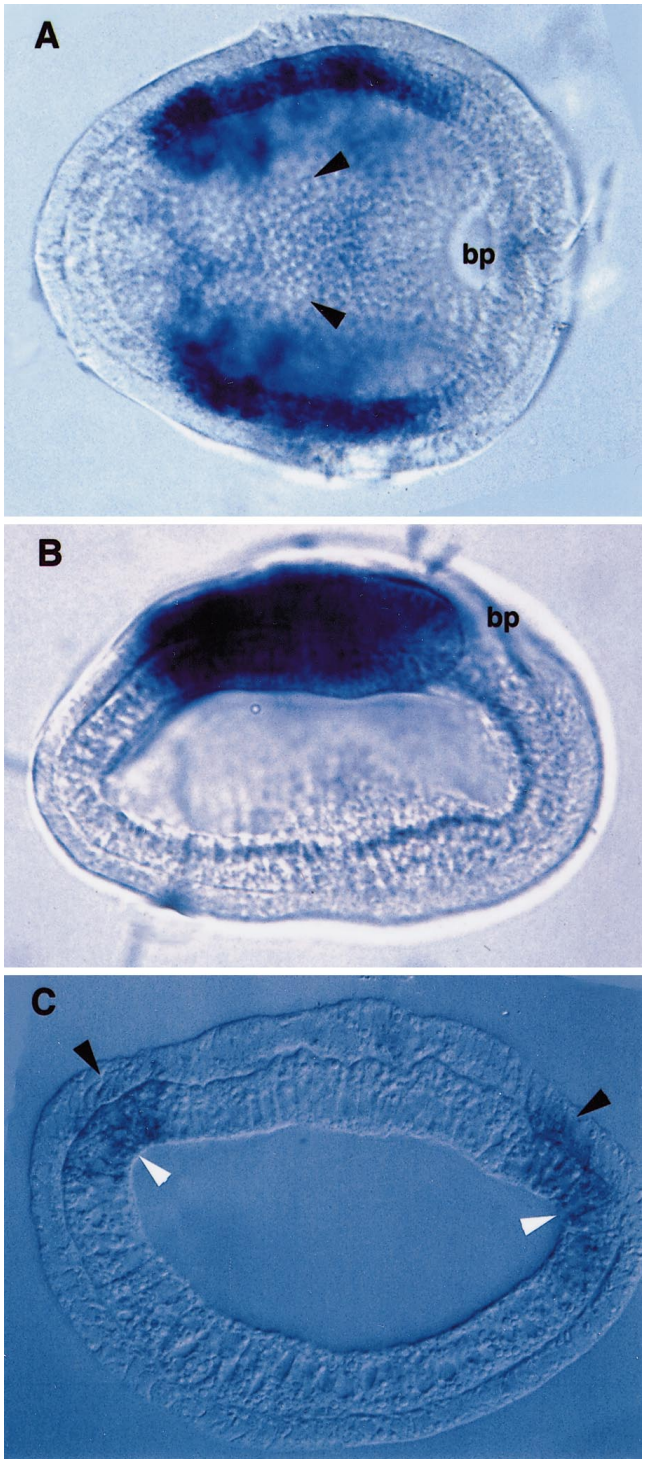


Fig. 4A–C By mid gastrula, *snail* expression is dorso-laterally restricted. **A** Whole-mount dorsal view of mid gastrula, approximately 8 h post-fertilization, oriented with anterior to the *left*. *Snail* expression is extinguished along the dorsal midline (*between arrows*). **B** Whole-mount lateral view of similarly staged embryo, with dorsal oriented *up*, illustrating the dorsal restriction of *snail* expression. **C** Transverse section of mid gastrula, with dorsal oriented *up*. *Snail* transcripts are localized to the presomitic mesoderm (*white arrows*) and patches of cells along the ventro-lateral edge of the neural plate (*black arrows*, *bp* blastopore)

this time, the archenteron has just formed and the gastrula consists of two cell layers (Fig. 1B). In whole-mount specimens (Fig. 3A) *snail* transcripts are restricted to roughly the dorsal third of the embryo. This orientation can be determined morphologically by the slight flattening that characterizes the dorsal gastrula. A sagittal section through a similarly staged embryo reveals that *snail* expression is restricted to the outer, ectodermal cell layer and extends from the blastopore lip to halfway to the anterior pole (Fig. 3B).

Morphogenetic cell movements are very dynamic during early gastrulation as the epibolic expansion of the ectoderm covers the presumptive endoderm and mesoderm, and the blastopore continues to close (Fig. 1C,D). *Snail* expression is equally dynamic during this time. By midgastrula, 8 h after fertilization, *snail* expression is extinguished along the dorsal midline (Fig. 4A). As can be seen in a lateral view of an 8-h embryo (Fig. 4B), *snail* expression is clearly localized dorsally, even though it is excluded from the midline. In addition, *snail* expression does not extend posteriorly beyond the blastopore, and has a well defined anterior boundary. A transverse section of an 8-h gastrula indicates that *snail* transcripts are primarily localized to the dorso-lateral aspect of the inner cell layer, which corresponds to the presumptive somitic mesoderm (Fig. 4C). Patches of *snail*-expressing cells can also be seen in the ectodermal cell layer, along the lateral edge of the neural plate.

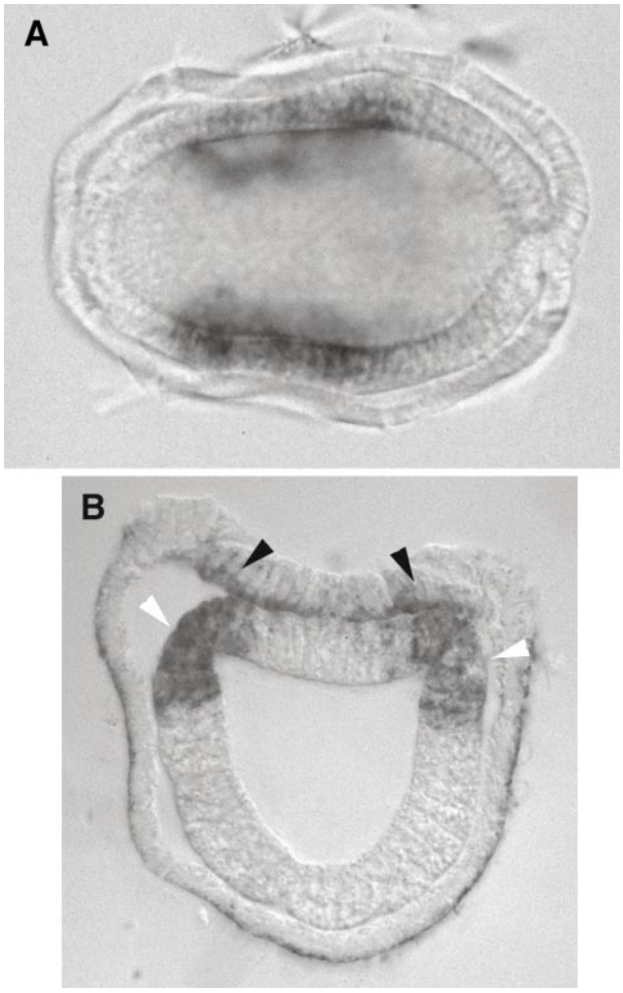


Fig. 5A, B *Snail* is expressed at high levels in presumptive somitic mesoderm, and at the ventro-lateral edge of the neural plate. **A** Whole-mount dorsal view of *Snail* expression in a late gastrula, approximately 10 h post fertilization. **B** Transverse section of a similarly staged embryo. Somitic mesoderm is beginning to evaginate and contains abundant *snail* transcripts (*white arrows*). Expression spreads along the ventral edge of the neural plate (*black arrows*), which is beginning to be overgrown by the epidermal ectoderm

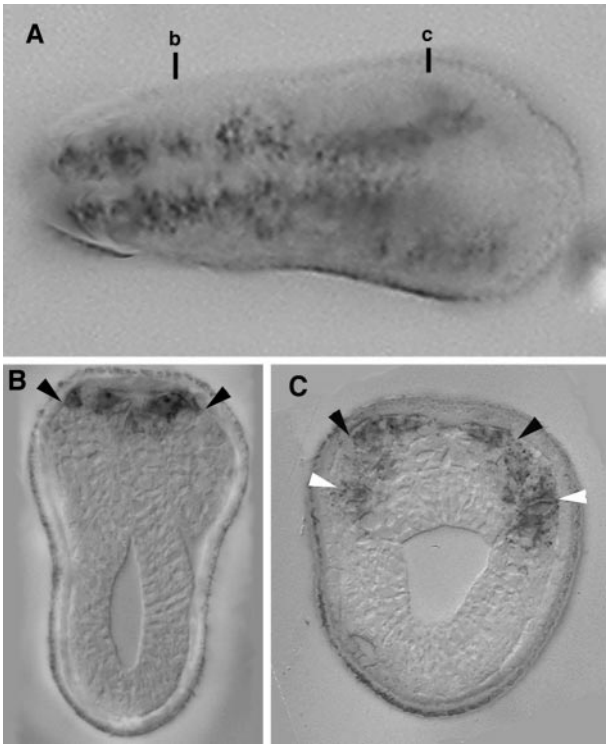


Fig. 6A, C During neurulation, *Snail* is down-regulated in somites, and up-regulated in the invaginating neural plate. **A** Whole-mount dorsal view of *Snail* expression in an early neurula, approximately 14 h post fertilization. Expression converges toward the midline. **B** Transverse section through *b* in **A**. *Snail* transcripts are detected throughout the invaginating neural plate, although remain higher at the lateral edges (*arrows*). Note that expression is extinguished in the definitive somites. **C** Transverse section through *c* in **A**. *Snail* expression persists at high levels in presumptive somitic mesoderm (*white arrows*), as well as the lateral edges of the neural plate (*black arrows*)

Neural plate expression of amphioxus *snail*

In the late gastrula, approximately 10 h after fertilization, the neural plate forms and epidermis begins to overgrow it (Fig. 1E). The somite mesoderm begins to separate by constriction laterally, as the notochord condenses and constricts mid-dorsally. *Snail* transcripts remain at high levels in the somitic mesoderm (Fig. 5) and expression continues along the ventro-lateral edge of the neural plate.

As neurulation commences, approximately 14 h after fertilization, the neural tube forms by the dorsal folding

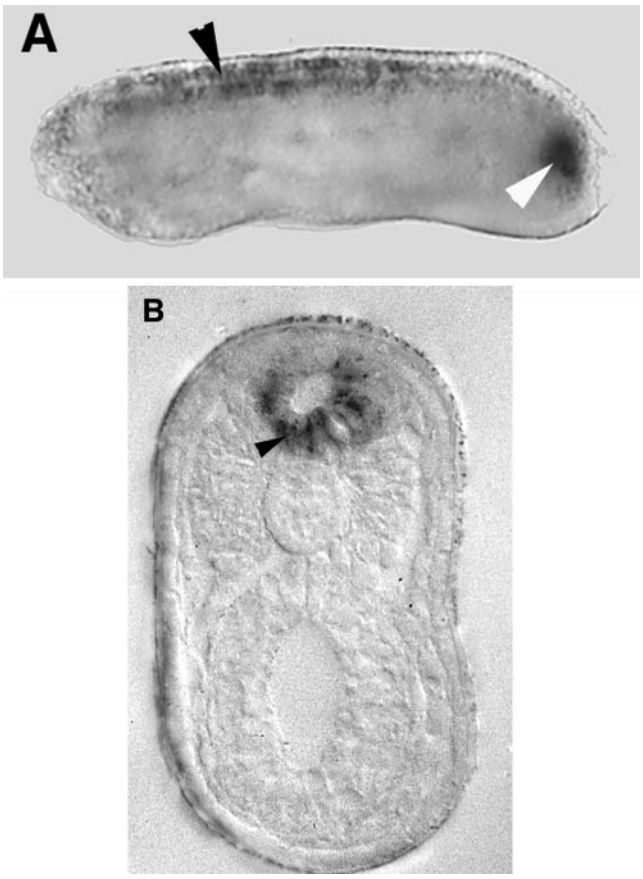


Fig. 7A, B By late neurula, *snail* transcripts are confined to the neural tube and the tail bud. **A** Whole-mount lateral view of late neurula, approximately 20 h post fertilization. *Snail* expression can be detected in the neural tube (*black arrow*), and a mass of cells in the tail bud (*white arrow*). **B** Transverse section of a similarly staged embryo showing cells in the neural tube with high levels of *Snail* transcripts (*arrow*)

of the lateral edges of the neural plate, and the somites complete their lateral constrictions from the notochord (Fig. 1F). During this process, *snail* expression is extinguished in the developing somites and expands throughout the neural plate. In whole-mount 14-h embryos (Fig. 6A) expression remains dorsally localized, but is closer to the midline. A transverse section through an anterior region where neurulation has commenced (Fig. 6B) indicates that *snail* transcripts are located throughout the invaginating neural plate, but are completely extinguished in the definitive somites. A transverse section through a posterior region where neurulation has not commenced (Fig. 6C) reveals that *snail* expression remains at high levels in the presumptive somitic mesoderm, as well as in the neural plate.

By the late neurula stage, 20 h after fertilization (Fig. 1G), *snail* expression can be detected dorsally along the length of the embryo and in a mass of cells in the tail bud (Fig. 7A). A transverse section of a similarly staged embryo (Fig. 7B) shows that the *snail*-expressing cells are confined to the neural tube.

Discussion

Amphioxus and comparative approaches to vertebrate origins

Despite more than a century of paleontological and embryological research, the origin of vertebrates remains obscure (Jeffries 1986; Gee 1996). There is widespread agreement that the cephalochordate amphioxus represents the sister group to the vertebrates; evidence for this view comes from both classical comparative embryology (Hatschek 1893; Conklin 1932; Nelson 1953) and molecular systematics (Wada and Satoh 1994). Given its unique phylogenetic position, amphioxus has been the subject of renewed comparative investigations now that specific genes and genetic mechanisms controlling vertebrate development have been identified.

Amphioxus has been shown to possess a single *Hox* cluster, suggesting that at the level of genome organization, it is far simpler than vertebrates (Garcia-Fernandez and Holland 1994). Additionally, while obvious structural homologies are not present between the well-differentiated vertebrate neural tube and the comparatively simple neural tube of amphioxus, expression studies of select *Hox* genes (Holland et al. 1992) as well as the head-specific gene *Otx* (Williams and Holland 1996) indicate that positional homologies can be drawn on the basis of gene expression. Furthermore, expression studies with amphioxus *Brachyury* (Holland et al. 1995) and *HNF3* (Shimeld 1997) homologs confirm that the homology of the vertebrate and cephalochordate notochords observed at the morphological level extends to the molecular level. These findings provide a strong foundation for using molecular markers to postulate homology between vertebrate and amphioxus embryos, and using such information to make evolutionary inferences. In using gene expression in this manner, it is important to appreciate that homology at the molecular level is not necessarily indicative of homology at the level of differentiated tissue (see Bolker and Raff 1996), and such arguments must be accompanied by other supporting lines of evidence.

Snail genes in amphioxus and vertebrates

We have cloned an amphioxus gene that is a clear *snail* homolog and appears to represent the sole *snail* homolog in the amphioxus genome. The phylogenetic tree produced from a comparative sequence alignment of known *snail* homologs provides strong support for amphioxus *snail* being an outgroup to the characterized vertebrate *snail* genes, which suggests that it may retain features of the ancestral vertebrate *snail* gene. At the structural level, this is clearly the case. Like most vertebrate *snail* genes, this amphioxus homolog possesses five zinc finger motifs. Although some vertebrate *snail* genes, including mouse *Sna* (Neito et al. 1992; Smith et al. 1992) and zebrafish *snail1* (Hammerschmidt and Nüsslein-Volhard 1993; Thisse et al. 1993) possess four zinc finger motifs, they are phylogenetically more closely related to

the vertebrate genes containing five fingers, than is amphioxus *snail*. Additionally, both *Drosophila snail*, as well as the *snail* homolog from the urochordate *C. intestinalis* (Corbo et al. 1997) are phylogenetic outgroups to cephalochordates and vertebrates, and also have five zinc fingers. These findings strongly suggest that the five finger motif represents the organization of the ancestral chordate *snail* gene, and that the vertebrate homologs with four zinc fingers are derived.

Snail as a marker of presomitic mesoderm

Somites are a shared-derived feature of the vertebrate+cephalochordate clade. The fate map of the amphioxus gastrula is comparatively simple (Hatschek 1893; Conklin 1932; see Fig. 1), and *snail* expression clearly correlates with presomitic mesoderm. *Snail* is known to be required for mesoderm formation in *Drosophila* (Alberga et al. 1991), and is also expressed in mesoderm in ascidians, which are chordates but do not form somites (Corbo et al. 1997). In a variety of vertebrates, *snail* genes have been shown to be expressed in presomitic mesoderm. Our finding is entirely consistent with this and indicates that *snail* expression was likely associated with somite formation in the common ancestor of vertebrates and cephalochordates. Thus in addition to having retained structural features of the ancestral vertebrate *snail* gene, amphioxus *snail* also appears to have retained developmental features of the ancestral vertebrate *snail* gene.

Snail expression in amphioxus is extinguished as the definitive somites form by lateral constriction. This pattern resembles that described for the chick *snail* homolog *Slug* (Neito et al. 1994), but is markedly different to the expression pattern of other vertebrate *snail* genes such as zebrafish *snail1*, (Hammerschmidt et al. 1993; Thisse et al. 1993), chick *cSnR* (Isaac et al. 1997) and mouse *Sna* (Neito et al. 1992; Smith et al. 1992), where expression persists in the definitive somites. The significance of these differences is unclear, but it may be the result of novel roles for *snail* genes following gene duplication events in the vertebrate lineage. *Snail* expression provides the earliest known molecular marker for somitic mesoderm in amphioxus, and should thus prove useful in studies aimed at elucidating the mechanisms of mesoderm formation and somitogenesis in amphioxus. Additionally, since muscle is one of the major somitic derivatives, this finding complements studies of downstream somitic markers such as myosin light chain I (Holland et al. 1995) and muscle specific bHLH genes (Araki et al. 1996) in piecing together the myogenic pathway in amphioxus.

Neurulation and the origin of the neural crest

Neural crest is thought to be a vertebrate synapomorphy and a key innovation during the origin of vertebrates (Gans and Northcutt 1983). Neural crest cells delaminate from the invaginating neural tube, migrate extensively, and differentiate into a variety of tissues including sensory ganglia, pigment cells, cartilage and bone; these tissues are absent from non-vertebrate chordates such as amphioxus and ascidians (reviewed in Bronner-Fraser 1995; Hall and Hörstadius 1988). In all vertebrates examined, *snail* (or *Slug*) expression is found in cells along the lateral edge of the neural plate and on the crest of the invaginating neural tube, and thus provide a consistent marker of presumptive neural crest. Our finding of *snail*-expressing cells in the neural plate and neural tube of amphioxus suggests that a population of cells homologous to the premigratory neural crest may have been present in the common ancestor of cephalochordates and vertebrates.

Based upon the expression of *Distal-less* (*Dll*) in amphioxus, Holland L.Z. et al. (1996) proposed that neural crest cells arose from the epidermal cells that overgrow the invaginating neural tube. However, while *Dll* is expressed in these cells in amphioxus, *snail* is not. Since, in vertebrates, *snail* expression correlates with premigratory neural crest cells, while *Dll* genes are expressed only after migration has begun (Dollé et al. 1992; Akimenko et al. 1994; Dirksen et al. 1994), our finding indicates that an alternative scenario must be considered. Specifically, the precursors of neural crest in the vertebrate ancestor may have resided within the neural tube itself. In this view neural crest cells would have arisen as a distinct population of cells prior to acquiring motility.

Various lines of evidence suggest that the chordate body plan arose as the result of a dorso-ventral axis inversion of a protostome ancestor (see DeRobertis and Sasai 1996). In *Drosophila*, *snail* is required not only for mesoderm formation, but also has a specific morphogenetic function in ventral furrow formation (Ip et al. 1994). After a dorso-vental axis inversion, the site of the ventral furrow would be the topological equivalent of the dorsal midline in a chordate. It is tempting to speculate that early in the chordate lineage, while the mesodermal role of *snail* was conserved, this morphogenetic role of *snail* was co-opted into the process of neural plate invagination. This view is supported by *snail* expression in ascidians (Corbo et al. 1997), which is also localized in the invaginating neural plate. The origin of true neural crest in vertebrates then must have been accompanied by the additional morphogenetic innovation of some *snail*-expressing cells acquiring motility [indeed, experimental evidence from antisense and overexpression studies of the *snail* homolog *Slug* in chicks (Nieto et al. 1994) suggest that *snail* genes also regulate the migratory behavior of neural crest cells], as well as the diverse array of developmental pathways characteristic of neural crest.

Further comparisons are clearly warranted to elucidate the origin of the vertebrate body plan. *Snail* is one of only two genes known to be associated with neural crest in vertebrates that have been examined in amphioxus. Other genes that might be examined include members of the *Wnt* (Ikeya et al. 1997) and *forkhead* (Dirksen and Jamsich 1995) families. In order to test speculations about the role of *snail* in neurulation, it will also be instructive to delve deeper into the chordate lineage and include not just amphioxus, as described here, and tunicates, but also to include larvaceans and even hemichor-

dates. Finally, since the fate(s) of the *snail*-expressing cells in amphioxus are presently unknown, the further development of cell marking procedures in this organism (see Zhang et al. 1997) may help to clarify whether any of the developmental pathways characteristic of vertebrate neural crest occur in amphioxus.

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