Genetic Correlates of Neural Crest Evolution

Thesis by

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In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

2003

(Defended July 17, 2003)

ACKNOWLEGEMENTS

I would like to thank first and foremost my thesis advisor, Marianne Bronner-Fraser, for allowing me to pursue this incredibly interesting line of research in her lab and for giving me just the right mix of support, friendship, advice, and independence I needed to bring the work to fruition. I also thank my committee members, Ellen Rothenberg, Scott Fraser, Kai Zinn and Eric Davidson, for the critical input that kept me focused on graduation. I am also grateful to Clare Baker, Dave McCauley, Nick Holland, Linda Holland, and Jr Kai Yu for constituting my immediate Evo/Devo community. The core of my limited knowledge about vertebrate Evo/Devo was gleaned from discussions with them.

I also acknowledge the positive impact of my undergraduate thesis advisor, Gert DeCouet, who not only taught me molecular biology, but helped me realize that biological research was really what I wanted to do with my life. Other folks at UH who helped hone my scientific skills and prepare me for graduate school at CALTECH are Alex Brown, Sue McCombs, and Stephen Saul. Of course, I must also thank my family and friends for their love, support, friendship, and above-average entertainment value—without which everything else is pretty much pointless.

ABSTRACT

Neural crest cells are unique to vertebrates and generate most of the adult structures that distinguish them from their closest invertebrate relatives, the cephalochordates. To elucidate the molecular bases of neural crest evolution, I analyzed the expression, function, and *cis*-regulation of amphioxus genes with vertebrate homologs having established roles in neural crest development. By comparing these amphioxus genes with their agnathan and gnathostome homologs, I have uncovered genetic changes coincident with, and potentially causal to, the origins of neural crest. I demonstrate that three transcriptional regulators involved with neural crest development, AP-2, Id, and SoxE, were recruited to the neural plate border early in vertebrate evolution— implying that genetic cooption of high-order transcription factors was a major driving force in neural crest evolution. I also show that the function of the Snail protein in establishing the neural plate border was not significantly altered during vertebrate evolution, although vertebrate Snail genes may have evolved novel domains necessary for later functions in neural crest cells. Finally, I began characterizing the *cis*-regulation of vertebrate and amphioxus Slug/Snail orthologs to determine if divergent aspects of Snail gene expression (i.e., expression in neural crest cells) are reflected in structural differences in Snail *cis*-regulatory DNA. Using this 3-tiered approach I have begun to define the novel genetic regulatory interactions that drove the evolution of neural crest cells in the vertebrate lineage.

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Chapter 1:

Introduction: The Evolution of Vertebrate Development

Understanding How Evolution Works

Metazoan diversity is the result of ontological variation, which, in turn, is the consequence of heritable differences in the developmental genetic programs of different phyla. A central question in the field of 'The Evolution of Development' is exactly how genetic changes alter ontogeny to yield novel morphologies. Given the conserved nature of the metazoan proteome, it is widely accepted that much of the developmental differences between animals are due to modifications in gene regulation (Davidson, 2001). While many of these changes are likely to be in the *cis*-regulatory DNA itself, growing evidence indicates that the role of coding sequence mutations in high-order transcriptional regulators has been underestimated (Hsia and McGinnis, 2003; Ronshaugen et al., 2002). Thus, it is becoming apparent that unraveling both the *cis*- and *trans*-regulatory history of developmentally important genes is critical to understanding how new forms arise.

By necessity, identifying the gene regulatory changes associated with a given evolutionary change is a "reverse-genetic" process-- dependent on, and guided by, detailed knowlege obtained from traditional model organisms (although in rare cases a forward genetic approach is also feasible, especially when the change is relatively recent and not accompanied by intractable reproductive barriers (Peichel et al., 2001)). It also necessitates the development of experimental techniques in diverse non-model species, which may or may not be tractable to experimental manipulation. In addition, it requires that one or a small number of critical mutations be chiefly responsible for the observed evolutionary novelty. For these reasons, identifying the specific genetic changes responsible for any single macro- or micro-evolutionary transition has proven difficult.

As expected, the handful of reports to do so convincingly have uncovered modifications in specific gene regulatory networks, either within *cis*-regulatory DNA (Belting et al., 1998) or the coding region of *trans*-acting transcriptional regulators (Ronshaugen et al., 2002), or both (Wittkopp et al., 2002).

Despite the inherent difficulty of identifying the exact genetic alteration(s) driving a particular evolutionary event, ample circumstantial evidence exists suggesting that regulatory modification is the dominant genetic mechanism through which evolution works. Most all of this information comes as gene expression pattern data from evolutionarily informative phyla which diverged near the time of some critical morphological shift. These kinds of comparative expression pattern studies are usually fairly tractable and require only access to the embryos of interest and optimization of in situ hybridization or immunostaining protocols. When thoughtfully and thoroughly done, such examinations have yielded provocative leads as to the genetic nature of many major evolutionary events. As the techniques for experimentally manipulating diverse non-model embryos mature, these comparisons are becoming the observational foundations of increasingly testable hypotheses regarding the regulatory history of developmentally and evolutionarily important genes.

Amphioxus, Lamprey, and Vertebrate Origins

The phylum Chordata is comprised of three subphyla, Urochordata (mostly sessile, barnacle-like filter feeders), Cephalochordata (fish-like, motile, and burrowing filter feeders), and Craniata (the vertebrates)(Fig. 1A). Despite the range of adult forms, chordate embryos are built upon a common body plan including a notochord, a dorsal

hollow nerve cord, a perforated pharynx, and a muscular post-anal tail. Formation of the chordate nerve cord proceeds in a more or less stereotyped fashion. During gastrulation a region of dorsal ectoderm is induced to become the neural plate. As neurulation begins, the neural plate may roll up to form the neural tube, or condense towards the dorsal midline. In the latter case the neural tube lumen then forms by cavitation of a solid neural rod or keel. The neural tube of every chordate shows some degree of dorso-ventral polarity (Saitou and Nei, 1987). In vertebrates, this is taken to the extreme, with an array of specialized cells forming at specific levels of the neural tube. At dorsal-most levels a population of cells arises called the neural crest. Neural crest cells are specified early in neurulation at the border of the neural plate and non-neural ectoderm. After specification, neural crest cells delaminate from the neuroepithelium and migrate throughout the embryo as multipotent stem cells, forming a variety of cell and tissue types.

Gans and Northcutt proposed that the evolution of craniates from an invertebrate chordate ancestor was driven by the evolution of the neural crest and neurogenic placodes (Gans and Northcutt, 1983; Northcutt and Gans, 1983). This assertion reflects the fact that most of the defining craniate characters (anterior paired sensory organs, cranial and peripheral ganglia, the cranium, the pharyngeal arch skeleton) are derived from the neural crest and epidermal placodes. Northcutt and Gans convincingly link the appearance of these tissues to a shift from filter feeding to active predation in the craniate lineage (Gans and Northcutt, 1983; Northcutt and Gans, 1983). Indeed, most neural crest and placodal derivatives appear to be adaptations for hunting and capturing live food, i.e., anterior paired sense organs, the facial skeleton supporting these organs, an enhanced

peripheral nervous system for more efficient locomotion, a cranium to protect the larger brain, and a pharyngeal skeleton with teeth to capture and hold prey. Consistent with this scenario, definitive neural crest cells and their derivatives have been described in the most basal craniates (agnathans) but not in their filter-feeding relatives, the protochordates (urochordates and cephalochordates).

Despite their importance, little is known about the evolutionary origins of neural crest cells. Contributing to this is a lack of living intermediates linking the protochordate and agnathan (lamprey and hagfish) body plans. In the most basal craniate embryos examined, those of the sea lamprey, a typically vertebrate neural crest generates most of the derivatives seen in gnathostomes (Langille and Hall, 1988). In contrast, the most vertebrate-like invertebrates, the cephalochordates, lack even a rudimentary neural crest.

Further obscuring the matter is a sparse and controversial fossil record. The Burgess Shale fossil *Pikaia*, widely interpreted as representing the ancestral chordate, does not appear until the middle Cambrian. A single fossil dating from the early Cambrian of the chordate *Cathaymyrus* has also been provisionally interpreted as a basal chordate (Shu et al., 1996). However, clearly vertebrate chordates such as *Haikouichthyes* appear before, or coeval with, these presumed vertebrate ancestors, pushing back the origins of the first craniates deep into the earliest Cambrian (Shu et al., 1999; Shu et al., 2003). The identification of fossil urochordates from the Lower Cambrian further support a very ancient divergence of the three chordate subphyla (Shu et al., 2001). Perhaps the best candidate for a pre- or proto-craniate chordate is the Lower Cambrian *Haikouella* which-- in addition to the basic chordate characters-- possesses a pharyngeal arch skeleton, pharyngeal denticles, and dorsal and ventral aortae (Chen et al.,

1999). These features suggest that *Haikouella* had pharyngeal arch neural crest, and a rudimentary neural crest derived dermal skeleton (in the form of denticles), but lacked the cephalic crest population that generates the cranium. Cladistic analyses place *Haikouella* as either a stem group vertebrate, or a sister group to the vertebrates (reviewed in (Holland and Chen, 2001)). While the fossil record offers some clues about the origins of neural crest, it is constrained by the physics of preservation, which are not conducive to ancient, soft bodied, and tiny remains. In addition, there are limits to what can be reasonably inferred about an organism's development by looking at its fossilized adult form. Similar adult structures can arise via very different developmental mechanisms, and many evolutionarily important tissue and cell types, such as the nervous system, are simply not preservable.

The most complete picture of any evolutionary event therefore comes from examining not only fossil forms, but salient extant ones. In the case of the invertebrate/vertebrate transition these are the cephalochordates and the agnathan craniates. The subphylum cephalochordata is considered the sister group to the craniates because the two clades share several key traits not shared by urochordates. These include 1) a nerve cord that extends the length of the body and ends in an anterior swelling (cerebral vesicle or brain), 2) a notochord which extends the length of the body and persists in some form as the adult axial skeleton, 3) a digestive caecum (primitive liver), 4) true segmented myomeres which develop from somites, and 5) a closed circulatory system with dorsal and ventral aortae. Based on these affinities, and the fact that these characters are also seen in the most ancient chordates and craniates, it is accepted that cephalochordates are good representatives of the pre-vertebrate chordate condition. This

relationship has been repeatedly supported in the past decade by developmental genetic studies showing that the cephalochordate amphioxus has homologs of essentially every vertebrate gene and that these homologs are used in similar ways during development (Holland and Holland, 1998). Similarities between cephalochordates and vertebrates extend grossly to the level of ecology and life history. The overall morphology and lifestyle of the adult amphioxus is strikingly similar to that of the larval lamprey in that both are fusiform, burrowing, filter feeders (Fig. 1B).

On the other side of the invertebrate/vertebrate transition are the agnathans, which are universally recognized as the most basal extant craniates. Agnathans diverged from the vertebrate lineage before the origins of jaws, and, accordingly, the most primitive craniate fossils, *Haikouichthys* and *Myllokunmingia,* are lamprey and hagfish -like agnathans (Shu et al., 1999). Ablation studies (Langille and Hall, 1988), and more recently, scanning electron microscopy (Horigome et al., 1999) and vital dye labelling (McCauley and Bronner-Fraser, 2003) have established that lamprey have cranial neural crest cells which behave similarly to those of gnathostomes-- save for generating jaws. Less is known about lamprey trunk neural crest except that it is necessarily less multipotent than gnathostome trunk crest since lamprey lacks sympathetic ganglia, a gnathostome neural crest derivative. The basal position of agnathans, their lack of gnathostome specializations, and their similarity to fossil forms suggest that they closely approximate the primitive vertebrate state.

Neural Crest Cell Biology

As in other chordates, the vertebrate neural plate forms from a broad domain of dorsal ectoderm around the time of gastrulation. Initial partitioning of neural and non-neural ectoderm is likely induced by signals emanating from underlying mesendoderm (reviewed by (Bally-Cuif and Hammerschmidt, 2003)). In *Xenopus*, inhibition of BMP signaling is sufficient to push ectoderm towards a neural fate, while Wnt, FGF, and IGF signals appear necessary for neural induction to in the embryo. In the non-neural ectoderm, high levels of BMP signaling activate expression of Dlx genes, suppressing neural fates and leading to epidermal differentiation (Feledy et al., 1999). Low levels of BMP activity in the neural plate upregulate the neural factors Sox2 and Zic, which in turn activate proneural genes such as Neurogenin and Achaete-Scute (Mizuseki et al., 1998). Conservation of the basic features of neural induction in amphioxus is suggested by expression of BMP 2/4 and Dlx gene homologs in the non-neural ectoderm (Holland et al., 1996; Panopoulou et al., 1998) and Sox2, Neurogenin, and Zic genes in the neural plate (Gostling and Shimeld, 2003; Holland et al., 2000).

The neural crest arises at the border of the neural plate and non-neural ectoderm. Several transcriptional regulators expressed in this domain have demonstrated functions in neural crest specification including Zic, Pax3/7, Msx, FoxD3, Twist, Snail/Slug, Id, Sox9 and AP-2. Some of these have been shown to respond to Wnt, BMP, and FGF signaling pathways and to cross- and auto- regulate (For reviews see (Aybar and Mayor, 2002; LaBonne and Bronner-Fraser, 1999). Among them Slug/Snail family factors and FoxD3 are both necessary and sufficient to induce expression of most neural crest markers, including Twist, FoxD3, Zic, AP-2 and Slug/Snail (LaBonne and Bronner-

Fraser, 2000; Sasai et al., 2001). Sox9 has been shown to be necessary, but not sufficient for expression of Twist, FoxD3, Snail, Pax3 and Msx (Spokony et al., 2002) while AP-2 has recently been shown to be necessary and sufficient for expression of Slug and Sox9 (Luo et al., 2003).

While the network of interactions required to specify the neural crest cell fate is being elucidated, how these interactions ultimately confer neural crest cell morphology and behavior is unclear. Evidence suggests that several genes have multiple roles in neural crest development, first in early induction and later in regulating migration and differentiation. For example, transcriptional repressors of the Snail/Slug family have established roles in neural crest induction, but also promote the epithelial to mesenchymal transition and migration (LaBonne and Bronner-Fraser, 2000), perhaps by down-regulating cadherins (Cano et al., 2000). Recent evidence from *Xenopus* suggests an early role for AP-2 genes in neural crest induction (Luo et al., 2003), while loss of function studies implicate AP-2 genes in turning on post-migrational differentiation programs in the mouse (MorrissKay, 1996; Schorle et al., 1996). Similarly, FoxD3 has been shown to affect the early stages of neural crest specification, and to have later roles in promoting neural and glial fates and suppressing melanogenesis (Dottori et al., 2001; Kos et al., 2001; Sasai et al., 2001). Sox9, while necessary for the expression of early neural crest markers in frog (Spokony et al., 2002), has a later role in chondrogenesis of pharyngeal arch crest in zebrafish (Yan et al., 2002).

Elucidating The Genetic Bases of Neural Crest Evolution

The aim of this work is to understand the molecular bases neural crest cell evolution. To accomplish this, I have used a 3-tiered experimental approach, and examined 1) the expression, 2) the function, and 3) the regulation of amphioxus genes with vertebrate homologs involved in neural crest development. By comparing these amphioxus genes with their lamprey and gnathostome counterparts, I have uncovered genetic changes coincident with, and potentially causal to, the origins of neural crest. Using in situ hybridization I analyzed the embryonic expression of three amphioxus genes and demonstrated that their deployment to the neural plate border is a vertebrate novelty coincident with the appearance of definitive neural crest cells. I also tested the functional capacity of the amphioxus Snail protein to induce expression of neural crest markers in a vertebrate embryo, given that amphioxus Snail is not coexpressed with most other neural crest marker homologs in amphioxus. I show that the function of the Snail protein in establishing the neural plate border was not significantly altered during vertebrate evolution, although vertebrate Snail genes may have evolved novel domains necessary for later functions in neural crest cells. Finally, I began characterizing the *cis*-regulation of vertebrate and amphioxus Slug/Snail orthologs to determine if divergent aspects of Snail gene expression (i.e., expression in neural crest cells) are reflected in structural differences in Snail *cis*-regulatory DNA. Using this 3-tiered approach I have begun to define the novel genetic regulatory interactions that drove the evolution of neural crest cells in the vertebrate lineage.

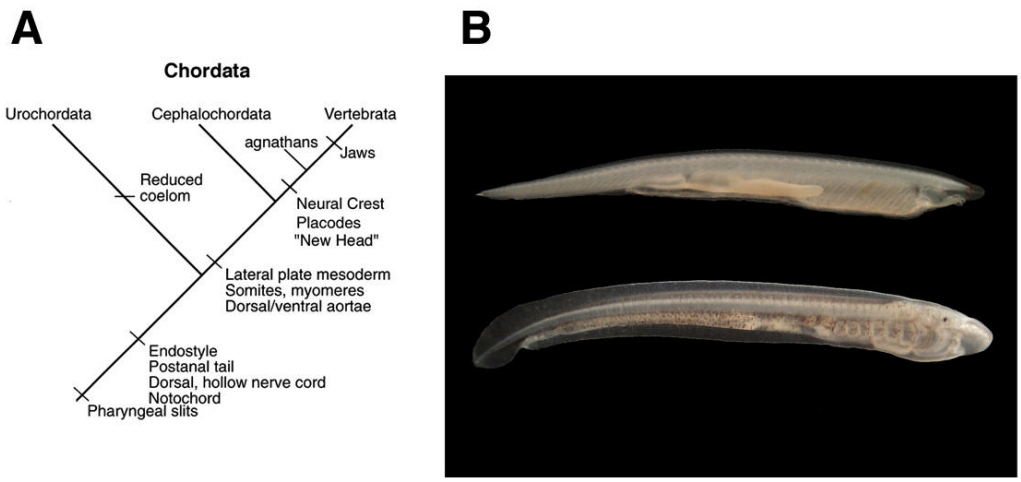

Figure 1. Phylogeny of the chordates. (A) An abbreviated phylogeny of the phylum Chordata showing the three subphyla and the agnathan vertebrates. Branch lengths are not indicative of divergence time as the subphyla first appear during the same period deep in the Cambrian. (B) A post-metamorphic amphioxus juvenile, above, and a lamprey ammocoete larva below. Despite lacking neural crest cells and placodes, amphioxus grossly resembles the larva of this basal vertebrate. Interestingly, amphioxus adults and lamprey ammocoetes also have similar lifestyles; spending most of their time in vertical burrows, filter feeding.

Chapter 2:

Amphioxus and Lamprey AP-2 Genes: Implications for Neural Crest Evolution and Migration Patterns

ABSTRACT

The neural crest is a uniquely vertebrate cell type present in the most basal vertebrates, but not in cephalochordates. Here, we study differences in regulation of the neural crest marker AP-2 across two evolutionary transitions: invertebrate to vertebrate, and agnathan to gnathostome. Isolation and comparison of amphioxus, lamprey, and axolotl AP-2 reveals its extensive expansion in the vertebrate dorsal neural tube and pharyngeal arches, implying co-option of AP-2 genes by neural crest cells early in vertebrate evolution. Expression in non-neural ectoderm is a conserved feature in amphioxus and vertebrates, suggesting an ancient role for AP-2 genes in this tissue. There is also common expression in subsets of ventrolateral neurons in the anterior neural tube, consistent with a primitive role in brain development. Comparison of AP-2 expression in axolotl and lamprey suggests an elaboration of cranial neural crest patterning in gnathostomes. However, migration of AP-2 expressing neural crest cells medial to the pharyngeal arch mesoderm appears to be a primitive feature retained in all vertebrates. Because AP-2 has essential roles in cranial neural crest differentiation and proliferation, the co-option of AP-2 by neural crest cells in the vertebrate lineage was a potentially critical event in vertebrate evolution.

INTRODUCTION

A defining event in vertebrate evolution was the appearance of neural crest cells (Gans and Northcutt, 1983). These cells form most of the structures that distinguish vertebrates from other chordates, including the skeleto-musculature of the cranium, face, jaws and pharynx and most of the peripheral nervous system. Despite their importance, little is known about the evolutionary origins of neural crest cells.

In the most basal vertebrate studied, lamprey, an essentially modern neural crest generates almost all of the derivatives seen in gnathostomes (Langille and Hall, 1988). In contrast, the most vertebrate-like invertebrates, the cephalochordates, appear to lack even a rudimentary neural crest. Furthermore, the fossil record offers no obvious intermediate forms that display features suggestive of a primitive neural crest.

Within the gnathostomes, the molecular mechanisms underlying neural crest induction are largely conserved (for review see LaBonne and Bronner-Fraser, 1999). Input from BMP, Wnt and FGF signaling pathways activate a complement of transcription factors at the neural plate border including Snail, Twist, Zic, Id, AP-2, FoxD3, Distalless, Msx, and Pax genes. A subset of these factors has been shown to cross- and autoregulate, such that a rough outline of their regulatory relationships is emerging (Sasai et al., 2001).

Amphioxus and lamprey are useful organisms for investigating neural crest evolution as they both diverged near the time neural crest first appeared. Amphioxus, a cephalochordate, separated from the vertebrate lineage before the origin of neural crest and is thought to approximate the ancestral pre-vertebrate chordate. Expression studies

in amphioxus reveal that some of the genetic machinery needed to create neural crest cells (including BMP-4, Snail, Pax-3, Wnt7B, Distalless and Msx) was in place before bona fide neural crest cells appeared (reviewed by Holland and Holland, 2001). Lamprey diverged from other vertebrates relatively soon after the neural crest arose and is thought to display primitive features lost or masked in gnathostomes.

In this study, we focus on the regulatory evolution of the transcriptional activator AP-2 as a starting point for dissecting the molecular history of neural crest cells. AP-2 is a robust neural crest marker shown to be essential for cranial neural crest development in vertebrates. The vertebrate AP-2 family consists of four genes (AP-2 alpha, beta, gamma, and delta) that have dynamic and largely overlapping patterns of expression during embryogenesis (reviewed by Hilger-Eversheim et al., 2000; for description of AP-2 delta see Zhao et al., 2001). At gastrula stages, AP-2 transcripts are initially observed in non-neural ectoderm. As neurulation proceeds, AP-2 expression is extinguished in non-neural ectoderm and up-regulated in the neural folds, marking neural crest cells before, during, and after their migration. AP-2 alpha is functionally important for neural crest cells, since null mice almost completely lack cranial neural crest derivatives (Schorle et al., 1996; Zhang et al., 1996). In addition, AP-2 is necessary for expression of HoxA2 in the neural crest, indicating an indirect role for AP-2 genes in neural crest patterning (Maconochie et al., 1999).

Here, we describe the isolation of amphioxus and lamprey AP-2 homologs and compare their expression patterns to that of AP-2 in the gnathostome, axolotl. Using this broad comparative base, we span two important evolutionary transitions: the divergence of vertebrates from invertebrates and the divergence of jawed vertebrates from agnathans.

Across each transition, we observe differences in the deployment of AP-2 genes suggestive of key genetic and developmental changes during early vertebrate evolution. Taken together our observations suggest a critical role for AP-2 during neural crest evolution.

MATERIALS AND METHODS

Embryo collection

Adult amphioxus (*Branchiostoma floridae*) were collected from Old Tampa Bay Florida and electrostimulated to induce gamete release. Eggs were fertilized and embryos cultured, staged, and fixed as described by Holland et al. (1996). Embryos of the lamprey *Petromyzon marinus* were collected, staged, and fixed at the Lake Huron biological station according to the methods of Tomsa and Langeland (1999) and provided as a gift by David McCauley. Albino embryos of the Mexican axolotl, (*Ambystoma mexicanum*) were obtained from the axolotl colony in Bloomington, Indiana and raised and staged as described by Epperlein et al. (2000).

AP-2 gene isolation

Amphioxus and lamprey embryonic cDNA libraries were the generous gifts of Jim Langeland. 500 and 200 bp fragments of amphioxus and lamprey AP-2 genes, respectively, were amplified directly from diluted lambda phage libraries by degenerate PCR with the following primers: for amphioxus, 5' primer GTRTTCTGYKCAGKYCCYGGICG and 3' primer GWKATVAGGKWGAAGTGSGTCA, for lamprey, 5' primer CCVCCIGARTGCCTSAAYGC and 3' primer GAAGTCICGVGCSARRTG. Amplified fragments were used to screen the libraries at high stringency (final wash 0.2XSSC) to isolate full-length clones. Phagemids were excised and inserts sequenced completely from both ends. Low stringency screens of the amphioxus cDNA library and an arrayed amphioxus genomic library were performed as described for Southern blot analysis.

Phylogenetic analysis

The conceptual protein products of the amphioxus and lamprey AP-2 transcripts were aligned with vertebrate and *Drosophila* AP-2 protein sequences. Axolotl AP-2 was not used for analysis as only a partial sequence is available. A phylogenetic tree was created within the ClustalX program (Thompson et al., 1997) using the neighbor-joining method of Saitou and Nei (1987). Bootstrap values were determined by 1000 resamplings of alignment data. Genbank accession numbers for the aligned sequences are; mouse AP-2 alpha, NP035677, mouse AP-2 beta, Q61313, mouse AP-2 gamma , Q61312, mouse AP-2 delta, AAL16940, chicken AP-2 alpha, AAB65081, chicken AP-2 beta, AAC26111, human AP-2 alpha, NP003211, human AP-2 beta, NP003212, human AP-2 gamma , XP009543, *Xenopus* AP-2 alpha, S34449, *Drosophila* AP-2, CAA07279.

Hox2 in silico *cis*-regulatory analysis

Genomic sequence surrounding the transcriptional start of *Drosophila* proboscipedia and AmphiHox2 were scanned for consensus AP-2 binding sites using the MatInspector v2.2 program. Core and matrix similarities were set at the default values of .75 and .85, respectively. Accession numbers are NG000110 for proboscipedia and AB050888 and AB050887 for AmphiHox2 genomic sequences.

In situ hybridization

In situ hybridization on amphioxus embryos was performed as described by Holland (1996) with the omission of deacetylation and RNAse treatments. In addition,

post-hybridization washes were in PBS-tween 0.1%, rather than SSC, and the blocking solution was 2mg/mL BSA/ 2% sheep serum in PBS-tween 0.1%. Riboprobes against the DNA binding/dimerization domain and full-length cDNA yielded identical staining patterns.

In situ hybridizations on axolotl and lamprey embryos were as described by Henrique et al. (1995) with the addition of an extra 12 hour wash in MAB-tween. Tween-20 concentrations for PBS and MAB solutions were increased to 0.2%. Proteinase K treatments were also adjusted to 50ug/ml for 15 minutes for lamprey embryos and 10ug/ml for 4 min for axolotl embryos. Hybridization was at 65°C. For lamprey, the riboprobe was generated against a 500 bp portion of the DNA binding/dimerization domain. The axolotl AP-2 riboprobe was prepared as previously described Epperlein et al. (2000).

Southern blot analysis

Genomic DNA from five adult amphioxus was purified and digested with four restriction enzymes (*Apa*I, *Cla*I, *EcoR*V, and *Hind*III). Genomic DNA from a single adult lamprey was isolated and digested with six restriction enzymes (*Apa*I, *EcoR*I, *Hind*III, *Nco*I, *Pst*I, and *Stu*I). Digests were electrophoresed on 0.7% agarose gels and blotted onto GeneScreen Plus filters (NEN Life Science Products). Homologous 200 bp probes were designed to intra-exonic regions of the DNA binding and dimerization domains of the amphioxus and lamprey AP-2 genes. Intron-exon boundaries were deduced from human AP-2 alpha genomic sequences (Bauer et al., 1994) and partial sequencing of amphioxus AP-2 cosmids. Southern blots were hybridized in 6xSSC/5% SDS/ 100

ug/mL sheared herring sperm DNA/5x Denhardt's solution at 60°C to ^{32}P-labeled probes. Washes were in 2xSSC, 0.5% SDS at 55°C.

Plastic sectioning

Embryos were dehydrated in ethanol and embedded in Epon-Araldite. After polymerization for 72 hours at 60°C, the embryos were sectioned to 10-15 µm using a glass knife, coverslipped in Gelmount, and photographed.

RESULTS

Isolation of AP-2 from amphioxus and lamprey

We used degenerate polymerase chain reaction to isolate AP-2 gene fragments from amphioxus and lamprey embryonic cDNA libraries. The fragments were then utilized to screen the libraries at high stringency for full-length clones. The longest cDNAs from each screen were completely sequenced. Within both cDNAs, open reading frames encoding proteins of exactly 498 amino acids were identified (Fig. 1A). The amphioxus and lamprey proteins were found to be 49 and 54 percent identical, respectively, to mouse AP-2 alpha, with 74 and 78 percent identity over the DNA binding and dimerization domains. A region of high sequence similarity was also seen in the proline-rich transactivation domain. An arrayed amphioxus cosmid library was screened at low stringency, and four hybridizing cosmids were partially sequenced.

Southern blot analysis

Low-stringency Southern blot analysis was used to estimate the number of AP-2 genes in the amphioxus and lamprey genomes. In both cases, probes were created that recognized part of the highly conserved DNA binding domain, but were likely to be intra-exonic based upon the genomic structure of human and amphioxus AP-2 genes.

Probing of genomic DNA from a single adult amphioxus revealed two strongly hybridizing fragments when digested with 7 of 8 enzymes (data not shown). This raised the possibility that there was more than one AP-2 family member in the amphioxus genome. To test this, we re-probed the cDNA library at low stringency and detected no

additional AP-2 cDNAs. Low stringency screening of an arrayed amphioxus genomic library also yielded no new AP-2 gene sequences. We then investigated whether the multiple fragments were due to polymorphism at the AP-2 locus. Genomic DNA from 5 individual adult amphioxus were digested with four enzymes. All 5 adults had different restriction fragment length profiles (Fig. 2A). For each enzyme, 2-4 different fragments were observed in total, with each animal possessing only one or two fragment types per enzyme. Collectively, the results are consistent with various homo- and heterozygotic combinations of several restriction fragment length alleles at a single highly polymorphic locus. Based upon this, and the fact that low-stringency screens of cDNA and genomic libraries consistently yielded a single gene, we conclude that there is a single AP-2 gene in the amphioxus genome.

Low-stringency Southern blot of genomic DNA from an individual adult lamprey showed a single band in 4 out of 5 digests. Probing of lamprey genomic DNA with an amphioxus AP-2 probe yielded no discernable signal above background (data not shown).

AP-2 gene phylogeny

Amphioxus, lamprey, mouse, chicken, frog, human and *Drosophila* AP-2 sequences were aligned, and a phylogenetic tree was generated using the neighbor joining method (Fig. 1B). Axolotl AP-2 was not used for analysis as only a partial sequence is available. The deduced phylogeny shows amphioxus AP-2 falling outside of the vertebrate AP-2 clade, which includes lamprey AP-2 and gnathostome AP-2 alpha, beta, and gamma. Within the vertebrate clade, lamprey AP-2 fails to group with any one

gnathostome AP-2 isoform. This general topology is maintained when the DNA binding/dimerization domain alone is used for alignment.

Unexpectedly, the recently described mouse AP-2 delta fails to group with vertebrate AP-2s when full-length sequences are aligned. When only the conserved DNA binding/dimerization domains are used for alignment, AP-2 delta also falls outside of the amphioxus/vertebrate clade (data not shown). Both phylogenetic positions are poorly supported by low bootstrap values and may reflect rapid evolution of AP-2 delta in gnathostomes or early divergence of AP-2 delta in the vertebrate lineage.

Pattern of AP-2 expression in amphioxus

Amphioxus development proceeds in a simplified vertebrate-like manner, with the neural plate forming from dorsal ectoderm at 8-9 hours post-fertilization. In 9-hour neurulae, AP-2 transcripts are detected throughout the non-neural ectoderm (Fig. 3A, B). No expression is seen in the open neural plate or mesendoderm. Following the onset of somitogenesis at 9.5-10 hours, non-neural ectoderm begins closing over the invaginating neural plate. In 11.5-hour neurulae, AP-2-expressing ectoderm cells appear to be migrating over the closing neural plate (Fig. 3C, D, G). Upon hatching at 12 hours, the neurula is covered in ciliated AP-2-positive epidermis. Neurulation is completed under the epidermis by hour 18. During this period, AP-2 ectodermal expression begins to recede from the anterior- and posterior- most ends of the larva (Fig. 3E). At 20 hours, a small spot of staining appears in the anterior gut, likely presaging formation of the left gut diverticulum. At 24 hours, this expression sharpens, marking the endodermal portion of the developing preoral pit (Fig 3F, H). Simultaneously, strong staining appears in the

ventro-lateral walls of the cerebral vesicle and expression in the epidermis fades (Fig. 3H, I, L). At 36 hours, the embryo has elongated to roughly twice its 18-hour length, and the mouth and first gill slit begin to form (Fig. 3K). Both the cerebral vesicle and pre-oral pit stainings become markedly reduced after this time (Fig. 3J), but persist weakly until 4 days.

Pattern of AP-2 expression in lamprey

At 4 days, the neural plate of the lamprey embryo is a flattened area of dorsal ectoderm. At this stage, AP-2 staining is observed in non-neural ectoderm (Fig. 4A, F). As the neural plate condenses towards the dorsal midline around day 5, AP-2 transcripts are detected at the edges of the neural plate and broadly in the adjacent ectoderm (data not shown). AP-2 is down-regulated in the non-neural ectoderm at ventral and lateral levels.

At six days, AP-2 is expressed solely in the dorsal neural rod (Fig. 4C, G), forming a stripe that is disrupted anteriorly by a gap in expression near the protruding head (Fig. 4B). While expression in non-neural ectoderm is extinguished in 6-day embryos, a new phase of epidermal expression begins at 7 days in the head (Fig. 4D). Scattered AP-2 positive cells appear throughout the head ectoderm, but are conspicuously absent from the otic placode. Also at 7 days, the anterior gap in neural rod expression sharpens, and sections reveal staining in surrounding head mesenchyme highly reminiscent of early migrating neural crest in other vertebrates (Fig. 4H).

At 7.5 days, separations in the head staining become discernable, suggestive of neural crest-free spaces between AP-2 positive streams (Fig. 4E). Horizontal sections

reveal the initial outpocketing of first arch endoderm at this time with AP-2 transcripts in the mesenchyme and dorsal neural tube (Fig. 4P). Sections at the level of the otic vesicle show accumulation of AP-2 signal in the space dorsal to the vesicle as well as in the mesenchyme below it, but never medial to the otic vesicle (Fig. 4I).

At 8 days, divisions in the head staining become more obvious, and three broad areas of AP-2 expression can be distinguished—an anterior band, and two more caudal swathes straddling the putative otic placode (Fig. 4K). Ventrally, in the region of the nascent pharyngeal arches, the two posterior streams fuse into one continuous mass, while the anterior stream splits into three smaller streams. The rostral-most stream sits just anterior to the area of the optic cup, and likely represents the ophthalmic neural crest stream (Fig. 6B). Around the mouth, the rest of the anterior stream forks, marking cells in the mandibular arch and maxillary (anterior lip) region. Horizontal sections at 8.5 days show formation of the first three pharyngeal arches with AP-2 transcripts detected in the ectoderm, superficial to the ectoderm, and adjacent to pharyngeal endoderm (Fig. 4Q). From 8.5-9 days, staining in the area of the pharyngeal arches accumulates (Fig. 4L, M). In the trunk, staining in the dorsal neural tube, dorsal fin, and weak staining between the somites is apparent (data not shown).

At 10-11 days, a new phase of AP-2 neural expression begins in a subset of cells in the anterior neural tube (Fig. 4O). From days 11 to 12, gaps appear in the AP-2 positive arch mesenchyme where the pharyngeal endoderm and ectoderm meet to create the pharyngeal slits (Fig. 4N). Horizontal sections at 12 days show the formed arches, with AP-2 signal present medial and lateral to the pharyngeal mesoderm (Fig. 4R).

Pattern of AP-2 expression in axolotl

To facilitate comparison of lamprey and amphioxus AP-2 gene usage with that of gnathostomes, a developmental series of axolotl embryos was probed for AP-2 transcripts. AP-2 expression in the axolotl has been described for stages just preceding and following neural crest migration and was found to mirror that of mouse, chicken and frog (Epperlein et al., 2000). Staining patterns in early neurulae, however, have not been previously described. At open neural plate stages, AP-2 transcripts are detected in the non-neural ectoderm, and are strongly expressed at the neural plate border (Fig. 5A, far right panel). As neurulation proceeds, AP-2 is further up-regulated in the protruding neural folds and down-regulated in the non-neural ectoderm. Upon neural tube closure, AP-2 staining in the dorsal aspect of the neural tube is maximal, while non-neural ectoderm has only a residual AP-2 positive signal (data not shown). Little or no staining is apparent in non-neural ectoderm at later stages.

DISCUSSION

In this study, we have utilized the expression of chordate AP-2 genes in two ways: 1) to suggest homology between structures where homology is well-supported by anatomy; and 2) as evidence of AP-2's regulatory history in the chordate lineage.

Chordate AP-2 genes

Single representatives of the AP-2 gene family were isolated from amphioxus and lamprey embryonic cDNA libraries. The presence of a single AP-2 gene in each genome was suggested by low-stringency genomic Southern blotting and phylogenetic analyses. This follows with gene numbers in amphioxus where a 1:3 or 1:4 correspondence of amphioxus to gnathostome gene homologues is usually observed, and supports the proposed scheme of two whole or partial genome duplications in the vertebrate lineage (for review see Holland, 1999). Limited data from lamprey indicate a homologue ratio closer to 1:2 when taking into account lamprey-specific gene duplication events (Sharman and Holland, 1998; Ueki et al., 1998; Ogasawara et al., 2000; Myojin et al., 2001; Neidert et al., 2001; Force et al., 2002). Thus, there is a chance that another lamprey AP-2 gene exists that was not detected. Furthermore, phylogenetic analysis leaves open the possibility that lamprey has an AP-2 delta, as lamprey AP-2 groups with gnathostome alpha, beta and gamma, but not AP-2 delta. Whether this is due to rapid evolution of AP-2 delta in mammals or early divergence of AP-2 delta in vertebrates is unclear. Alternately, a second lamprey AP-2 may have been lost during evolution or double duplication of an ancestral AP-2 gene occurred after the divergence of agnathans.

Outside of phylum chordata, it is likely that having one AP-2 gene is the primitive condition for bilateria, as only a single AP-2 gene is found in *Drosophila* (Bauer et al., 1998; Monge and Mitchell, 1998). Overall, our data are consistent with the vertebrate genome double-duplication hypothesis, but are inconclusive as to the timing of these duplications relative to gnathostome origins.

Early non-neural ectoderm AP-2 expression is ancestral

A striking feature of AP-2 expression common to amphioxus, lamprey, and axolotl, is robust expression in non-neural ectoderm at open neural plate stages (Fig. 5A). Similar early ectodermal expression has been reported for chick and mouse (Shen et al., 1997; Mitchell et al., 1991). These data suggest an ancient role for AP-2 in the chordate non-neural ectoderm, and strong conservation of an early ectodermal regulatory element in the AP-2 promoter. Interestingly, Dlx and BMP-4 are also co-expressed in the non-neural ectoderm of gnathostomes and amphioxus (Panopoulou et al., 1998; Holland et al., 1996), and a regulatory relationship between the two has been proposed in frog (Feledy et al., 1999). It is possible that all three genes interact in an evolutionarily ancient pathway for specification of non-neural ectoderm in chordates.

Neural tube expression differs between amphioxus and vertebrates

Before neural tube formation in vertebrates, AP-2 expression at the neural/non-neural interface increases (Mitchell et al., 1991; Chazaud et al., 1996; Moser et al., 1997; Shen et al., 1997). Simultaneously, expression in the remaining non-neural ectoderm begins to fade. By the completion of neurulation, AP-2 positive cells have become

incorporated into the dorsal neural tube and epidermal staining is reduced. AP-2 expression in the neural folds and neural tube at these stages mirrors that of neural crest markers Snail, Slug, and Id-2, and overlaps with the cranial neural crest markers Dlx-2 and Msx-1,2 (Robinson and Mahon, 1994; Martinsen and Bronner-Fraser, 1998; Sefton et al., 1998; Bendall and Abate-Shen, 2000).

During parallel stages in amphioxus, the non-neural ectoderm has closed over the forming neural tube and AP-2 transcripts are detected strongly throughout the epidermis. After neurulation, AP-2 is down-regulated in the epidermis, but no AP-2 positive cells become incorporated into the dorsal neural tube. Subsequent AP-2 expression includes only a few cells in the ventro-lateral cerebral vesicle and preoral pit. During these stages, the amphioxus homologs of Snail and Msx are expressed at the edges of the neural plate and then expand throughout the neural tube (Langeland et al., 1997; Sharman et al., 1999). Unlike vertebrates, neither AmphiSnail or AmphiMsx gene expression overlaps with AP-2 in the dorsal neural tube.

Differences in amphioxus and vertebrate AP-2 neural expression imply divergent modes of AP-2 regulation in the two subphyla. A simplistic explanation is the presence of a neural crest enhancer in vertebrate AP-2 gene promoters that is absent in the homologous amphioxus promoter. Candidate regulators for a putative novel enhancer would include genes like Snail and Msx, which are coexpressed with AP-2 in vertebrate neural crest, but not in the amphioxus neural tube. Provocatively, the murine Msx-1 promoter contains a consensus AP-2 binding site (Kuzuoka et al., 1994). Thus, it is possible that new regulatory relationships between these genes resulted in novel deployment of AP-2 to neural crest early in vertebrate evolution. An alternative

explanation is novel deployment of AP-2's upstream regulators in vertebrates. Furthermore, secondary loss of a neural enhancer, or differential deployment of trans-acting regulators may have resulted in a loss of AP-2 expression in the amphioxus dorsal neural tube. Expression data from the third chordate subphylum, Urochordata, may clarify the direction of this evolutionary change.

Given its essential role in cranial neural crest cell differentiation and proliferation, it is tempting to speculate that cooption of AP-2 by the dorsal neural tube was a critical event in neural crest evolution. Knockout studies of AP-2 alpha demonstrate the necessity of AP-2 activity in post-migratory cranial neural crest (Morriss-Kay, 1996; Schorle et al., 1996; Zhang et al., 1996). Mice lacking AP-2 have relatively normal neural crest migration, but most neural crest derivatives in the head, including the rostral-most parts of the skull, the first and second arch cartilages, and cranial sensory ganglia, are missing or reduced. Thus, AP-2 expression in the dorsal neural tube may have been a prerequisite for the evolution of neural crest cells

If AP-2 usage in the dorsal neural tube is indeed a vertebrate apomorphy, an intriguing question is whether its new roles in neural crest involved evolution of the AP-2 protein itself, or simply redeployment of a functionally conserved gene. While all described AP-2 protein sequences are highly conserved in the DNA binding/dimerization domains, novel motifs in the more divergent transactivation domain may confer additional regulatory properties onto vertebrate AP-2 genes. *In vivo* and *in vitro* comparisons of amphioxus and lamprey AP-2 gene function may shed light on the biochemical features important for AP-2 function in the neural crest.

Later AP-2 expression in amphioxus

Shortly after expression has faded from the epidermis in amphioxus, AP-2 is up-regulated in cells of the ventro-lateral cerebral vesicle and forming preoral pit. In axolotl and lamprey, AP-2 has a potentially homologous late phase of expression in neurons of the anterior neural tube (Fig. 5B). AP-2 expression in the developing cerebellum of mouse also has been reported (Moser et al., 1997). Furthermore, AP-2 expression in the developing fly brain is a prominent feature of *Drosophila* AP-2 expression (Monge and Mitchell, 1998), suggesting an ancient function for AP-2 genes in the anterior nervous system of bilaterians.

Enrichment in the developing pre-oral pit is harder to relate to any aspect of vertebrate expression. It may reflect, however, an evolutionarily conserved regulatory relationship between AP-2 and Hox2 genes. AmphiHox2 expression temporally and spatially overlaps with AP-2 in the preoral pit (Wada et al., 1999), and AP-2 genes are essential for HoxA2 expression in cranial neural crest (Maconochie et al., 1999). Amphioxus AP-2 may similarly regulate Hox2 in the preoral pit, as two consensus AP-2 binding sites are present in the 5' genomic sequence of AmphiHox2. AP-2 expression in *Drosophila* also overlaps with proboscipedia (Hox2) (Monge and Mitchell, 1998). Three consensus AP-2 binding sites are found clustered within a 1 kilobase intronic region shown to direct reporter expression to the maxillary lobe. Taken together, these observations suggest an ancient role for AP-2 in Hox class 2 gene regulation.

AP-2 and neural crest migration patterns in lamprey

Our results show that AP-2 expression in lamprey closely resembles AP-2 expression in axolotl, chicken, and mouse. Early deployment in ectoderm is followed by expression in the neural folds and dorsal neural tube. AP-2 transcripts are then seen throughout the head mesenchyme, in a pattern consistent with expression in early migrating neural crest. Later, AP-2 staining in the head is confined to apparent streams or blocks of cells. Finally, lamprey AP-2 appears in mesenchyme surrounding pharyngeal arch mesoderm. The similarity of lamprey AP-2 and gnathostome AP-2 staining, together with the anatomical context of lamprey AP-2 expression, strongly suggests that AP-2 marks neural crest cells in lamprey.

Utilizing AP-2 as a marker gives valuable insight into the migration patterns of neural crest cells in a basal vertebrate. Previous studies have analyzed lamprey neural crest migration using scanning electron microscopy, molecular markers for subsets of crest cells (Otx, Dlx) (Tomsa and Langeland, 1999; Neidert et al., 2001), or limited DiI labelling (Horigome et al., 1999). The current study is the first time expression of a pan-neural crest marker has been analyzed in lamprey. Comparing lamprey and axolotl AP-2 expression patterns illustrates that lamprey cranial neural crest migrates in typical vertebrate fashion. Three broad areas of AP-2 expression can be discerned in the lamprey head which appear equivalent to the trigeminal, hyoid, and branchial streams in gnathostomes (Fig. 6B, E). Furthermore, the hyoid and branchial streams appear to lie on either side of the otic vesicle, as in gnathostomes. This contradicts previous scanning electron microscopy analyses suggesting that lamprey hyoid neural crest migrates directly

under the otic vesicle (Horigome et al., 1999). In sections through lamprey embryos, no AP-2 positive cells are observed interior to the otic vesicle (Fig. 4I).

Interestingly, during the early stages of neural crest migration (6-7days), a gap in AP-2 expression appears in the neural tube just anterior to the otic placode (Fig. 6A). In gnathostomes, similar gaps correspond to rhombomeres 3 and 5, which are depleted of neural crest (Fig. 6D). We cannot be sure if this gap corresponds to a rhombomere as no molecular or anatomical rhombomeric markers are available for this stage in lamprey. However, the presence of only one gap is suggestive of reduced patterning in the early migrating neural crest of lamprey.

At later stages, the putative trigeminal stream appears to divide into the ophthalmic stream rostrally, and "maxillo-mandibular" caudally (Fig. 6B). The maxillo-mandibular then splits around the mouth, filling the maxillary (anterior lip) and mandibular regions. This subdivision mimics streaming patterns in gnathostomes as illustrated by AP-2 staining in axolotl (Fig. 6E) and supports homology of lamprey and gnathostome mandibular segments. This finding, along with recent studies of engrailed and Otx expression, lend molecular support to the idea that gnathostome jaws evolved from the pumping organ of an agnathan ancestor, rather than anterior gill arch cartilage.

While initial subdivision of putative neural crest cells in lamprey closely mimics that of gnathostomes, later ventral migration into the nascent pharyngeal arches is somewhat different. Conspicuously, coherent streaming of lamprey cranial neural crest is not maintained as the cells move ventrally, and the three streams appear to fuse as they fill the pharyngeal region (compare Fig. 6B and 6E). Subsequent partitioning of pharyngeal arch neural crest appears to occur only after migration as the outpocketing

endoderm divides both the paraxial mesoderm and overlying neural crest (Fig. 4N). This difference in streaming pattern may reflect a heterochrony in arch formation relative to neural crest migration between lamprey and gnathostomes.

A long-recognized difference between lamprey and gnathostome cranial neural crest is its final destination in the arches (Graham, 2001; Kimmel et al., 2001). In gnathostomes, cartilages derived from cranial neural crest lie medial to the arch mesoderm. In lamprey, this support tissue lies lateral to the arch mesoderm. This is reflected by AP-2 staining in axolotl showing neural crest cells internal to the arch mesoderm (Fig. 6F). We find that in lamprey, AP-2 transcripts are similarly distributed internal to the pharyngeal arch mesoderm (Fig. 6C), suggesting medial movement of pharyngeal arch neural crest does indeed occur in lamprey, although to a lesser degree than in gnathostomes.

CONCLUSIONS

In this study, we have documented differences in AP-2 regulation across the evolutionary transitions from invertebrate to vertebrate and agnathan to gnathostome. AP-2 expression in amphioxus and vertebrates imply cooption of AP-2 by neural crest cells in the vertebrate lineage. This was a potentially critical event in vertebrate evolution as AP-2 has essential roles in cranial neural crest differentiation and proliferation. AP-2 deployment in the neural tube may have potentiated neural crest evolution by promoting transcription of downstream effectors of cranial neural crest differentiation. AP-2 expression patterns in lamprey and axolotl demonstrate an increase in neural crest patterning in gnathostomes, and elaboration of neural crest migratory

behavior that may relate to the timing of pharyngeal arch formation. As in situ hybridization is merely a series of static observations of a single gene's usage, conclusive proof of these differences await the results of detailed cell tracking experiments. Taken together, the regulatory history of AP-2 genes in the chordate lineage suggest molecular and developmental mechanisms for the evolution of the vertebrate head.

ACKNOWLEDGEMENTS

We thank Linda and Nick Holland for making amphioxus collection possible, John Lawrence for lab space in Tampa, Jim Langeland for generously providing excellent cDNA libraries, Dave McCauley for supplying lamprey embryos, and Robert Cerny for invaluable discussion. This work was supported by NASA grant 98-HEDS-02 to MBF.

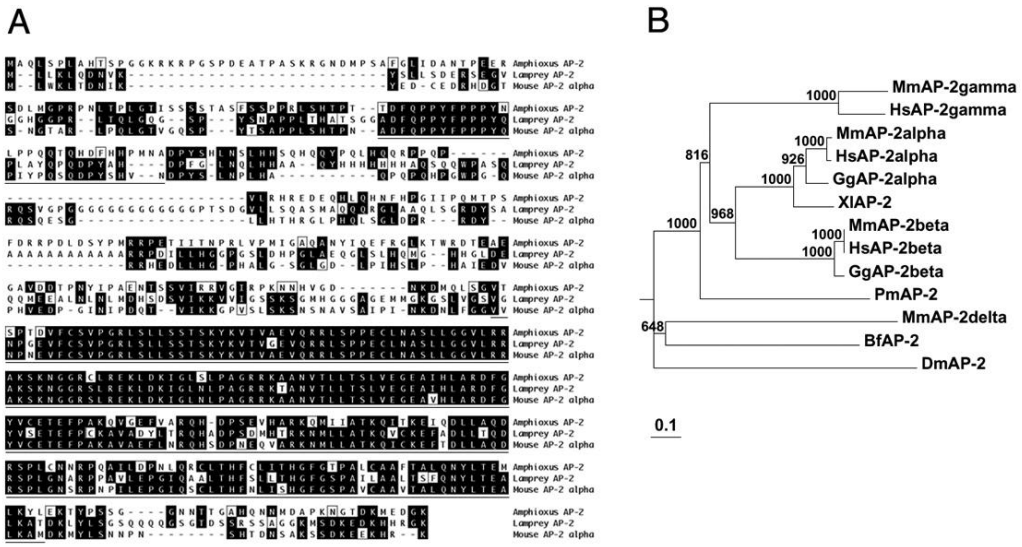

Figure 1. Sequence analysis of amphioxus and lamprey AP-2 proteins. (A) Clustal alignment of amphioxus AP-2, lamprey AP-2 and mouse AP-2 alpha. Identical residues are shaded black, biochemically similar residues are boxed. Underlined regions of mouse AP-2 alpha represent the proline-rich transactivation domain (N-terminal) and the DNA binding/dimerization domain (C-terminal). The regions of highest homology between the three sequences are within these functionally important domains. (B) Neighbor-joining phylogenetic tree of vertebrate and amphioxus AP-2 protein sequences. *Drosophila* serves as an outgroup. Numbers at branch points are confidence values derived from 1000 bootstrap resamplings of the alignment data. Sequence distance is indicated to the bottom left as substitutions per base. Lamprey AP-2 is an outgroup to the gnathostome AP-2s and has no affinity for any one AP-2 family member, consistent with there being a single lamprey AP-2 gene. Amphioxus AP-2 falls outside the vertebrate clade. The divergent mouse AP-2 delta groups with amphioxus AP-2 at low bootstrap values, and its phylogenetic relationship to the other vertebrate AP-2 family members is unclear. Genbank accession numbers for the aligned sequences are listed in the Materials and Methods. Abbreviations; Dm, *Drosophila melanogaster* , Bf, *Branchiostoma floridae*, Pm, *Petromyzon marinus*, Hs, *Homo sapiens*, Mm, *Mus Musculus*, Gg, *Gallus gallus*, Xl, *Xenopus laevis*.

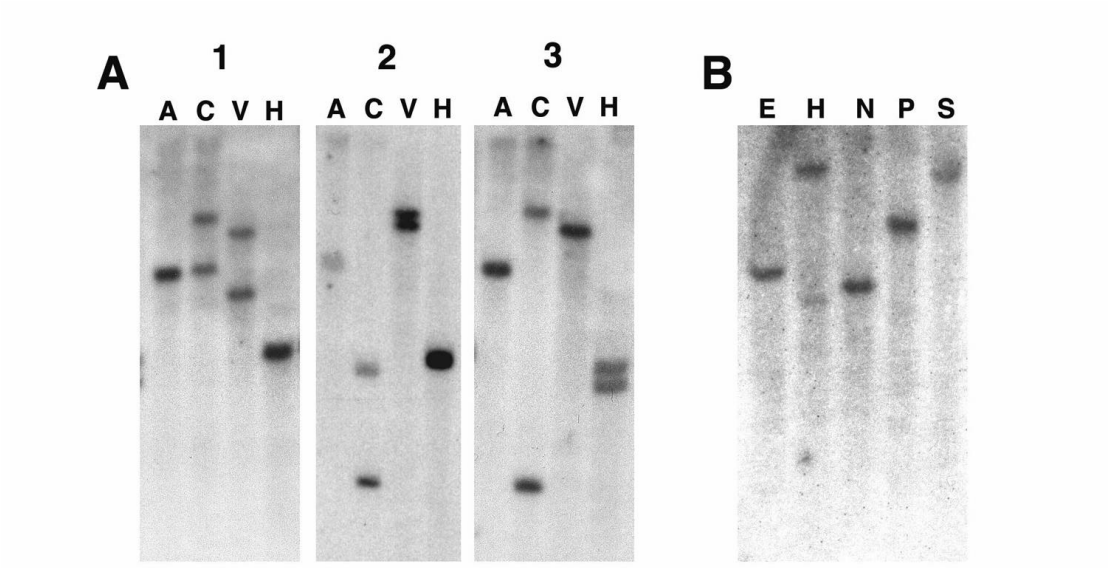

Figure 2. Reduced stringency southern blot analysis to estimate AP-2 gene number in the amphioxus and lamprey genomes. (A) Genomic DNA from 5 individual adult amphioxus was digested with four restriction enzymes and analyzed on the same Southern blot. Lanes from three representative individuals are shown. Interestingly, each animal gives a different banding pattern. For a particular enzyme, an individual has no more than two band types, and most of these bands are shared between individuals. For example, individuals 2 and 3 share lowest *Cla*I band, while all three individuals share a single *Apa*I band. Overall, the banding patterns are most consistent with various hetero- and homozygotic combinations of restriction fragment length polymorphisms at a single locus, suggesting there is one amphioxus AP-2 gene. (B) Genomic DNA from a single adult lamprey digested with 5 restriction enzymes. 4 of 5 digests yield a single band, consistent with there being a single lamprey AP-2 gene. Abbreviations; A, *Apa*I, C, *Cla*I, V, *EcoR*V, H, *Hind*III, E, *EcoR*I, N, *Nco*I, P, *Pst*I, S, *Stu*I.

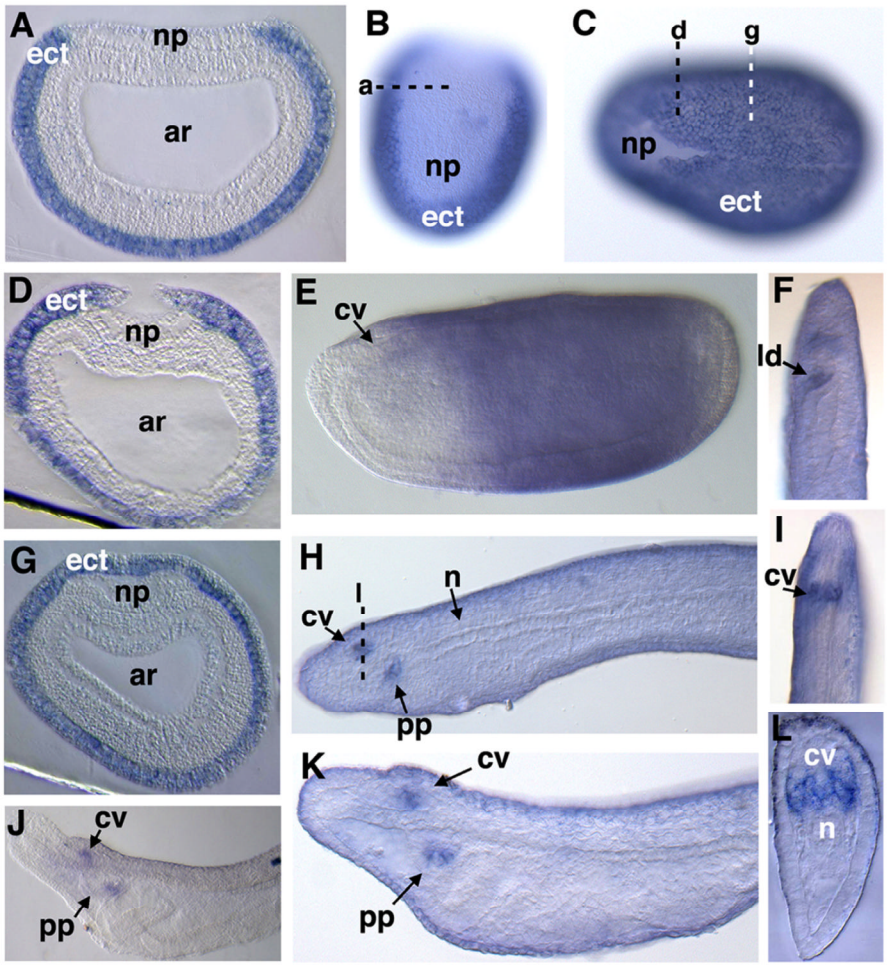

Figure 3. AP-2 expression in amphioxus. (A) Cross section through the 9-hour neurula depicted in B at a showing AP-2 trancripts in the non-neural ectoderm .(B) Dorsal anterior view of 9-hour neurula showing exclusion of AP-2 from the neural plate. (C) Dorsal view of 11.5-hour neurula, anterior is to the left. AP-2 is expressed in the epidermis overgrowing the neural plate. (D) Cross section through the embryo in C at d. (E) Side view of 18-hour neurula. AP-2 expression in the epidermis is being extinguished from the anterior and posterior -most ends of the embryo. (F) Optical horizontal section of 24-hour embryo through the gut with anterior towards the top. A spot of AP-2 positive cells is apparent in the left gut diverticulum, the endodermal portion of the pre-oral pit (arrow). (G) Cross section of 9-hour neurula in C at g. At more posterior levels, the AP-2 positive epidermis has completely covered the open neural plate. (H) Side view of 24-hour embryo with anterior to the left. AP-2 expression is seen in the cerebral vesicle and pre-oral pit, but has largely faded from the epidermis. (I) Optical horizontal section of 24-hour embryo in H at the level of the neural tube showing symmetrical AP-2 staining in the cerebral vesicle. Anterior towards top. (J) 2-

day larva with AP-2 expression persisting in the cerebral vesicle and pre-oral pit. (K) 36-hour larva with AP-2 expression in cerebral vesicle and pre-oral pit. (L) Cross section through 24-hour embryo in H at l. AP-2 expression in the cerebral vesicle is limited to ventro-lateral levels. ar, archenteron; ect, non-neural ectoderm/epidermis; np, neural plate; n, notochord; cv, cerebral vesicle; pp, pre-oral pit; ld, left gut diverticulum.

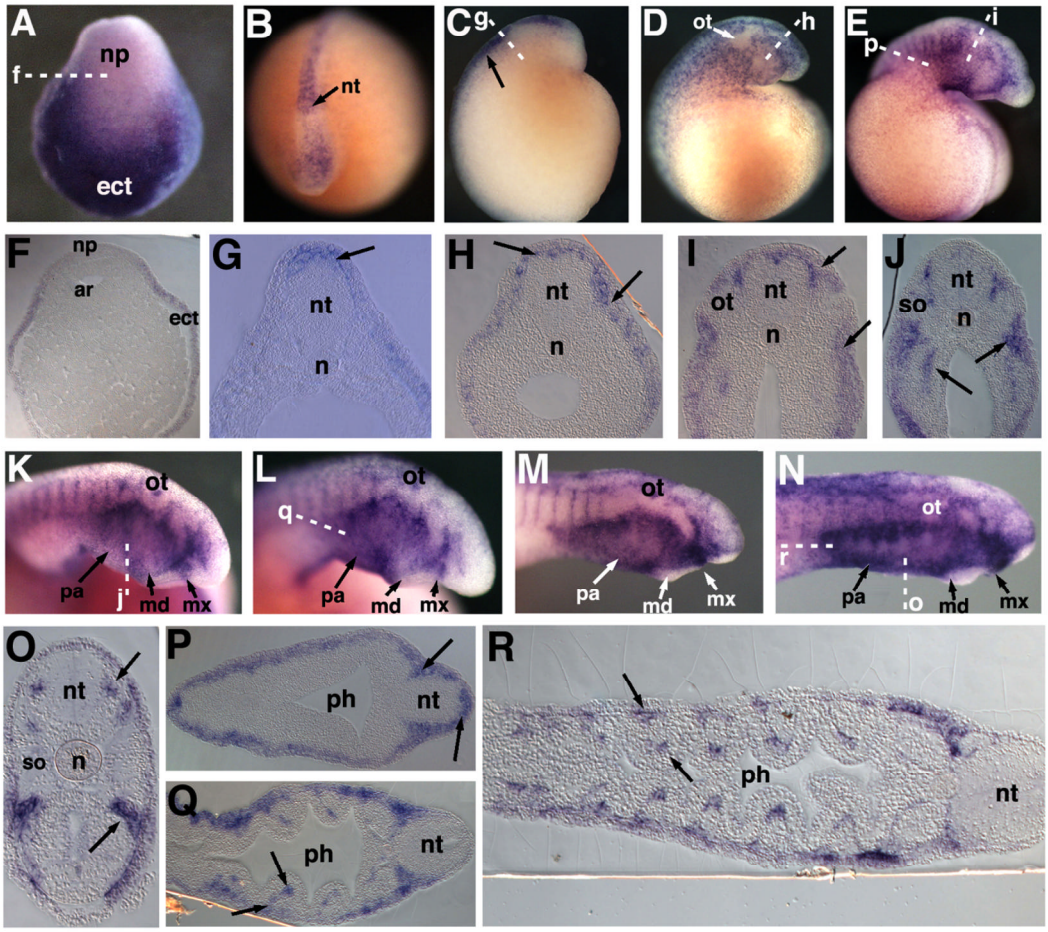

Figure 4. AP-2 expression in lamprey. (A) Dorsal anterior view of 4 day neurula. AP-2 expression is observed in the non-neural ectoderm at the exclusion of the neural plate. (B) Dorsal anterior view of 6-day neurula showing expression confined to the neural rod. An AP-2- free gap disrupts the neural rod signal anteriorly (C) Side view of embryo in B with head protruding upward. Dorsal neural rod expression is indicated by the arrow. (D) Side view of 7-day embryo, anterior is towards the right. AP-2 expression is enhanced in the head epidermis and mesenchyme, but is excluded from the otic cup. (E) 7.5-day embryo showing AP-2 expression throughout the head. (F) Cross section through the neurula in A at f. AP-2 transcripts are absent from the neural plate. (G) Cross section through 6 day neurula in C at g showing AP-2 expression in the dorsal neural tube (arrow). (H) Cross section through 7-day embryo in D at h showing AP-2 staining in dorsal neural tube, head mesenchyme and epidermis (arrows). (I) Cross section of 7.5-day embryo in E through the otic vesicle at i. AP-2 transcripts are detected in the dorsal neural tube and in mesenchyme above and below the otic vesicles (arrows). No staining is apparent medial to the vesicle where it approximates the neural tube. (J) Cross section through 8-day embryo in K at the level of the first somite (j). AP-2 expression is observed in mesenchyme superficial to the ectoderm and adjacent to the gut (arrows). (K) AP-2 expression in the head of an 8-day embryo. Staining is most intense in the region

of the forming pharyngeal arches. (L) AP-2 expression in the head at 8.5 days, anterior is to the right. AP-2 positive cells are localized more ventrally than at 8 days. (M) AP-2 expression in the head at 9 days showing extensive staining in the pharyngeal region. (N) Side view of 11-day embryo showing AP-2 expression in the pharyngeal arches and strongly in the anterior lip. (O) Cross section through the 11-day embryo in N at o. AP-2 mRNA is detected in a cluster of neurons in the lateral neural tube and in mesenchyme surrounding the pharynx (arrows). (P) Horizontal section through a 7.5-day embryo at the approximate level of p in E, showing expression in the dorsal neural tube, mesenchyme and ectoderm (arrows). (Q) Horizontal section through the head of the embryo in L at q. Anterior is to the right. AP-2 expressing cells are seen in the mesenchyme surrounding the pharyngeal mesoderm (arrows). (R) Horizontal section through an 11-day embryo at about the level of r in N. Expression is observed in the mesenchyme of the pharynx (arrows). ar, archenteron; np, neural plate; ect, non-neural ectoderm; n, notochord; ot, otic pit/vesicle; nt, neural tube/rod; ph, pharynx; pa, pharyngeal arches; md, mandibular arch; mx, maxillary region (anterior lip); so, somite.

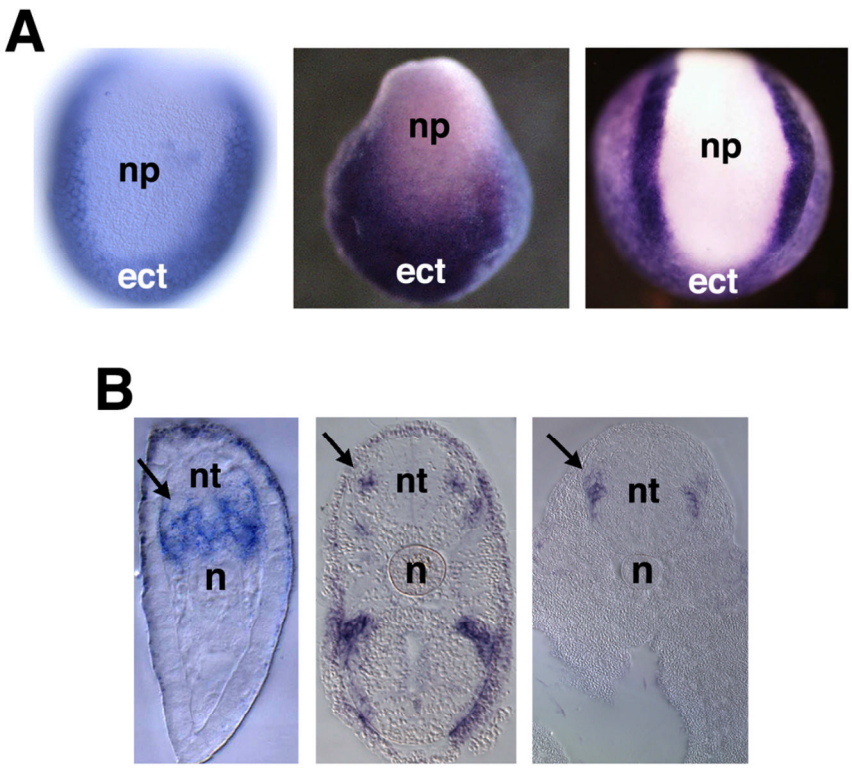

Figure 5. Conserved aspects of AP-2 expression in amphioxus, lamprey, and gnathostomes. (A) Dorsal anterior view of similarly staged amphioxus (left panel), lamprey (middle panel) and axolotl (right panel) neurulae. In all three embryos, AP-2 is upregulated in the non-neural ectoderm and excluded from the neural plate. (B) Cross sections through the anterior neural tube of amphioxus (left panel), lamprey (middle panel) and axolotl (right panel). All three embryos show AP-2 staining in the lateral walls of anterior neural tube (arrows) after neurulation. n, notocord.; np, neural plate; ect, non-neural ectoderm; nt, neural tube.

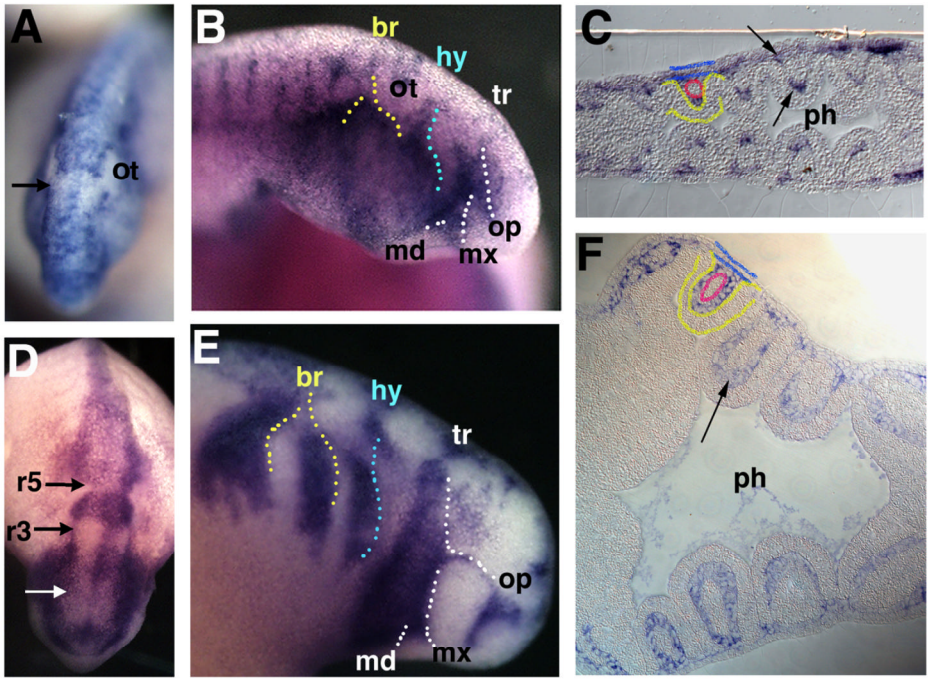

Figure 6. Comparison of AP-2 expression in the head of lamprey and axolotl. (A) Dorsal anterior view of 7-day lamprey embryo showing AP-2 expression in neural tube, early migrating neural crest, and ectoderm. AP-2 positive cells are excluded from a discreet region of the neural tube (arrow) and the otic cup. (B) Side view of an 8-day lamprey embryo head showing AP-2 staining in cranial neural crest cells. Putative streams of migrating cells are bordered by dotted lines. Rostrally, white dots indicate the anterior edge of the trigeminal stream, which splits into the ophthalmic (rostralmost white line), maxillary and mandibular streams (ventral white lines). Blue dots border the rostral edge of the putative hyoid crest stream, and yellow dots indicate the position of the common branchial stream. (C) Horizontal section through the pharynx of an 11-day lamprey embryo showing AP-2 positive cells in the mesenchyme medial and lateral to the arch mesoderm (arrows). For reference, colored lines outline the three germ layers in a single arch; mesoderm is red, ectoderm blue and endoderm yellow. (D) Dorsal anterior view of approximately st. 21 axolotl embryo showing AP-2 staining in early migrating neural crest. AP-2 expression is reduced in rhombomeres 3 and 5 (black arrows) and a portion of rhombomere 1 (white arrow). Compared with the lamprey embryo in A, axolotl shows greater patterning of AP-2 expression along the anterior neural tube. (E) Side view of approximately st. 25 axolotl embryo showing AP-2 expression in cranial neural crest streams. Streams are marked with dotted lines as in B. White dots border the trigeminal stream which splits into the ophthalmic, maxillary and mandibular streams. Blue dots border the hyoid stream, and yellow dots border the first branchial and

common 2/3/4 branchial streams. Overall, the distribution of AP-2 positive cells in the axolotl head resembles that of lamprey (B), except ventrally, where the divisions between putative neural crest streams are much less defined in lamprey than in axolotl. (F) Horizontal section through the pharynx of approximately st. 30 axolotl embryo. Germ layers are marked as in C. AP-2 positive cells are observed in the mesenchyme surrounding the arch mesoderm (arrow). Both lamprey (C) and axolotl have AP-2 positive cells lying medial to the pharyngeal arch mesoderm. ot, otic pit/vesicle; md, mandibular neural crest stream; mx, maxillary neural crest stream; op, ophthalmic neural crest stream; br, branchial neural crest stream; hy, hyoid neural crest stream; tr, trigeminal neural crest stream; ph, pharynx; r3, rhombomere 3; r5, rhombomere 5.

Chapter 3:

Id Expression in Amphioxus and Lamprey Highlights the Role of Gene Cooption During Neural Crest Evolution

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ABSTRACT

Neural crest cells are unique to vertebrates and generate many of the adult structures that differentiate them from their closest invertebrate relatives, the cephalochordates. Id genes are robust markers of neural crest cells at all stages of development. We compared Id gene expression in amphioxus and lamprey to ask if cephalochordates deploy Id genes at the neural plate border and dorsal neural tube in a manner similar to vertebrates. Furthermore, we examined whether Id expression in these cells is a basal vertebrate trait or a derived feature of gnathostomes. We found that while expression of Id genes in the mesoderm and endoderm is conserved between amphioxus and vertebrates, expression in the lateral neural plate border and dorsal neural tube is a vertebrate novelty. Furthermore, expression of lamprey Id implies that recruitment of Id genes to these cells occurred very early in the vertebrate lineage. Based on expression in amphioxus we postulate that Id cooption conferred sensory cell progenitor-like properties upon the lateral neurectoderm, and pharyngeal mesoderm-like properties upon cranial neural crest. Amphioxus Id expression also supports an evolutionary relationship between the anterior neurectoderm of amphioxus and the presumptive placodal ectoderm of vertebrates. We relate these observations to previous studies and propose that neural crest evolution was driven in large part by cooption of multi-purpose transcriptional regulators from other tissues and cell types.

INTRODUCTION

Gans and Northcutt proposed that the evolution of vertebrates from a cephalochordate-like ancestor was driven in large part by the evolution of the neural crest and neurogenic placodes (Gans and Northcutt, 1983; Northcutt and Gans, 1983). This assertion reflects the fact that neural crest and placodes give rise to adult structures that define the vertebrate clade, including the cranial and peripheral ganglia, pharyngeal arch skeletomusculature, and cranium. Consistent with this, definitive neural crest cells have been described in the most basal extant vertebrates, the agnathans, but not in their closest living invertebrate relatives, the cephalochordates.

Neural crest cells form at the border of the neural and non-neural ectoderm and become incorporated into the dorsal neural tube during neurulation. Around the time of neural tube closure, they delaminate and begin to migrate extensively throughout the embryo. They remain in an undifferentiated and proliferative state until they reach their destinations whereupon they activate a range of tissue specific differentiation programs. Neural crest cells are highly multipotent and can form neurons, glia, melanocytes, bone, cartilage, smooth muscle, and chromaffin cells.

Several transcriptional regulators of neural crest formation have been identified including Zic, Pax3/7, Msx, FoxD3, Twist, Snail/Slug, and AP-2 genes. Some of these have been shown to respond to Wnt, BMP, and FGF signaling pathways and to cross- and auto- regulate (For reviews see Aybar and Mayor, 2002; LaBonne and Bronner-Fraser, 1999). While the network of interactions required to specify the neural crest cell fate is being elucidated, how these interactions ultimately confer neural crest cell morphology and behavior is unclear. Evidence suggests that several genes have multiple roles in

neural crest development, ranging from early induction to regulating migration and differentiation. For example, transcriptional repressors of the Snail/Slug family have established roles in neural crest induction, but also promote the epithelial to mesenchymal transition and migration (LaBonne and Bronner-Fraser, 2000), perhaps by down-regulating cadherins (Cano et al., 2000). Recent evidence from frog suggests an early role for AP-2 genes in neural crest induction (Luo et al., 2003), while loss of function studies implicate AP-2 genes in turning on post-migrational differentiation programs in the mouse (MorrissKay, 1996; Schorle et al., 1996). Similarly, FoxD3 has been shown to affect the early stages of neural crest specification, and to have later roles in promoting neural and glial fates and suppressing melanogenesis (Dottori et al., 2001; Kos et al., 2001; Sasai et al., 2001).

An important feature of neural crest cells is that they persist in an undifferentiated and proliferative state well into embryogenesis. How they sustain this stem cell–like condition is largely unknown. A possible explanation lies in the observation that potent helix-loop-helix (HLH) transcriptional regulators of the Id family (for inhibitors of DNA binding or differentiation) are expressed robustly in pre- and migratory neural crest cells (Dickmeis et al., 2002; Jen et al., 1997; Kee and Bronner-Fraser, 2001a; Kee and Bronner-Fraser, 2001b; Kee and Bronner-Fraser, 2001c; Martinsen and Bronner-Fraser, 1998; Tzeng and de Vellis, 1998; Zhang et al., 1995). Id genes, and their *Drosophila* homolog, extramacrochaete, act as dominant negative inhibitors of basic helix-loop-helix (bHLH) transcription factors and are utilized throughout development to inhibit differentiation and promote proliferation (For reviews see Campuzano, 2001 and Yokota, 2001). Though structurally similar, different Id proteins demonstrate different affinities

for specific bHLH transcription factors in vivo, suggesting they have non-redundant functions (Langlands et al., 1997). Furthermore, certain Id proteins have been shown to inhibit DNA binding by Pax 2, 5, and 8 proteins (Roberts et al., 2001) and to antagonize Ets 1 and 2 activity (Ohtani et al., 2001). Limited experimental evidence from chicken suggests that overexpression of Id-2 may promote neural crest cell formation and inhibit ectodermal cell fates (Martinsen and Bronner-Fraser, 1998).

The closest living relative of the vertebrates, amphioxus, lacks neural crest cells, but expresses homologs of some genes implicated in neural crest specification at the neural plate border, including Pax3/7, Msx, Snail and Zic (Gostling and Shimeld, 2003; Holland et al., 1999; Langeland et al., 1997; Sharman et al., 1999). Interestingly, other factors shown to be critical for later neural crest development and differentiation, such as Twist (Yasui et al., 1998), FoxD3 (Yu et al., 2002) and AP-2 (Meulemans and Bronner-Fraser, 2002), are not present in neural plate border cells.

In this study, we analyzed the expression of Id genes in amphioxus, lamprey, and gnathostomes and asked whether or not cephalochordates, like vertebrates, deploy Id genes in the neural plate border region and dorsal neural tube. Furthermore, we examined whether expression of Id genes at the neural plate border, dorsal neural tube, and neural crest is a basal vertebrate trait, or a derived feature of gnathostomes. We found that unlike vertebrates, amphioxus does not express Id genes at the lateral neural plate border or in the dorsal neural tube, though expression in other tissues is conserved. In lamprey, we observed that Id expression is largely confined to the neural plate border and neural crest cells, indicating Id genes were coopted by these cells very early in the vertebrate lineage. Based on these expression patterns, we postulate that Id cooption in

vertebrates conferred sensory cell progenitor-like properties upon the lateral neurectoderm, and pharyngeal mesoderm-like properties upon cranial neural crest. We relate these findings to earlier studies and propose that neural crest evolution was driven in large part by cooption of multi-purpose high-order transcriptional regulators from other germ layers and tissues. We hypothesize that this cooption involved the evolution of novel interactions between these regulators and factors already in place at the neural plate border of a cephalochordate-like ancestor.

MATERIALS AND METHODS

Embryo Collection

Amphioxus adults (*Branchiostoma floridae*) were collected from Tampa Bay, Florida and electrostimulated to induce gamete release. Eggs were fertilized, and embryos were cultured and fixed per the methods of Holland et al. (1996). Embryos of the sea lamprey *Petromyzon marinus* were collected and fixed at the Hammond Bay Biological Station as described by McCauley and Bronner-Fraser (2002) and staged according to Tahara (1988) .

Id Gene Isolation

The following degenerate primer was designed in the 3' direction against the HLH region of all known Id genes and *Drosophila* Extramacrochaetae (EMC): GTA GTC RAT SAC GTG CTG IAK RAT YTC. Using a standard T7 promoter primer as the 5' primer, approximately 600 bp fragments of Id cDNAs were amplified from amphioxus and lamprey Lambda Zap II embryonic cDNA libraries kindly provided by Jim Langeland. For amphioxus Id amplification, diluted library was used directly as the PCR template. For lamprey Id amplification, library DNA was phenol/chloroform extracted and ethanol-precipitated prior to use. The libraries were then plated and screened at high stringency with vector-trimmed PCR fragments to isolate full-length Id gene clones. Clones were fully sequenced from both ends.

Phylogenetic Analysis

Full-length cDNAs were translated and their conceptual protein products were aligned to published Id and extramacrochaetae sequences. A bootstrapped Neighbor-Joining tree (Saitou and Nei, 1987) was then constructed using the ClustalX program (Thompson et al., 1997). The distantly related basic-helix-loop-helix protein zebrafish ASHa was included as an outgroup. GenBank accession numbers for the aligned sequences are as follows: Human Id3, Q02535, Mouse Id3, NP032347, RatId3, NP037190, Zebrafish Id3, NP694499, *Drosophila* Id1a, I51278, Newt Id3, BAA76632, Chicken Id3, AY040528, Newt Id2, AB019514, *Drosophila* Id2, CAB38648, Trout Id2, Y08369, Chicken Id2, AF068831, Human Id2, D13891, Rat Id2, D10863, Mouse Id4, NP112443, Human Id4, NP001537, Human Id4H, AAA82882, Chicken Id4, AY040529, Chicken Id4b, AY040530, Chicken Id1, AY040527, Trout Id1, CAA69656, Zebrafish Id6, AAB62940, Human Id1, P41134, Rat Id1b, NP036929, Mouse Id1, A34690, Rat Id1, JC2111, *Drosophila* EMC, P18491, Zebrafish ASHa, NP571294.

Southern Blots

Genomic DNA from a single adult amphioxus was purified and digested with the following restriction enzymes; *Apa*I, *Cla*I, *EcoR*V, and *Hind*III. Genomic DNA from a single adult lamprey was similarly prepared and restricted with *Nco*I, *Pst*I, *Stu*I, and *Xho*I. Digests were electrophoresed on a 0.7% agarose gel, blotted onto Gene Screen Plus filters (NEN Life Science Products), and probed with P^{32}-labelled DNA fragments. For amphioxus Id, a 440 bp *Xho*I-*Nar*I fragment of the original cDNA clone was utilized for probe synthesis. For lamprey Id, a 234 bp PCR fragment corresponding to the HLH

domain was used. Southern blots were hybridized in 6XSSC/5% SDS/100µg/ml sheared herring sperm DNA /5X Denhardts solution at 50˚C. Low stringency washes were in 2XSSC/.2%SDS at 40˚C.

In situ Hybridization

In situ hybridizations to amphioxus and lamprey embryos were as described previously (Meulemans and Bronner-Fraser, 2002). Riboprobes made against the entire transcript or just the coding region gave identical results.

RESULTS

Isolation of Id Genes from Amphioxus and Lamprey

Degenerate PCR was used to isolate Id gene fragments from amphioxus and lamprey cDNA libraries. Full-length cDNAs were obtained by library screening. When translated, both sequences were found to include the helix-loop-helix (HLH) domain characteristic of Id genes (Fig. 1A). In addition, both genes demonstrated obvious affinity to gnathostome Ids, with amphioxus and lamprey Id being 35 and 39 percent identical, respectively, to newt Id2. Over the HLH domain, higher identity was observed, with amphioxus Id showing 79% identity to human Id4, and lamprey Id showing 68% identity to rat Id1.

Full length and HLH domain sequences were aligned to gnathostome Ids, *Drosophila* EMC, and zebrafish ASHa. The alignments were then used to construct Neighbor-Joining phylogenetic trees (Fig. 1B). Full-length alignment yields a tree that places amphioxus Id within the Id clade, but outside any one gnathostome Id paralogy group. On the same tree, lamprey Id groups with gnathstome Id4s, but at low confidence values. This general topology is maintained when only HLH regions are used for alignment, except that lamprey Id instead groups with Id3 paralogs at low bootstrap values (data not shown).

Southern Blot Analysis

Low stringency southern blotting was used to estimate Id gene number in amphioxus and lamprey. Hybridization and wash conditions were chosen that would allow for cross-species hybridization of related sequences (Langeland et al., 1997). Only a single hybridizing fragment was recognized by the amphioxus Id probe in 3 of 4 amphioxus genomic digests (Fig. 2A). In the fourth digest, cleavage with *Hind*III yielded two hybridizing fragments. Sequence analysis revealed a *Hind*III site within the Id probe sequence, accounting for the observed doublet.

Probing of lamprey DNA with a lamprey Id probe under similar conditions yielded a single weakly hybridizing fragment in each digest, with high background (Fig. 2B). Higher stringency wash conditions gave only a nominal reduction in background signal. We speculate that high overall GC content, high frequency of repeat elements, and low stringency hybridization conditions contributed to low signal-to-noise.

Id Expression in Amphioxus Embryos and Larvae

Amphioxus Id is expressed throughout development in a dynamic pattern spanning all three germ layers. In the 6-hour cup-shaped gastrula, moderate levels of Id transcripts are detected in the mesendoderm (Fig. 3A). Sagittal sections reveal this early Id expression is restricted to the anterior two-thirds of the embryo (Fig. 3E). As the blastopore narrows and neurulation begins (about 9 hours), a novel domain of Id expression is observed in the anterior neurectoderm (Fig. 3B). Horizontal sections show this expression is more intense than the signal in the underlying mesendoderm (Fig. 3F). Between early neurula stages and hatching at 11.5 hours, Id expression increases in the

dorsal mesendoderm while weaker signal persists ventrally. The narrow band of anterior ectoderm expression first observed at 9 hours is also maintained (Fig. 3C). A dorsal view reveals that the dorsal mesendoderm staining has sharpened into two antero-posterior stripes in the middle of the embryo (Fig. 3D). From this angle, the anterior patch of Id-positive neurectoderm is also apparent. A horizontal section through the anterior of a similarly staged embryo shows strong neurectodermal expression overlying the weakly stained mesendoderm (Fig. 3G). More posteriorly, neurectodermal staining is absent and Id-positive cells bordering the prospective axial mesoderm form the two stripes seen in Fig. 3D. Diffuse staining in the ventral mesendoderm is also observed at this level (Fig. 3H). A horizontal section through the posterior of the same neurula shows only weak mesendodermal expression (Fig. 3I).

From hatching until about 15 hours, the mesendoderm segregates into endodermal and mesodermal components as the presomitic mesoderm and chordamesoderm pinch off from the gut. Concurrently, anterior neurectodermal staining is largely extinguished and endodermal expression becomes restricted ventrally (Fig. 4A). In the mesoderm, Id signal expands into the medial somites and nascent notochord (Fig. 4E). In the 18-hour larva, endodermal expression is lost from the hindgut (Fig. 4B) but persists in the ventral foregut, notochord, and medial somites (Fig. 4F). Between 18 and 20 hours the neural tube finishes closing under the epidermis and the lateral walls of the somites expand ventrally, forming the mesothelial lining of the perivisceral coelom. At this stage, virtually all mesodermal and neural expression is lost, while endodermal expression persists in the presumptive pharynx (Fig. 4C). At 24 hours, lateral out-pocketings of the gut have formed the left and right gut diverticulae. Id expression is observed in the left

diverticulum and foregut (Fig. 4D). Sections reveal that Id is no longer expressed in the notochord at this stage (Fig. 4G, H). A horizontal section through the head shows Id expression througout the left gut diverticulum and anteriormost pharyngeal endoderm (Fig. 4G). In the caudal pharynx and midgut, Id transcripts are more abundant ventrally than dorsally (Fig. 4H).

Over the next several hours, the larva elongates. At about 36 hours the mouth and first gill slit form on the left and right sides of the pharynx, respectively. At this point, Id expression in the endoderm is limited to the left gut diverticulum and mouth (Fig. 5B, D). In addition, new mesodermal expression appears in the mesothelium lining the perivisceral coelom of the first gill bar (Fig. 5A-D). Similar mesothelial expression is observed in the narrow coelom surrounding the mouth (Fig. 5D, C). At 2 days, the second gill slit forms on the right side, and Id-positive mesothelial cells can be seen filling the nascent second and third gill bars (Fig. 5E). At 4 days, two gill slits have formed in line with the mouth on the left side. Id transcripts are detected in first formed gill bars on the left side (data not shown).

Embryonic and Larval Expression of Lamprey Id

Lamprey Id transcripts are detected at all stages examined in a pattern consistent with pre-, post-, and migratory neural crest cells. In the early neurula (stage 17), lamprey Id expression is seen in two broad domains on either side of the open neural plate (Fig. 6A). Horizontal sections reveal this staining is confined to the neurectoderm (Fig. 6F) As the neural plate condenses around stage 19, Id messages mark the neural folds (Fig. 6B) and dorsal neural rod (Fig. 6C, G). When the head process begins to protrude at stage 21,

scattered Id expression is seen in the ventral head ectoderm (Fig. 6C, D). Sections through the head of a late stage 21 embryo also show Id-positive head mesenchyme abutting the neural tube (Fig. 6I). Additional weak expression is observed in the newly formed somites (Fig. 6H). At stage 23, lamprey Id transcripts are apparent as swathes of expression in the head (Fig. 6E).

In late stage 23 embryos, strong Id staining is observed in the pharynx, head, and dorsal neural tube (Fig. 7A). Sections show Id transcripts marking head mesenchyme overlying the condensing trigeminal ganglia (Fig. 7D). By stage 24, lamprey Id is expressed throughout the pharynx and head (Fig. 7B). Horizontal sections show transcripts in mesenchyme just under the pharyngeal ectoderm (Fig. 7E). At stage 25, a similar distribution of Id messages is observed in the pharynx and head (Fig. 7C). Horizontal sections at the level of the neural tube show Id positive mesenchyme surrounding the forming cranial ganglia (Fig. 7G). Cross sections through the pharynx reveal Id-positive pharyngeal mesenchyme (Fig. 7F).

DISCUSSION

Metazoan diversity is the result of ontological variation which, in turn, is the consequence of heritable differences in the developmental genetic programs of different phyla. Given the conserved nature of the metazoan proteome, it is widely accepted that much of the developmental differences between animals are due to modifications in gene regulation (Davidson, 2001). Thus, unraveling the *cis*-regulatory history of developmentally important genes can shed light on how new forms arose.

An essential first step toward understanding the regulatory evolution of a given gene is a thorough description of its deployment in the embryos of related phyla. Comparisons of related gene expression patterns then become the observational foundations of testable hypotheses regarding the regulatory history of a gene or gene family in a particular lineage.

This study is part of an effort to understand the evolutionary origins of a critical vertebrate apomophy, neural crest cells. As a starting point, we are examining the embryonic expression of amphioxus and lamprey genes with gnathostome homologs having suspected roles in the development of neural crest cells. These observations are then utilized to construct testable hypotheses regarding their regulatory relationships. Using this approach we seek to define the novel genetic regulatory interactions that drove the evolution of neural crest cells in the vertebrate lineage. We chose amphioxus and lamprey for these comparisons because they diverged near the time of vertebrate origins and thus likely approximate the pre-vertebrate and basal vertebrate conditions.

Id Genes in Amphioxus and Lamprey

Vertebrate Id genes are robust markers of neural crest cells and have suspected roles in neural crest development. Using a degenerate primer designed against all known Id genes and *Drosophila* extramacrochaete, we amplified fragments of Id homologs from amphioxus and lamprey. These fragments were then used to screen cDNA libraries for full-length sequences. Single representatives of the Id gene family were isolated from both amphioxus and lamprey. Neighbor-Joining analysis clearly groups amphioxus Id within the Id/EMC gene family, but shows it has no particular kinship to any vertebrate Id paralog. In addition, low stringency southern blotting suggests this is the only member of the Id family present in amphioxus. These data are consistent with theories of multiple whole or partial genome duplications in the vertebrate lineage (reviewed by Holland, 1999).

Neighbor-Joining analysis groups lamprey Id with vertebrate Id4 genes at low bootstrap values. However, when only the HLH region is used for alignment, lamprey Id groups weakly with Id3 genes. Furthermore, unlike chick and mouse Id4, lamprey Id is expressed in the neural plate border, somites, and migratory neural crest. Low stringency Southern blotting shows a single weakly hybridizing fragment in each genomic digest. However, high background, perhaps due to the GC-rich nature of the lamprey genome and the reduced stringency wash conditions, may be occluding weaker bands. The lack of a clear relationship to any particular Id subfamily may reflect divergence of lamprey Id before the duplications that created the four gnathostome paralogs. Alternately, lamprey Id may be a divergent Id4 or Id3 ortholog. Based on these findings, we conclude that

amphioxus likely has a single Id gene, and leave open the phylogenetic position of lamprey Id relative to the gnathostome paralogy groups.

The differing capacities of the four vertebrate Id paralogs to inhibit specific bHLH transcription factors imply that they have some non-redundant functions during development (Langlands et al., 1997). The existence of only one amphioxus Id gene, whose expression pattern is largely a composite of vertebrate Id gene expression (see discussion below), is consistent with evolutionary subfunctionalization as described by Force et al. (1999). This scenario predicts that amphioxus Id has the combined properties of the four vertebrate paralogs. It would be interesting to test this hypothesis by assaying the ability of amphioxus Id to interact with different Id-binding bHLH transcription factors. Similar functional examination of lamprey Id may clarify its evolutionary relationship to the other vertebrate Id genes.

Id Endodermal Expression is Conserved between Amphioxus and Gnathostomes

Amphioxus Id is expressed in the endoderm at all stages examined. Transcripts are first observed in the gastrula mesendoderm and persist in the ventral gut until larval stages. In late larvae, Id is largely extinguished from the endoderm, except around the mouth and within the left gut diverticulum. In gnathostomes, extensive expression of Id genes in endodermal derivatives has been reported. At primitive streak stages in chick, Id4 is expressed in broad swathes of ingressing mesendoderm (Kee and Bronner-Fraser, 2001a). Concurrently, Id3 transcripts are observed in proamnionic endoderm (Kee and Bronner-Fraser, 2001c). During early neurulation both Id3 and Id4 become highly expressed in the anterior intestinal portal (the edge of the developing gut). At later stages,

chick Id3 transcripts are seen in the ventral foregut and stomodeum. At primitive streak stages in the mouse, Id1 and Id3 messages are expressed in extraembryonic endoderm, and Id1 transcripts are observed in ingressing mesendoderm (Jen et al., 1997). All mouse Id genes are deployed in the gut at later stages (10-14 d.p.c.), with Id1, 2, 3, and 4 expressed in the caudal foregut and Id1, 2, and 3 observed in the mid- and hindgut (Jen et al., 1996). Overall, the broad endodermal deployment of gnathostome and amphioxus Id genes is reminiscent of fly extramacrochaete expression and may represent a primitive function for Id/EMC genes in endoderm formation (Cubas et al., 1994; Ellis, 1994). In contrast to both gnathostomes and amphioxus, no endodermal Id expression is seen in lamprey. This suggests the presence of unidentified lamprey Id genes with endodermal expression domains, or the independent loss of endodermal Id expression in agnathans.

Early Mesodermal Expression of Id is Conserved in Amphioxus and Vertebrates

Amphioxus somites are formed as dorso-lateral outpocketings of the archenteron. After detaching from the mesendoderm, the lateral somite wall thins and expands ventrally to surround the gut. The medial somite wall abuts the notochord and eventually forms the myomere, and is thus homologous in position and fate to the vertebrate somite proper. In the amphioxus neurula, Id is upregulated in two stripes at the border of the axial and paraxial mesoderm. As neurulation progresses, expression expands into the medial wall of the somites and is upregulated in the forming notochord. This phase of mesodermal Id expression ends abruptly around the time of neural tube closure (18-20 hours). Somitic expression appears to be a feature of Id deployment conserved in amphioxus and vertebrates, since lamprey, zebrafish, mouse, chicken and frog all express

one or more Id genes in the developing somites. This aspect of expression likely relates to the demonstrated function of Id genes as dominant-negative inhibitors of myogenic bHLH factors (Melnikova and Christy, 1996; Rescan, 2001). The rapid down-regulation of amphioxus Id in the somites between 18 and 20 hours may therefore mark the beginning of muscle cell differentiation as myogenic bHLH function is released from Id inhibition. Consistent with this, two myogenic bHLH factors (AmphiMRF1 and 2) have been identified in amphioxus and the expression of both overlap with amphioxus Id in the medial somite wall (Schubert et al., 2003). Like Id, AmphiMRF2 is downregulated at about 20 hours, but AmphiMRF1 persists into late larval stages.

Expression of Id genes in the nascent notochord does not appear to be a widely conserved aspect of Id expression in vertebrates. None of the Id genes isolated from lamprey, mouse, chick, or frog are expressed in chordamesoderm. However, transcripts of the recently described zebrafish Id3 are detected in the nascent notochord (Dickmeis et al., 2002). Several scenarios could account for the phylogenetic distribution of Id notochord expression. The simplest explanation is loss of Id notochord deployment in the amniote lineage. A loss predicts either the expression of an unidentified lamprey Id gene in the notochord, or independent loss of this expression domain in agnathans.

Conservation of dorso-anterior ectoderm expression suggests amphioxus has a functional equivalent of the vertebrate anterior neural plate border

In the early neurula, amphioxus Id is expressed in a narrow patch of dorso-anterior ectoderm. This expression overlaps with deployment of amphioxus Pax6 and Distalless in the anterior-most neural plate and epidermis (Glardon et al., 1998; Holland

et al., 1996). During equivalent stages, chick Id1 is similarly deployed at the anterior neural plate border where it colocalizes with Pax6 and Dlx5 transcripts in the presumptive placode region (Li et al., 1994; Yang et al., 1998). In amphioxus larvae, the dorsal anterior ectoderm is rich in sensory cells, including photoreceptors in the neural plate and putative chemoreceptors in the adjacent epidermis. We speculate that coexpression of Id, Pax6, and Dlx genes at the anterior neural plate border marks a field of sensory cell specification equivalent to the placodal domain of the vertebrate anterior neurectoderm.

Neurectodermal expression of amphioxus and vertebrate id genes implies cooption by lateral neural plate and dorsal neural tube cells early in the vertebrate lineage

As the epidermis closes over the neural plate, amphioxus Id is extinguished from the anterior neurectoderm. No significant neural or ectodermal expression is observed at later stages. During vertebrate neurulation, Id transcripts mark cells at the lateral neural plate border and persist in the neural folds and dorsal neural tube. This expansion of Id expression in the neurectoderm apparently occurred early in the vertebrate lineage as high levels of lamprey Id transcripts are observed at the lateral neural plate border, neural folds, dorsal neural tube, and neural crest. Novel lateral neural plate deployment of Id may reflect the recruitment of genetic programs from anterior sensory cell progenitors like those marked by Id, Pax6, and Distalless in amphioxus. Recent work suggests similar cooptions of AP-2, FoxD, and Twist genes from ectodermal and mesodermal tissue derivatives (Meulemans and Bronner-Fraser, 2002; Yasui et al., 1998; Yu et al., 2002). The accumulation of genetic pathways from other cells and tissues may have

bestowed novel properties upon the lateral neural plate of an amphioxus-like ancestor, potentiating the evolution of definitive neural crest cells (Fig. 9).

The apparent role of cooption in neural crest evolution raises the question of how, mechanistically, these novel factors were recruited to the neural plate border. In general, there are two ways by which a gene might gain a novel expression domain: 1) changes in its *cis*-regulatory sequence, such as the addition of an enhancer module or removal of a repressor module and 2) changes in the deployment of its trans regulators. In the former case, the gene gains new responsiveness to pre-existing factors. In the latter, it retains regulatory relationships to upstream factors which have themselves gained new domains of expression. Both of these mechanisms could have driven the cooption of Id genes by the evolving vertebrate neural plate border. Several factors involved in vertebrate neural plate border specification are expressed in the lateral neural plate of amphioxus, including Pax3/7, Msx, Zic, and Snail (Gostling and Shimeld, 2003; Holland et al., 1999; Langeland et al., 1997; Sharman et al., 1999). Novel regulatory interactions between these pre-positioned factors and Id may have driven its recruitment to the neural plate border. Other genes coexpressed with Id in the amphioxus embryo appear to have been coopted by the vertebrate dorsal neural tube. Conserved regulatory relationships between these genes and Id could potentially drive Id expression in this novel domain. The transcription factor FoxD is coexpressed with amphioxus Id in the nascent notochord and medial somite (Yu et al., 2002) and could regulate Id expression in the vertebrate dorsal neural tube. Similarly, Notch expression overlaps with Id in the somites, notochord, and pharyngeal endoderm of amphioxus (Holland et al., 2001) and is expressed in the dorsal neural tube of vertebrates (Williams et al., 1995). Consistent with a role for Notch in Id

gene regulation, evidence from *Drosophila* suggests that Notch signaling directly controls Id3 expression (Reynaud-Deonauth et al., 2002).

Id expression in amphioxus pharyngeal mesothelial cells and vertebrate cranial neural crest may reflect the cooption of migratory and/or chondrogenic programs from mesodermal derivatives

While the expansion of Id into the lateral neurectoderm may reflect the cooption of sensory cell specification programs, it does not suggest why vertebrate Id expression persists in pharyngeal arch neural crest, which mostly gives rise to cartilage. The functional significance of this later expression may be inferred by examining similar, and possibly related, expression domains in amphioxus. Interestingly, amphioxus Id transcripts are observed in mesoderm lining the coelom of the forming pharyngeal gill bars. These cells originate as ventral outpocketings of the somites, and migrate between the pharyngeal endoderm and overlying ectoderm into the nascent gill bars. Although their fate has not been demonstrated experimentally, these cells lie in a position later occupied by cartilagenous skeletal rods (Azariah, 1973; Rahr, 1982). A similar distribution of Id transcripts is seen in the vertebrate pharynx where Id positive neural crest cells migrate between pharyngeal arch endoderm and overlying ectoderm to ultimately generate pharyngeal cartilage (see side-by-side comparison in Fig. 8). These observations imply similar roles for Id genes in amphioxus gill bar mesothelial cells and vertebrate cranial neural crest, and may reflect the transference of migratory and/or chondrogenic capacities from the gill-bar mesothelial cells of a cephalochordate ancestor.

It will be interesting to see if amphioxus homologs of genes with defined functions in vertebrate pharyngeal chondrogenesis are also deployed in the gill bar mesothelium.

Late expression of lamprey id in cranial neural crest

Unlike amphioxus Id, lamprey Id expression is largely restricted to the neurectoderm in a pattern consistent with neural crest cells. This interpretation is supported by equivalent expression of gnathostome Id genes, overlapping deployment of neural crest markers AP-2 and Sox10, and DiI labelling (McCauley and Bronner-Fraser, 2003). However, there are aspects of late Id localization that differ from described marker expression and DiI distribution. It has been shown with AP-2, Sox10, and DiI labelling that lamprey cranial neural crest cells migrate medial to the pharyngeal mesoderm (McCauley and Bronner-Fraser, 2003; Meulemans and Bronner-Fraser, 2002). We find that lamprey Id transcripts are absent from these cells. This may be a conserved feature of Id deployment as chick Id1 pharyngeal expression is similarly limited to subepidermal neural crest.

Lamprey Id also marks mesenchyme surrounding the condensing cranial ganglia. In gnathostomes, similarly positioned late migrating neural crest cells contribute to these ganglia. Recent DiI studies of early migrating neural crest have not demonstrated incorporation of lamprey neural crest into the cranial ganglia. Further DiI labelling in older embryos may establish if these Id-positive cells are homologous to late migrating neural crest which populates the cranial ganglia of gnathostomes.

CONCLUSIONS

We have isolated Id gene homologs from amphioxus and lamprey and compare their sequence and expression patterns to those of gnathostome Id genes. Based on phylogeny and low stringency Southern blotting, we conclude amphioxus has a single Id gene, while the relationship of lamprey Id to gnathostome Id paralogs is unclear. Overall, Id expression in the endoderm and mesoderm appears conserved in amphioxus and vertebrates. However, no single vertebrate Id gene displays the complete amphioxus expression pattern, suggesting subfunctionalization of vertebrate Id paralogs. Early expression of Id genes in the dorsal anterior ectoderm also appears to be a conserved feature and implies that amphioxus has a field of sensory cell progenitors similar to the presumptive placodal domain of vertebrate embryos. Unlike vertebrate Id genes, however, amphioxus Id is not expressed at the lateral neural plate border or dorsal neural tube. Deployment of lamprey and gnathostome Id genes in these cells implies genetic cooption early in the vertebrate lineage. This likely involved novel regulatory interactions between Id genes and factors deployed at the vertebrate neural plate border. We propose that expression of Id genes at the lateral neural plate border reflects the expansion of sensory cell progenitor properties restricted to the anterior ectoderm in an amphioxus-like ancestor. Furthermore, we postulate that later expression in pharyngeal neural crest reflects cooption of ventral mesodermal programs from pharyngeal mesoderm. Thus, cooption of Id functions from anterior ectoderm and pharyngeal mesoderm conferred new properties upon the evolving lateral neural plate border and neural crest.

ACKNOWLEGEMENTS

We thank Nick Holland, Linda Holland, and John Lawrence for making amphioxus collection possible, Jim Langeland for providing us with superb cDNA libraries, and Roger Bergstedt and the staff of Hammond Bay Biological Station for assisting with lamprey embryo collection. This work was supported by USPHS grant DE13223 and NASA NAG 2-1585 to MBF.

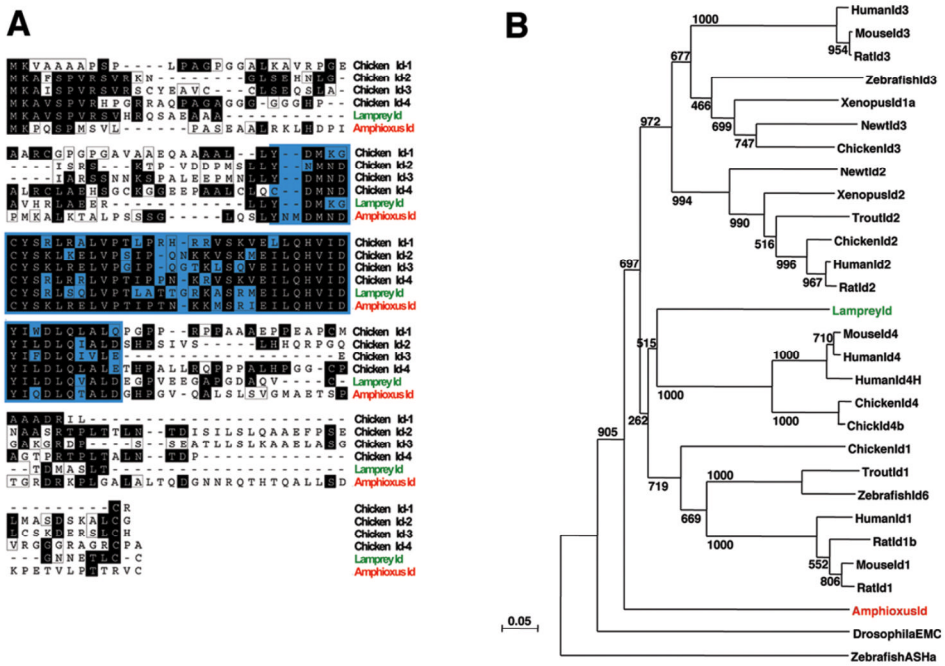


Figure 1. Sequence analysis of amphioxus and lamprey Id proteins. (A) Clustal alignment of chicken, lamprey and amphioxus Id proteins. Identical residues are shaded black, biochemically similar residues are boxed. The conserved HLH region is highlighted in blue. (B) Phylogenetic tree of Id and EMC (extramacrochaete) proteins created using the Neighbor-Joining method with zebrafish ASHa (achaete-scute homolog a) as the outgroup. Numbers at branch bases are confidence values derived from 1000 bootstrap resamplings of the alignment data. Sequence distance is indicated to the bottom left as substitutions per base. Lamprey Id groups with the gnathostome Id-4 proteins at low bootstrap values, while amphioxus Id falls within the Id/EMC gene family but outside the vertebrate Id clade.

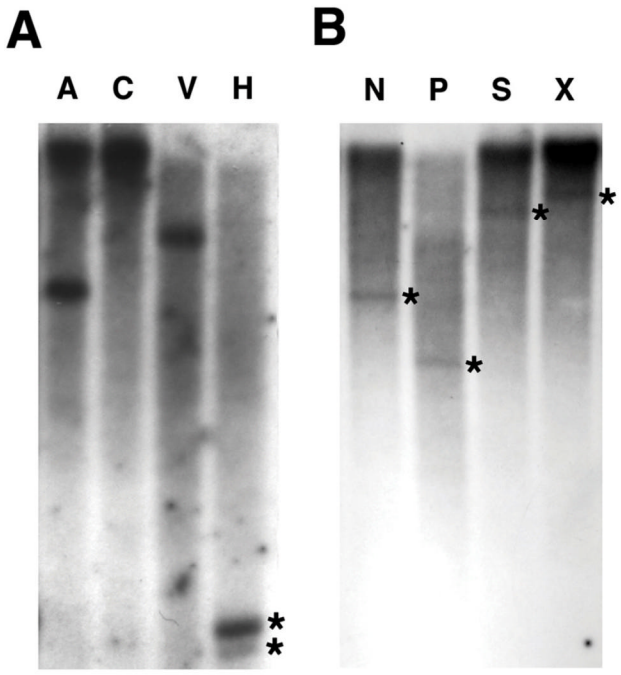


Figure 2. Low stringency southern blot analysis to estimate Id gene number in amphioxus and lamprey. (A) Genomic DNA from a single adult amphioxus was digested with four restriction enzymes and probed with a 440 bp fragment of the amphioxus Id cDNA. Single bands in lanes A, C, and V suggest there is a single amphioxus Id gene. The two hybridizing bands in lane H (asterisks) are created by cleavage at a HindIII site within the exonic sequence binding the probe, and also support the existence of only one gene. (B) Genomic DNA from a single adult lamprey digested with 4 restriction enzymes and probed with a 234 bp fragment of lamprey Id. Faint single bands (asterisks) in all lanes are consistent with there being a single lamprey Id gene. Abbreviations; A, ApaI, C, ClaI, V, EcoRV, H, HindIII, N, NcoI, P, PstI, S, StuI, X, XhoI.

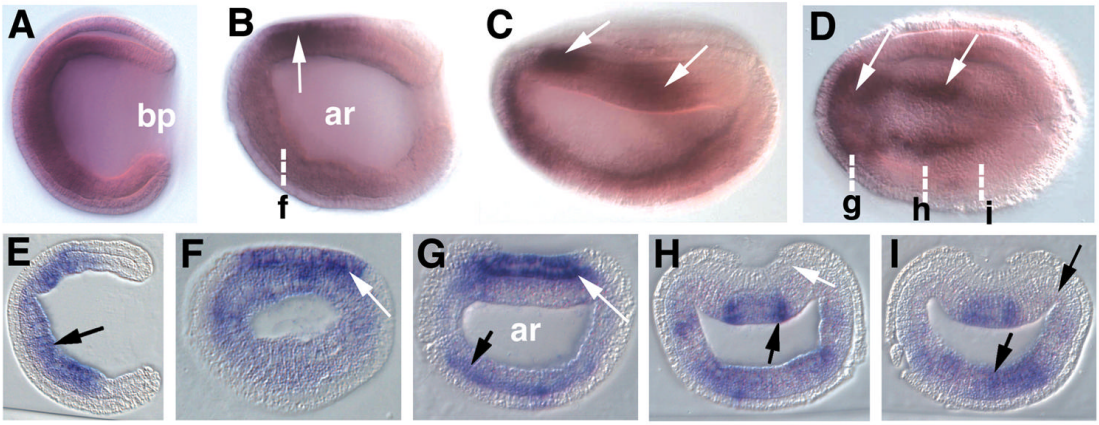


Figure 3. Expression of amphioxus Id at gastrula and early neurula stages. (A) Side view of a 6-hour cup-shaped gastrula. Id is expressed in the anterior mesendoderm (arrow). (B) Side view of 9-hour early neurula. Strong Id expression appears in a spot of anterior dorsal ectoderm (arrow) and persists at lower levels in the mesendoderm. (C) Side view of 11-hour neurula. Id is maintained at the anterior border of the neural plate (top arrow) and endoderm while it is upregulated in the mesoderm (lower arrow). (D) Dorsal view of embryo in C showing strong Id expression at the anterior neural plate border (left arrow), and in two stripes of underlying mesoderm (right arrow). (E) Sagittal section through the gastrula in A showing Id staining in mesendoderm (arrow). (F) Cross section through the anterior of the neurula in B at f. Expression is seen in the neurectoderm (arrow) and less intensely in the underlying mesendoderm. (G) Cross section through the neurula in C and D at g showing anterior neural plate (white arrow) and mesendoderm staining (black arrow). (H) Cross section through the middle of the neurula in C and D at h. At this level, the neural plate (white arrow) has no detectable Id transcripts, while Id is upregulated in cells bordering the axial mesoderm (black arrow). (I) Cross section through the neurula in C and D at i. In the posterior third of the neurula, the stripes of increased Id expression bordering the axial mesoderm are less pronounced. Staining is seen in the endoderm and axial mesoderm (bottom arrow), but is excluded from the evaginating somitic mesoderm (top arrow). ar, archenteron; bp, blastopore.

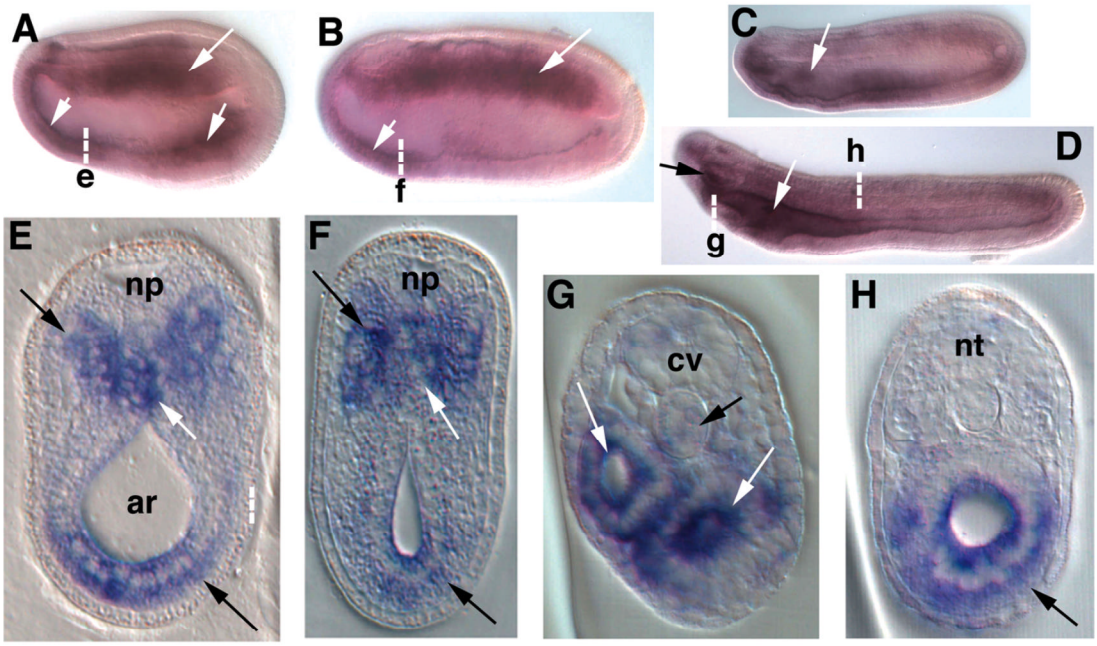


Figure 4. Expression of amphioxus Id at late neurula and early larval stages. (A) Side view of 15-hour larva. Id staining persists in the mesoderm (top arrow) and ventral endoderm (bottom arrows). (B) Side view of 18-hour larva. Id is expressed in the mesoderm (top arrow) and presumptive pharyngeal endoderm (bottom arrow), but is downregulated in the posterior endoderm. (C) Side view of 20-hour larva. Between 18 and 20 hours, Id expression is rapidly extinguished from the mesoderm, but expands throughout the pharyngeal endoderm (arrow). (D) Side view of a 24-hour larva. Expression is observed in the pharyngeal endoderm (white arrow), and the left gut diverticulum (black arrow). (E) Cross section through a 15-hour larva at e in A. Between 12 and 15 hours, Id is upregulated in the medial wall of the forming somites (top black arrow), and in the nascent notochord as it pinches off from the gut (white arrow). Staining in the ventral aspect of the gut is also observed (bottom black arrow). (F) Cross section through an 18-hour larva at approximately the level of f in B. Id expression in the medial wall of the somites (top black arrow), notochord (white arrow), and ventral endoderm of the developing pharynx (bottom black arrow). (G) Cross section through the anterior of a 24-hour larva at the level of g in D. Id expression in the notochord (black arrow) and somites is extinguished at this stage, while staining persists in the pharyngeal endoderm (right white arrow) and the left gut diverticulum (left white arrow). (H) Cross section through a 24-hour embryo at the level of h in D showing staining in the pharyngeal endoderm (arrow). ar, archenteron; np, neural plate; cv, cerebral vesicle; nt, neural tube.

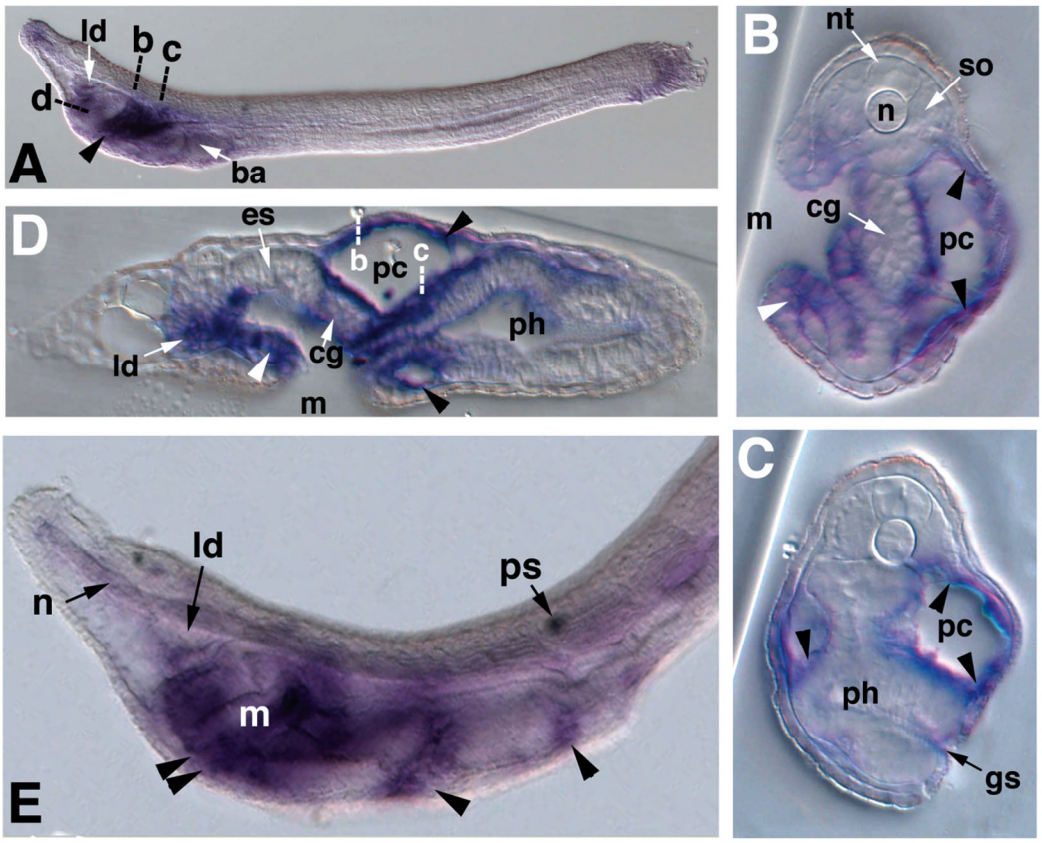


Figure 5. Expression of amphioxus Id at late larval stages. (A) Side view of a 36-hour larva with the plane of focus on the right side. Between 24 and 36 hours, Id expression in the pharyngeal endoderm is largely extinguished. Strong staining is seen in the first formed gill bar (black arrowhead). Slightly out of focus is staining in the left gut diverticulum (white arrow). Largely occluded is staining in the mouth, which is on the left side of the larva. (B) Section through the larva in A at b. The plane of section passes through the mouth on the left, and the first gill bar on the right. Staining is seen in the endoderm just inside the mouth (white arrowhead). On the right side, mesothelial cells lining the perivisceral coelom of the first gill bar express Id (black arrowheads). These cells arise as an outpocketing of the somite, which expands ventrally to line the gill bar coelom. (C) Section through the larva in A at c. The plane of section passes through the first gill slit on the right side of the larva. Id-positive mesothelial cells line the perivisceral coelom of the first gill bar (right arrowheads) and the coelomic space surrounding the mouth (left arrowhead). (D) Horizontal section through a 36-hour embryo at approximately the level of d in A. Id expression in the cells lining the perivisceral coelom of the first gill bar (top black arrowhead) and the coelom bordering the mouth (bottom black arrowhead). Endoderm just inside the mouth (white arrowhead) is also positive for Id transcripts, as well as the left gut diverticulum (ld). The dashed white lines indicate the approximate planes of section of B (b) and C (c). (E) Side view of a 48-hour larva. Staining is apparent in the second and third forming gill bars on the right side (single arrowheads). Out of focus is staining in the first gill bar and mouth

(double arrowhead). ph, pharynx; cg, club shaped gland; es, endostyle; gs, gill slit; ld, left gut diverticulum; m, mouth; n, notochord; nt, neural tube; pc, perivisceral coelom; ps, pigment spot; so, somite.

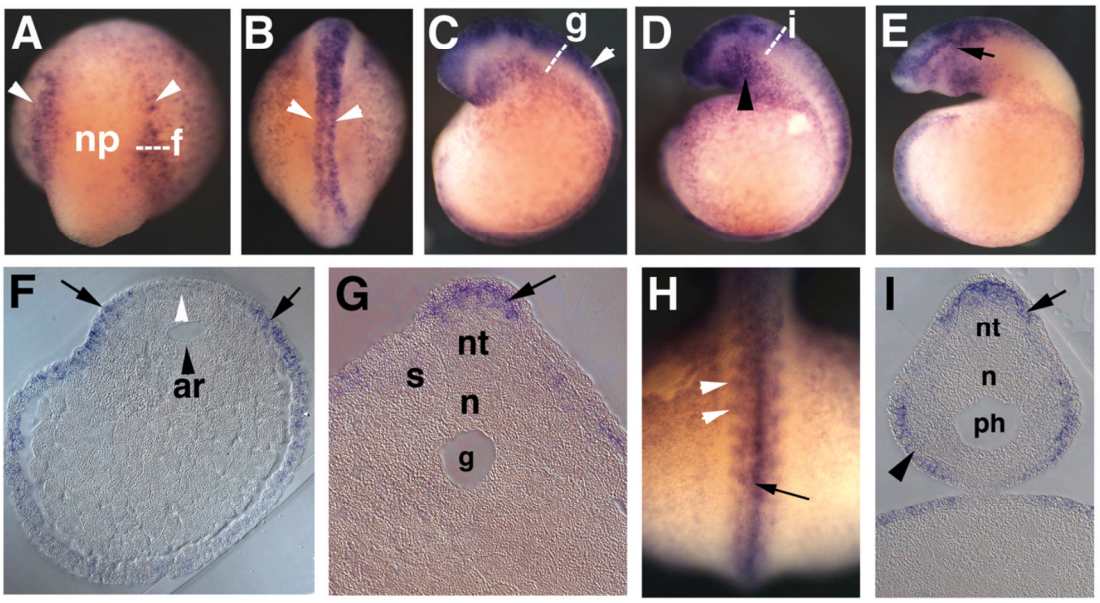


Figure 6. Id expression in stage 17 to stage 23 lamprey embryos. (A) Dorsal view of a stage 17 neurula, anterior is top. Staining is seen at the lateral borders of the neural plate (white arrowheads). (B) Dorsal view of a stage 19 neurula, anterior towards top. The Id expressing cells (white arrowheads) in the neural folds approximate as the neural plate condenses to form the neural rod. (C) Side view of a stage 21 embryo. The neural plate has condensed to form the neural rod. Id staining is observed in the dorsal aspect of the neural rod (arrowhead). (D) Side view of a stage 21+ embryo. Staining persists in the dorsal neural tube. Scattered cells in the ventral head ectoderm also express Id (arrowhead). (E) Side view of a stage 23 embryo. Id staining in the head is consistent with migrating cranial neural crest (arrow). Ventral ectoderm staining has diminished (F) Cross section through a stage 17 neurula at approximately the level of f in A. Id staining (black arrows) borders the open neural plate (white arrowhead). (G) Cross section through the stage 21 embryo in C at g. Id staining is observed in the dorsal neural rod. (H) Dorsal view of the stage 21+ embryo in D showing Id staining in the somites (white arrowheads) and dorsal neural rod (black arrow). (I) Cross section through the stage 21+ embryo in D at i. Id stains cells in the mesenchyme lateral to the neural tube (arrow), a position consistent with early migrating neural crest. Some cells in the ventral head ectoderm also express Id (arrowhead). np, neural plate; ar, archenteron; s, somite; nt, neural tube or neural rod; n, notochord; g, gut; ph, pharynx.

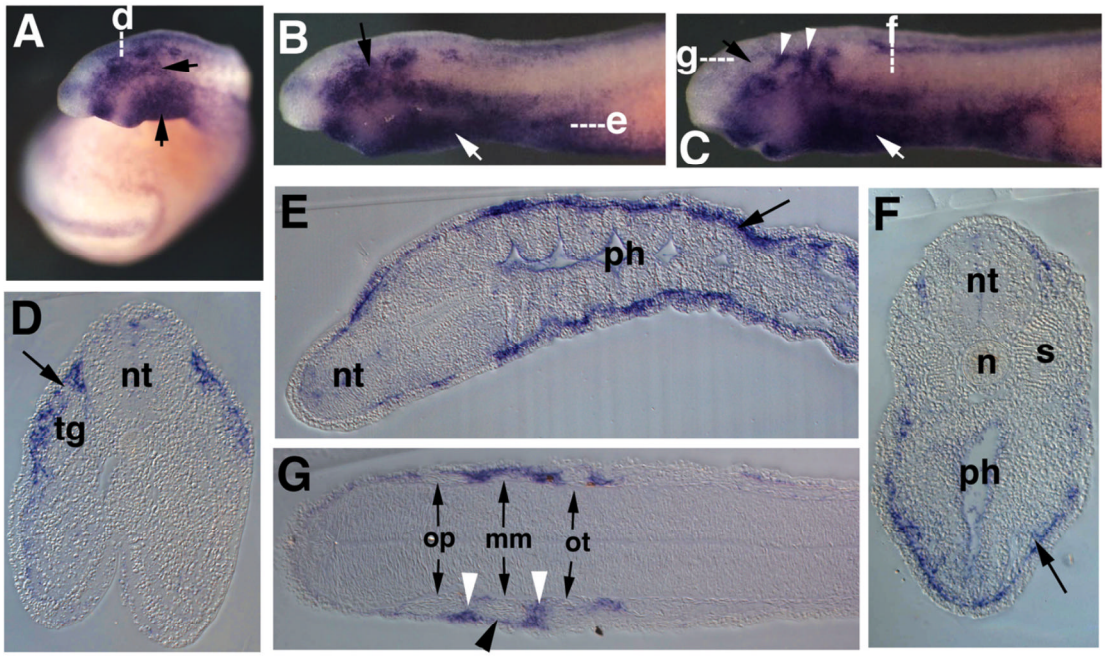


Figure 7. Id expression in stage 23+ to stage 25 lamprey embryos. (A) Side view of a stage 23+ embryo. Id staining in the pharyngeal region (bottom arrow) and lateral to the neural tube (top arrow) is suggestive of migrating neural crest cells. (B) Side view of a stage 24 embryo. Id is expressed in the pharyngeal arches (white arrow) and ventrolateral to the neural tube (black arrow). (C) Side view of a stage 25 larva. Id transcripts are detected in the pharyngeal arches (white arrow) and in distinct masses ventrolateral to the neural tube (white arrowheads). Id-positive cells appear to be occluded from the lobes of the trigeminal ganglia (black arrow). (D) Cross section through the stage 23+ embryo in A at d. Id positive cells in the mesenchyme lateral to the neural tube and superficial to the trigeminal ganglia (arrow). (E) Horizontal section through the anterior of a stage 24 embryo at about the level of e in B. Id staining is seen just underneath the ectoderm in the pharyngeal arches (arrow). (F) Cross section through a stage 25 embryo at about the level of f in C. Id expression in cells just underneath the pharyngeal arch ectoderm. (G) Slightly angled horizontal section through the stage 25 larva in C at about g. The plane of section passes through the middle of the neural tube and the dorsal portions of the cranial ganglia (black arrows). Id-positive cells appear occluded from the ganglia and lie in masses between them (white arrowheads). Some Id-positive cells are also seen superficial to the ganglia, just under the epidermis (black arrowhead). s, somite; nt, neural tube; n, notochord; ph, pharynx; tg, trigeminal ganglia; op, ophthalmic lobe of the trigeminal ganglia; mm, maxillomandibular lobe of the trigeminal ganglia; ot, otic capsule.

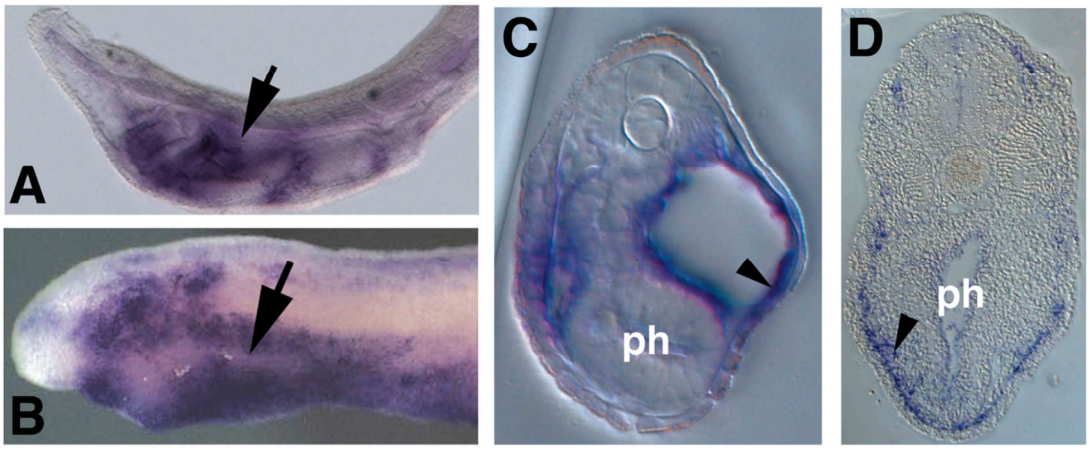


Figure 8. Side by side comparison of Id expression in the developing pharynx of amphioxus and lamprey. (A) Id expression in the mouth and gill bars of a 2-day amphioxus larva (arrow). Similar pharyngeal expression is seen in lamprey during roughly equivalent stages (B, arrow). (C) Cross section through the gill bar of a 36 hour amphioxus larva. Id expression in mesothelial cells lining the perivisceral coelom of the gill bar (arrowhead). These cells underlie the pharyngeal ectoderm and cover the basal surface of the pharyngeal endoderm. Id-positive neural crest cells occupy an equivalent position in the lamprey pharynx (D, arrowhead). ph, pharynx.

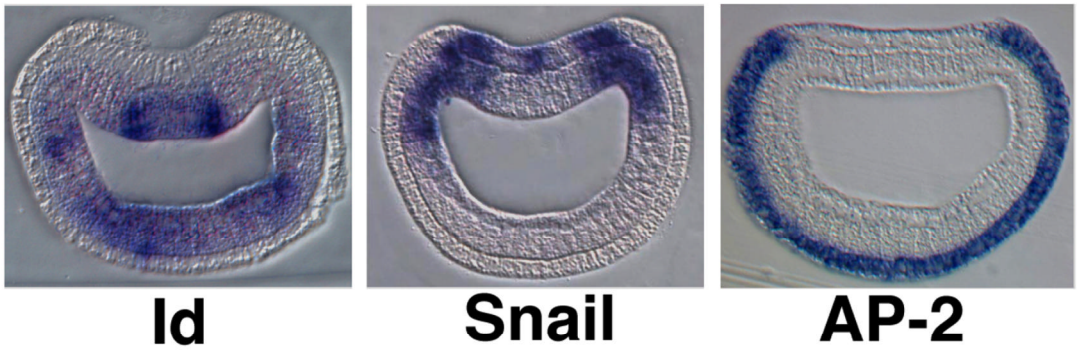


Figure 9. Expression of neural crest marker homologs in amphioxus neurulae. In vertebrates, Id, Snail, and AP-2 genes are coexpressed in presumptive neural crest cells at the neural plate border. In amphioxus, these genes have almost completely non-overlapping patterns of expression; Id is expressed in the endoderm and axial mesoderm, Snail is expressed in the paraxial mesoderm and neural plate and AP-2 is expressed in the non-neural ectoderm.

Chapter 4:

Expression of an Amphioxus SoxE Homolog: Implications for the Evolution of the Vertebrate PNS and Pharyngeal Arch Skeleton

ABSTRACT

Vertebrate SoxE genes mark neural crest cells at all stages of development and are required for neural crest formation and differentiation into cartilagenous and PNS derivatives. By degenerate PCR and low stringency library screening, I isolated a single SoxE homolog from amphioxus and examined its embryonic expression pattern. Early expression of amphioxus and vertebrate SoxE genes in the nascent notochord appears conserved and may relate to the demonstrated function of SoxE genes in regulating collagen expression. Unlike vertebrate Sox9, though, amphioxus SoxE is not expressed at the neural plate border, implying that the early function of Sox9 in neural plate border/neural crest specification is a vertebrate novelty. Later expression of amphioxus SoxE in the neural tube, but not the PNS, implies that the vertebrate PNS evolved from the CNS of a cephalochordate-like ancestor. Finally, SoxE deployment in the pharynx suggests that the chondrogenic potential of pharyngeal neural crest was coopted from pharyngeal mesoderm and that a vertebrate-like visceral skeleton evolved before definitive neural crest.

INTRODUCTION

The vertebrate embryo differs fundamentally from other chordate embryos by the presence of neural crest cells. Neural crest cells delaminate from the dorsal neural tube and migrate throughout the embryo to generate a range of neural and non-neural derivatives including enteric neurons, peripheral glia, pigment cells, cartilage, and

muscle. The cephalochordate amphioxus resembles a vertebrate, but has no neural crest cells, and is thus thought to approximate the ancestral pre-vertebrate chordate. Though lacking this embryonic cell type, amphioxus does have adult cells and tissues potentially related to certain neural crest derivatives (Northcutt and Gans, 1983). Of particular interest are the enteric nervous system and pharyngeal gill bar skeleton which, in vertebrates, are entirely neural crest derived. Bone (1961) describes the enteric nerve plexus of amphioxus as dense with cell bodies, but does not speculate as to its embryonic origins. Descriptions of the amphioxus pharyngeal skeleton report it as collagenous, chitinous, and cartilagenous (reviewed by Azariah, 1969), though most modern analyses point to a predominantly cartilagenous composition (Azariah, 1969; Rahr, 1982). Interestingly, amphioxus gill bar mesoderm also expresses at least one marker of chondrogenic neural crest, Id (Chapter 3 of this thesis). Given the similarity of the amphioxus enteric nervous system and pharyngeal gill bar skeleton to their neural crest-derived vertebrate counterparts, critical questions become: 1) from what embryonic tissues do these derivatives arise in amphioxus?, 2) how are these tissues related to neural crest?, and 3)what does this reveal about neural crest origins? To begin to answer these questions I looked at the expression of Sox group E genes in amphioxus embryos.

In vertebrates, the Sox group E genes (Sox8, 9 and 10) mark post-migratory neural crest cells in the pharyngeal arches and enteric nervous system as well as pre- and migratory neural crest cells. Sox9 is the earliest expressed group E gene and marks the neural plate border, dorsal neural tube, and migrating cranial neural crest (Spokony et al., 2002). Functional studies in *Xenopus* have demonstrated that Sox 9 is necessary for neural crest formation (Spokony et al., 2002), while data from zebrafish show that it is

required for the differentiation of chondrogenic pharyngeal arch neural crest (Yan et al., 2002). Outside its roles in neural crest cells, Sox9 functions generally as a chondrogenic factor and directly regulates expression of type II collagen during joint formation (Ng et al., 1997).

The closely related Sox10 is initially coexpressed with Sox9 in early migrating neural crest. Later, the two expression patterns diverge as Sox10 transcripts persist in trunk neural crest (Cheng et al., 2000) and Sox9 predominantly marks chondrogenic cranial neural crest. Sox10 expression continues in late and post-migratory neural crest cells that generate the dorsal root, sympathetic and enteric ganglia (Cheng et al., 2000; Southard-Smith et al., 1998). Functional studies indicate that it is essential for formation of peripheral neurons and glia, as well as melanocytes (Britsch et al., 2001; Kapur, 1999; Kim et al., 2003; Kuhlbrodt et al., 1998; Potterf et al., 2001; Southard-Smith et al., 1998). Consistent with these findings, Sox10 is a direct regulator of c-Ret in enteric neurons (Lang et al., 2000; Lang and Epstein, 2003) and dopachrome tautomerase in neural crest derived melanocytes (Potterf et al., 2001). Expression of the third Sox group E gene, Sox8, overlaps broadly with both Sox9 and Sox10 in the pharyngeal arches, dorsal root, and enteric ganglia (Bell et al., 2000; Schepers et al., 2000). Unique expression of Sox8 is observed in the dermomyotomal portion of the somite (Bell et al., 2000).

In this study I isolated a Sox group E gene from amphioxus and examined its expression during early development. Phylogenetic analysis and exhaustive low stringency library screening suggests it diverged before the duplication events that generated the vertebrate SoxE genes and is the only Sox group E member in amphioxus. In situ hybridization reveals expression in the mesoderm from early neurula stages that

resolves into staining of the ventral notochord in late neurulae. Expression throughout the neural tube begins in the late neurula, and novel mesodermal or endodermal expression is seen in forming pharyngeal gill bars of late larvae. Together these results suggest that 1) expression in the notochord is a conserved feature of Sox group E expression— as frog Sox9 is also a notochord marker 2) neurons and glia equivalent in function to vertebrate peripheral neurons and glia may reside in the amphioxus neural tube 3) the chondrogenic potential of vertebrate cranial neural crest may have been coopted from the pharyngeal mesoderm of a cephalochordate ancestor.

MATERIALS AND METHODS

Amphioxus collection

Amphioxus adults (*Branchiostoma floridae*) were collected from Tampa Bay, Florida and electrostimulated to induce gamete release. Eggs were fertilized, and embryos were cultured and fixed per the methods of Holland et al. (Holland et al., 1996).

Isolation of amphioxus SoxE

The following pairs of completely degenerate nested primers were designed against the conserved HMG box of all known vertebrate Sox group E proteins:
SoxE5'1: TACGAYTGGWCIYTNGTNCCIATGCC,
SoxE3'1:GGCTGRTAYTTRTAITCIGGRTRRTC,
SoxE5'2:AAGCCBCAYGTIAARMGNCCIATGAA,
SoxE3'2:TAITCIGGGTRRTCYTTYTTRTGYTG.

Approximately 270 and 220 bp PCR fragments were amplified from a diluted amphioxus Lambda Zap II embryonic cDNA library kindly provided by Jim Langeland. Nine fragments of each size were sequenced and all but one were found to code for the same putative amphioxus Sox group E gene. This fragment was then used to screen the plated library at low stringency (2XSSC/.1%SDS at 40˚C) for full length cDNAs. Four phagemid clones were isolated, excised, partially sequenced, and found to encode the same Sox group E gene. The largest of these was completely sequenced from both ends.

Phylogenetic analysis

Full-length cDNAs were translated and their conceptual protein products were aligned to published *Drosophila* and vertebrate Sox group E sequences. A bootstrapped Neighbor-Joining tree (Saitou and Nei, 1987) was then constructed using the ClustalX program (Thompson et al., 1997). The related Sox group F gene, Human Sox17, was included as an outgroup.

In situ Hybridization

In situ hybridizations were as described previously (Meulemans and Bronner-Fraser, 2002). Riboprobes were made against the entire transcript.

RESULTS

Isolation of amphioxus SoxE

Completely degenerate primers were designed to recognize all published vertebrate Sox group E gene sequences. Out of 18 resulting PCR fragments, 17 were identical and corresponded to the same Sox group E protein. The remaining fragment, when translated, showed high sequence similarity with Sox group B2. The SoxE fragment was used to screen the same library for full-length clones at low stringency. Again, only a single Sox group E gene was found. The full coding sequence was translated, aligned , and used to construct a phylogenetic tree. The amphioxus SoxE sequence grouped at perfect bootstrap values with vertebrate SoxE genes (Fig. 1) and showed no particular affinity to any particular vertebrate Sox group E ortholog.

Embryonic expression of amphioxusSoxE

Amphioxus SoxE is first detected at 11 hours in two stripes of dorsal mesendoderm confined to the anterior half of the embryo (Fig. 2A). An optical cross section (Fig. 2B) shows that the SoxE positive cells are positioned medial to the evaginating pre-somitic mesoderm (arrowhead), and border the axial mesoderm (arrow). By hatching at 12 hours, SoxE expression has begun to extend caudally (Fig. 2C). This expansion continues until, at 15 hours, mesodermal staining extends the entire length of the embryo (Fig. 2D, arrow). Also at 15 hours, SoxE expression appears abruptly

throughout the neural tube (Fig. 2D, arrowhead). An optical cross section through a bissected 15-hour neurula shows the paired stripes seen in younger neurulae have converged (Fig. 2F, arrowhead). These SoxE positive cells now form the ventralmost portion of the notochord abutting the newly formed gut. Strong staining is also apparent throughout the neural tube (Fig. 2F, arrow). At 18 hours, SoxE transcripts begin to disappear from the notochord (Fig. 2E, arrow). By 24 hours, notochord staining has been completely extinguished (data not shown). Optical cross section through a bissected 24-hour late neurula shows persistent neural tube staining, but no notochord signal (Fig. 2G). Between one and two days, the mouth and first pharyngeal gill slit form. By two days, SoxE expression in the neural tube has ceased, while weak signal appears in the area of the pharynx (data not shown). In 3-day larvae, strong staining is observed in the gill bars bordering the mouth and gill slits (Fig. 2G, arrows).

DISCUSSION

SoxE genes in amphioxus

Completely degenerate primers were designed against the HMG box of all known vertebrate SoxE genes. It would be expected that such primers should amplify all SoxE genes present in amphioxus— although in practice, degenerate primer mixes are always biased towards certain sequences, and not all target sequences are amplified with the same efficiency. However, the fact that a single amphioxus SoxE gene as well as a divergent SoxB gene were isolated suggests that the primers were indeed degenerate enough to have amplified any other SoxE sequences in the library. Furthermore, low

stringency library screening with the amphioxus SoxE fragment yielded no other SoxE family members, though the wash conditions permitted cross-hybridization of the SoxB fragment with two different SoxB genes (see appendix). These facts, and the phylogenetic placement of amphioxus SoxE, strongly suggest that it is the only SoxE gene in amphioxus, and supports theories of whole or partial genome duplications in the vertebrate lineage (Holland, 1999). Interestingly, lamprey appears to have a definitive Sox9 gene (David McCauley, personal communication), suggesting diversification of the vertebrate SoxE family happened near the time of vertebrate origins.

SoxE expression in the notochord is conserved in amphioxus and vertebrates

Amphioxus SoxE expression is first seen in cells flanking the prospective axial mesoderm. As the notochord forms by evagination of the chordamesoderm , the two bands of SoxE expressing cells are brought into contact and form the ventral notochord. This domain is also marked by amphioxus Id expression at early neurula stages, though Id subsequently expands to label the entire notochord (see chapter 3). Interestingly, both SoxE and Id are extinguished from the notochord between 18 and 20 hours, suggesting they are influenced by a common regulatory mechanism in this structure. Deployment of SoxE genes appears to be a conserved aspect of notochord development as frog Sox9 is also expressed in the nascent notochord (Spokony et al., 2002). This conserved expression may be related to the role of Sox9 in regulating collagen expression (Ng et al., 1997), as both cephalochordate and vertebrate notochords become collagenous early in development.

Amphioxus SoxE is not expressed at the neural plate border, implying a novel function in specifying this domain in vertebrates

In *Xenopus*, Sox9 is necessary for the specification of neural crest cells (Spokony et al., 2002). This role may be separate from its function in chondrogenesis, as the zebrafish ortholog Sox9a is required for cartilage formation but not neural crest specification (Yan et al., 2002). Consistent with a role in neural crest formation, *Xenopus* Sox9 is expressed at the neural plate border from neurula stages, and is required for the expression of the neural crest markers Slug, Snail, Pax3, FoxD3, Msx-1 and Twist. In amphioxus, SoxE not expressed at the neural plate border at early neurula stages and is upregulated in the neural tube only near the end of neurulation. Consequently, it is not coexpressed with amphioxus Pax3/7, Msx, Snail, twist or FoxD at the neural plate border (Holland et al., 1999; Langeland et al., 1997; Sharman et al., 1999; Yasui et al., 1998; Yu et al., 2002). Thus, this early role in specifying the neural plate border region and/or neural crest is likely unique to vertebrate SoxE genes. Interestingly, amphioxus SoxE expression overlaps with Snail and Msx expression in the neural tube at later stages. This may indicate a conserved regulatory relationship between these factors that potentiated the recruitment of SoxE to the neural plate border early in vertebrate evolution.

Amphioxus SoxE expression in the neural tube suggests the vertebrate PNS evolved from CNS elements

Vertebrate Sox10 is expressed in dorsal root, sympathetic, and enteric ganglia, and is required for their formation (Britsch et al., 2001; Kapur, 1999; Kim et al., 2003; Kuhlbrodt et al., 1998; Southard-Smith et al., 1998). Together with the sense organs and

cranial ganglia , these derivatives comprise the bulk of the vertebrate peripheral nervous system (PNS). Importantly, all of these components are derived from the neural crest and neurogenic placodes. In cephalochordates, there are no peripheral ganglia. Instead, the cephalochordate PNS consists of individual neurons forming dense nerve nets. The homology of these nerve plexi to the vertebrate PNS have been debated for over a century (Bone, 1961).

This controversy has been exacerbated by a lack of knowledge regarding the ontogeny of the cephalochordate PNS, and, in particular, its enteric component. This unusually dense plexus is of unique interest since it lies on the gut and away from any potentially neurogenic ectoderm. Lacking migratory neural crest cells, the amphioxus embryo must therefore generate this network by some other mechanism. The possibilities include 1) de novo from the visceral endoderm or mesoderm, 2) de novo from the epidermis with migration to the gut, 3) de novo from the CNS with migration to the gut, or 4) de novo from the CNS with only processes covering the gut. In the last case one would have to presume that the original descriptions misidentified cell bodies. If the cephalochordate enteric nervous system indeed consists of true peripheral neurons and arises from ectoderm, individual neuroblasts must migrate inward to populate the gut. This would imply they have some properties of a primitive neural crest cell. The same question could apply to the remainder of the cephalochordate PNS though its subepidermal location implies it simply arises from epidermal ectoderm as in other invertebrates. Consistent with this idea, recent vital dye labelling show sensory neurons apparently arising de novo in the larval epidermis (Holland and Yu, 2002).

Thus, the unique embryonic origins of the vertebrate PNS and its equivocal homology to the cephalochordate PNS have made it difficult to reconstruct its evolutionary history. I isolated amphioxus SoxE to elucidate the ontogeny of the amphioxus PNS and to clarify its relationship to the vertebrate PNS. Interestingly, amphioxus SoxE is expressed in the neural tube from soon after neurulation until sometime before the embryo becomes a feeding larva. This result implies that the vertebrate PNS is derived from cell types originally residing in the CNS, or that it recruited genetic programs originally used only in the CNS of a cephalochordate-like ancestor. Consistent with the former scenario, Bone (Bone, 1960) describes CNS cells likely related to vertebrate dorsal root ganglion neurons which send processes out through segmentally arranged dorsal roots.

Expression of amphioxus SoxE also has implications for the development of the amphioxus enteric nerve plexus and its homology to the vertebrate gut nervous system. CNS deployment of SoxE may indicate that amphioxus enteric neurons originate from the CNS, rather than endoderm, ventral mesoderm, or epidermis. If this is the case, these neurons must migrate to the gut, or remain embedded in the CNS and send processes to the gut. These possibilities could be distinguished with vital dye labelling of the embryonic neural tube. Alternately, a lack of SoxE expression in amphioxus enteric neurons may indicate that they are not homologous to vertebrate enteric neurons. Expression of c-Ret, a marker for enteric neurons in *Drosophila* and vertebrates may clarify this issue (Hahn and Bishop, 2001).

Amphioxus SoxE expression in the pharyngeal arches supports an evolutionary relationship between chondrogenic neural crest and amphioxus gill bar mesoderm

An intriguing property of the cephalic neural crest is its ability to generate typically mesodermal tissues such as cartilage and bone. These derivatives are significant from an evolutionary standpoint as they give rise to two diagnostic vertebrate features, the cranium and the pharyngeal arch skeleton. Understanding how this mesodermal potential was acquired by the cephalic neural crest is thus of critical importance to reconstructing the evolutionary history of vertebrates. A clue to the genetic basis of this novelty may be found by examining the regulatory evolution of chondrogenic genes.

Loss-of-function studies in zebrafish and *Xenopus* demonstrate that Sox9 is a key regulator of pharyngeal cartilage formation. Furthermore, expression of Sox9 in lamprey pharyngeal arch neural crest(McCauley and Bronner-Fraser, 2003) implies this is an ancient and conserved function of vertebrate SoxE genes. In amphioxus, the presence of cartilagenous pharyngeal skeletal rods composed of sulfated acid mucopolysaccharides have been confirmed by biochemical assays and scanning electron microscopy (Azariah, 1969; Azariah, 1973; Rahr, 1982). The presence of cartilage in amphioxus gill bars suggests some degree of homology between these structures and the neural crest-derived pharyngeal arch skeleton of vertebrates. Consistent with this, the neural crest marker Id is expressed by chondrogenic pharyngeal arch neural crest in lamprey and the gill bar mesoderm of amphioxus (Chapter 3 of this thesis). Expression of amphioxus SoxE in the nascent gill bars in 3 day old larvae further supports an evolutionary relationship between these two structures. Like Id, amphioxus SoxE pharyngeal expression may reflect

genetic cooption of chondrogenic programs from arch mesoderm to the evolving cephalic neural crest. An evolutionary implication of this hypothesis is that a vertebrate-like cartilagenous pharyngeal skeleton evolved before definitive neural crest. Its functions were only later transferred to neural crest cells, leaving the mesoderm to generate the arch musculature. This scenario should be taken into account when considering fossils such as *Haikouella* whose proposed vertebrate affinities rest largely upon possession of a pharyngeal skeleton, and by inference, neural crest.

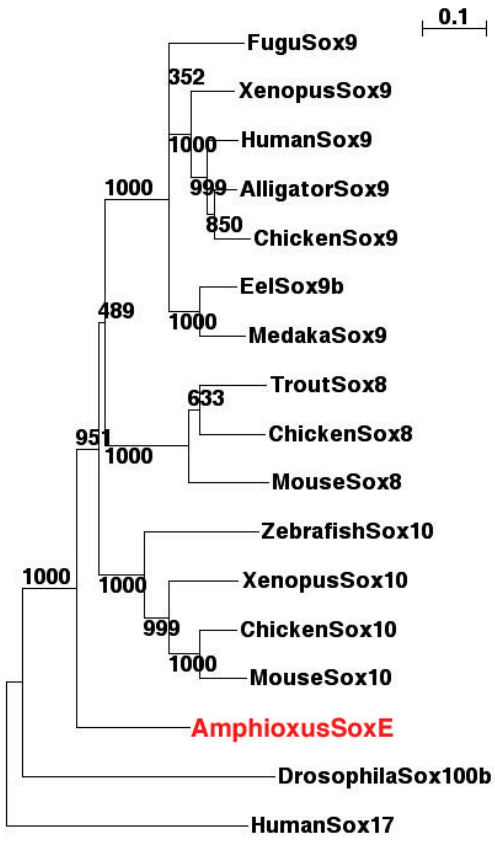


Figure 1. Phylogenetic tree of SoxE proteins created using the Neighbor-Joining method with human Sox17 as the outgroup. Numbers at branch bases are confidence values derived from 1000 bootstrap resamplings of the alignment data. Sequence distance is indicated at the top right as substitutions per base. Amphioxus SoxE clearly falls within the SoxE gene family.

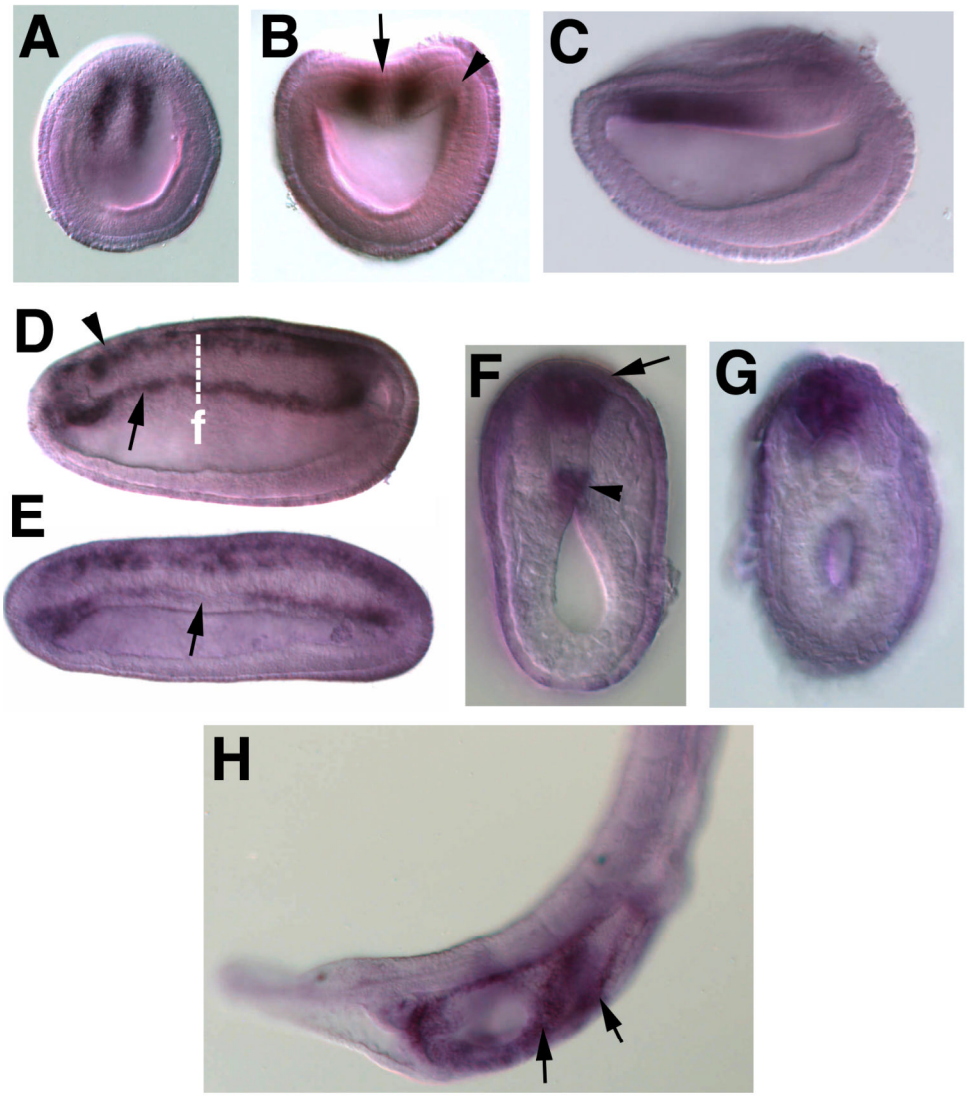


Figure 2. Embryonic and larval expression of amphioxus SoxE. (A) Dorsal view of 11-hour neurula, anterior is towards the top. SoxE expression is first observed as two stripes in the anterior half of the embryo. (B) Optical cross section through the 11-hour neurula in A. SoxE expressing cells are confined to the mesendoderm at the boundary of the somitic (arrowhead) and axial (arrow) mesoderm. (C) Side view of a 12-hour post-hatching neurula, anterior to the left. The mesendodermal expression domain has expanded slightly caudally. (D) Side view of a 15-hour neurula, anterior to the left. Mesendodermal expression now extends throughout the length of the embryo (arrow). High levels of SoxE transcripts also appear in the neural tube at this time. (E) Sideview of 18-hour neurula, anterior is to the left. SoxE transcripts are still observed in the neural

tube, but mesodermal expression begins to fade (arrow). (F) Optical cross section through a bissected 15-hour larva. High levels of staining are apparent in the neural tube (arrow). The row of SoxE-expressing mesodermal cells seen at early neurula stages have converged and now lie in the ventral notochord (arrowhead) and/or dorsal gut. (G) Optical cross section through a bissected 24-hour larva. Notochord expression has been extinguished, while SoxE expression persists in the neural tube. (H) Anterior half of a 3-day larva, staining is observed bordering the mouth and gill slits (arrows).

Chapter 5:

Functional Comparisons of Amphioxus and *Xenopus* Snail Proteins Reveal Possible Neo- or Subfunctionalization of Vertebrate Snail Genes

ABSTRACT

Snail genes are expressed at the neural plate border and dorsal neural tube in both vertebrate and amphioxus embryos. In vertebrates, Snail homologs are key regulators of neural crest formation and are necessary and sufficient for the expression of several neural crest markers. Amphioxus lacks neural crest cells, and amphioxus Snail is not coexpressed with most of the genes regulated by Snail homologs in vertebrates. I examined the ability of amphioxus Snail to phenocopy the overexpression phenotype of vertebrate Slug in *Xenopus* embryos using in situ hybridization and quantitative PCR. I found that ectopic expression of amphioxus Snail could expand expression of neural crest markers with an efficiency equal to, or greater than, that of *Xenopus* Slug. I also found that, unlike *Xenopus* Slug, amphioxus Snail was able to suppress expression of the neural marker ß-tubulin in animal caps, implying the two proteins are not completely functionally equivalent. I discuss evolutionary scenarios that could account for the observed differences, including subfunctionalization and neofunctionalization of vertebrate Snail paralogs, and experiments that will discriminate between these possibilities.

INTRODUCTION

Metazoan diversity is the result of ontological variation, which, in turn, is the consequence of heritable differences in developmental genetic programs. Given the conserved nature of the metazoan proteome, it is widely accepted that much of the developmental differences between animals are due to modifications in gene regulation (Davidson, 2001). While many of these changes are likely to be in the *cis*-regulatory DNA itself, growing evidence indicates that the role of coding sequence mutations in high-order transcriptional regulators has been underestimated (Hsia and McGinnis, 2003; Ronshaugen et al., 2002). Thus, unraveling both the *cis*- and *trans*-regulatory history of developmentally important genes is critical to understanding how new forms arise.

Most of the adult features that define the vertebrate subphylum are derived from two embryonic cell populations, the neural crest and neurogenic placodes. Recent studies comparing amphioxus and vertebrate developmental gene expression indicate that some factors involved in neural crest development, like Msx (Sharman et al., 1999), Pax3/7 (Holland et al., 1999), and Snail (Langeland et al., 1997), were in place at the neural plate border before the evolution of definitive neural crest. Others, like AP-2 (Meulemans and Bronner-Fraser, 2002), FoxD3 (Yu et al., 2002), Twist (Yasui et al., 1998), Id (chapter 3 of this thesis), and Sox9 (chapter 4 of this thesis) were probably recruited to these cells from various non-neural tissues early in vertebrate evolution (summarized in Fig. 1). The mechanisms behind these cooptions are unknown. One possibility is that novel regulatory relationships between these genes and factors already expressed at the neural plate border drove their cooption. Provocatively, Snail/Slug genes

are necessary and sufficient for the expression of many of these putatively coopted factors, including Twist and FoxD3 (LaBonne and Bronner-Fraser, 2000; Sasai et al., 2001).

Two closely related Snail genes are present in vertebrates; Snail and Slug, while only a single ascidian (Corbo et al., 1997) and a single amphioxus Snail gene have been described (Langeland et al., 1997). This is likely due to gene duplications in the vertebrate lineage, which gave rise to Snail and Slug (Sefton et al., 1998) and, Snail 1 and Snail 2 in teleosts (Smith et al., 2000; Thisse et al., 1995; Thisse et al., 1993). Snail and Slug have been shown to be necessary and sufficient for neural crest formation in vertebrate models. Slug anti-sense oligonucleotides and RNA disrupt neural crest cell migration in chickens and *Xenopus* (Carl et al., 1999; Nieto et al., 1994). In addition, expression of a dominant-negative Slug inhibits the formation of crest precursors and crest cell migration in frogs (LaBonne and Bronner-Fraser, 2000) (Sasai et al., 2001). A Slug knockout mouse has no neural crest phenotype, but this is likely due to functional redundancy of Snail and Slug (Jiang et al., 1998). Overexpression of Slug or Snail is sufficient to cause an expansion of neural crest markers and overproduction of melanocytes (LaBonne and Bronner-Fraser, 1998) (del Barrio and Nieto, 2002; Sasai et al., 2001). This activity is dependent on their function as transcriptional repressors since chimeric molecules where the Snail/Slug DNA binding region is combined with the repressor domain of *Drosophila* Engrailed function like the wildtype protein (LaBonne and Bronner-Fraser, 2000).

The amphioxus Snail gene is virtually identical to vertebrate Snail and Slug genes in the carboxy-terminal DNA-binding domain, while the N-terminus is less conserved

(Fig. 2). In this study I asked whether structural differences between the amphioxus and vertebrate Snail genes reflect functional differences related to the role of vertebrate Snail in neural crest development. To test this, I assayed the ability of amphioxus Snail to phenocopy the overexpression phenotype of vertebrate Slug in *Xenopus* embryos using in situ hybridization and quantitative PCR. I found that ectopic expression of amphioxus Snail could expand expression of neural crest markers with an efficiency equal to, or greater than, that of *Xenopus* Slug. I also found that, unlike *Xenopus* Slug, amphioxus Snail was able to suppress expression of the neural marker ß-tubulin in animal caps, implying the two proteins are not completely functionally equivalent. Several evolutionary scenarios could account for the observed differences, including, 1) evolutionary neofunctionalization of amphioxus Snail, 2) partitioning of ancestral Snail functions (subfunctionalization) between the vertebrate Snail paralogs, or 3) evolution of an 'attenuation' domain in vertebrate Snail genes that conditionally suppresses its activity. I discuss these possibilities and propose experiments to discriminate between them.

MATERIALS AND METHODS

Xenopus embryo injections and animal cap explants

Wildtype and albino *Xenopus* were harvested, fertilized, dejellied in 2% cysteine, and cultured in .1xMMR according to standard methods (LaBonne and Bronner-Fraser, 1998). At the two-cell stage, the embryos were moved to 1xMMR/3% ficoll for injection with in vitro synthesized mRNA. 5' capped synthetic mRNAs were generated

using the mMessage mMachine kit from Ambion and purified by phenol/chloroform extraction and LiCl precipitation. For analysis by whole mount in situ hybridization, 10 nl of diluted transcript was injected into one blastomere of 2-cell embryos. 100 ng bgal mRNA was included as a lineage tracer. For animal cap assays, all blastomeres of 2-8 cell embryos were injected with a total of 10 nL per embryo. 100 ng nuclear GFP was used as a lineage tracer. For all experiments, Wnt3a was injected at 100 ng, while *Xenopus* Slug and amphioxus Snail were injected at 500 ng. Animal caps were isolated in 1XMMR at Nieuwkoop and Faber stage 10-10.5, and cultured in .75XNAM until age-matched siblings reached stage 17.

ßgal visualization and in situ hybridization

Embryos were cultured until stage 17 in .1XMMR, then fixed for 1 hour in MEMFA. In situ hybridizations were as previously described (Knecht et al., 1995). Endogenous Slug transcripts were detected with an in situ probe against the *Xenopus* Slug 3' UTR (LaBonne and Bronner-Fraser, 1998). Visualization of β gal was done per standard protocols in 10 mM K3Fe(CN)6/10 mM K4Fe(CN)6/1 mM MgCl2 in PBS, with 1.5 mg/ml X-gal.

Total RNA isolation and Quantitative PCR analysis

Total RNA was isolated from animals caps using the RNAqueous kit from Ambion, then DNAse treated and LiCl precipitated for reverse transcription with SuperScript II reverse transcriptase (Invitrogen). Typically, 20-40 animal caps were processed per experiment. 3 ug total animal cap RNA were used per 20uL reverse

transcription reaction. After cDNA synthesis, water was added to bring the reaction volume to 30 uL. .5 uL of the diluted reverse transcription reactions were then used as templates in 25 uL PCR reactions containing 12.5 uL 2X SYBR Green PCR Master Mix (Perkin-Elmer), and 3 pmol gene-specific primers. Quantitative PCR was performed on an ABI Prism 7700 real-time PCR machine (Applied Biosystems) using SYBR Green as the detector dye. The following qPCR primers were designed using the Primer Express v2.0 software package (Applied Biosystems):

EF1a911F, AATCTGTTGAAATGCACCATGAA, EF1a976, RCGTTAAAGCCGACGTTGTCA, betaTubulin862F, GCCTTTCCCTCGATTGCA, betaTubulin926R, GTTGGCTGCCACGACTTGT, XFoxD3a1372R, GCGCAAGAGTGACACAACTGA, XSluga1150F, TGGTCCTTAAATACGCCCTATTTC, XSluga1225R, TGTCTAGGCAAGAATTGCTCTTTACA, XAP-2a 1366F, GCTCGAGTGAACAGAACGTGTT, XAP-2a 1448R, GGACCGGGCAATGTTCTAGA, XTwist-538F, CGACGAGCTGGACTCCAAA, XTwist-601R, GGCATAGCTGAGCCTCTCATG, XSox9-443F, AAGTTCCCCGTGTGCATCA, XSox9-511R, CGGTACCAGGGTCCAATCAT.

Relative quantities of target cDNAs were determined by the Standard Curve method with each reaction done in triplicate to control for pipetting inconsistency. Quantities were normalized against EF1alpha to compensate for variations in starting RNA concentration and reverse transcription efficiencies. Dissociation curves for each amplicon showed that none of the primer pairs formed primer dimers under these conditions.

RESULTS

Ectopic amphioxus Snail causes an expansion of Slug expression in *Xenopus* embryos

Several studies have demonstrated that ectopic expression of vertebrate Snail/Slug genes is sufficient to cause expansion of neural crest markers in whole embryo assays (del Barrio and Nieto, 2002; LaBonne and Bronner-Fraser, 2000). A particularly robust marker for neural crest cells is Slug itself. Thus, like the neural crest markers Twist and FoxD3, endogenous Slug expression is increased in response to ectopic synthetic Slug mRNA. To determine if amphioxus is functionally similar to vertebrate Slug in this regard, I tested the ability of amphioxus Snail to expand the domain of Slug expression in vivo. Injection of 500 pg of amphioxus Snail mRNA into one blastomere of a 2 cell *Xenopus* embryo lead to a dramatic expansion in Slug expression on the injected side at stage 17 (n=40), identical to that reported for *Xenopus* Slug (Fig. 3).

Quantitative comparison of the effects of ectopic *Xenopus* Slug and amphioxus Snail on neural crest marker expression in animal cap explants.

In *Xenopus* blastulae and early gastrulae it is possible to isolate prospective ectoderm before it has received inductive signals from the mesendoderm. This more-or-less naïve ectodermal tissue, termed the animal cap, can be pushed to adopt a variety of developmental fates by the injection of specific factors at early cleavage stages. The animal cap is particularly amenable to quantitative analyses of neurectodermal gene expression levels as it is free of extraneous tissues which could dampen ectoderm-

specific effects. It has been demonstrated that animal caps injected with a combination of BMP inhibitors and Wnt molecules form neural crest cells, while animal caps injected with either of these factors alone do so only at very low efficiencies (LaBonne and Bronner-Fraser, 1998; Saint-Jeannet et al., 1997). Similarly, ectopic Slug is insufficient to induce significant neural crest formation, but will do so when coinjected with either a Wnt or BMP inhibitor (LaBonne and Bronner-Fraser, 1998).

I tested the ability of amphioxus Snail to mimic the effects of *Xenopus* Slug when coinjected with Wnt3a into animal caps using quantitative PCR. This approach allowed quantitation of subtle differences in the expression levels of several neural crest markers, and one neural differentiation marker, in response to ectopic amphioxus Snail and *Xenopus* Slug. 2-8 cell embryos were injected with either 100 pg Wnt3a mRNA alone, 100 pg Wnt3a and 500 pg *Xenopus* Slug mRNA, or 100 pg Wnt3a and 500 pg amphioxus Snail mRNA. The results are summarized in Fig. 4 as fold differences in expression levels relative to Wnt3a-only injected animal caps. As expected from whole embryo data, amphioxus Snail/Wnt3a and *Xenopus* Slug/Wnt3a injections cause similar increases in endogenous Slug expression (about 25-30 fold). For the markers FoxD3, Sox9, Twist, and AP-2, a similar magnitude upregulation is observed in response to *Xenopus* Slug and amphioxus Snail. However, in general, amphioxus Snail is a slightly more efficient inducer of neural crest markers. For FoxD3 in particular, amphioxus Snail was twice as efficient in inducing expression as *Xenopus* Slug (14-fold over control caps versus 7-fold overcontrol caps). Interestingly, for the neural differentiation marker β-tubulin, amphioxus Snail resulted in an 8-fold decrease in expression versus *Xenopus* Slug/Wnt3a or Wnt3a injection alone.

DISCUSSION

Evolution is likely driven by alterations in developmental gene regulation, rather than functional changes in downstream effector genes. Substantial evidence for such changes comes from comparative gene expression studies demonstrating that alterations in morphology are often accompanied by dramatic changes in developmental gene expression patterns. Exactly how the genome must be modified to affect these changes is not known, though the possibilities are limited. A gene's expression pattern is the computational output of interactions between its *cis*-regulatory DNA and *trans*-acting factors. Thus, gene expression will be modified by functional changes in either element. In recent years the idea that *cis*-regulatory change is the main mechanism by which evolution alters gene expression has gained wide acceptance (Davidson, 2001). The core rationale behind this assertion is that *cis*- regulatory DNA is more likely to withstand evolutionary tinkering than protein-coding DNA. However, recent experimental work and theoretical considerations have argued that *trans*-acting DNA may be more plastic than previously thought, and the concept of a static 'toolkit' of developmental transcription factors may be an oversimplification (Hsia and McGinnis, 2003). Indeed, experimental evidence has shown that both kinds of regulatory alterations can lead to macroevolutionary changes (Belting et al., 1998; Ronshaugen et al., 2002).

The evolution of neural crest coincided with the recruitment of several classes of transcription factors to the neural plate border, including AP-2, FoxD, Twist, and SoxE. Other factors critical for neural crest formation were already present in this domain

before the presence of neural crest. One of these, Snail, is necessary and sufficient for the expression of several of the aforementioned 'coopted' neural crest factors, including, Twist and FoxD3 in vertebrates. The ability of Snail to regulate genes in vertebrates that it cannot regulate in amphioxus (because they are not deployed in the same cells) suggests that Snail has evolved novel regulatory properties in the vertebrate lineage. This could be due to the evolution of new Snail responsive sites in the *cis*-regulatory DNA of 'coopted' neural crest factors and/or the evolution of novel *trans*-regulatory capabilities in the Snail protein itself. In this study, I have begun to test this later possibility by examining the ability of amphioxus Snail to replicate the overexpression phenotype of its vertebrate homolog.

Initial assays using whole embryos showed that amphioxus Snail could cause an expansion of endogenous Slug expression in a manner similar to that of *Xenopus* Slug. The fact that Slug functions as a repressor indicated that this expansion was not due to a simple auto-regulatory feedback mechanism. At the minimum, amphioxus Snail participated in a two step interaction whereby it repressed genes which repress endogenous Slug expression.

This functional conservation lead me to more rigorously compare the abilities of *Xenopus* Slug and amphioxus Snail to upregulate neural crest markers using a sensitized animal cap assay and quantitative PCR. Utilizing this approach, I examined the effects of ectopic amphioxus Snail and *Xenopus* Slug on endogenous Slug, Twist and FoxD3 expression levels. In addition, I analyzed their effects on two other potential targets of Slug, AP-2 and Sox9. As expected, *Xenopus* Slug caused an upregulation of endogenous Slug, FoxD3 and Twist. *Xenopus* Slug was also shown to upregulate Sox9 (by roughly 11

fold) and AP-2 (by 2 fold). Amphioxus Snail showed similar upregulation of all neural crest markers, indicating functional conservation of the two genes extended beyond just upregulating endogenous Slug. Interestingly, in some cases— particularly with FoxD3 and Sox9— amphioxus Snail overexpression elicited a larger response that *Xenopus* Slug. This may merely reflect a difference in translation efficiency between the two transcripts. If this were the case, though, one would expect to see proportional differences in upregulation for every marker. However, for endogenous Slug and AP-2 *Xenopus* Slug is at least as efficient as amphioxus Snail. Alternately, it may reflect subfunctionalization of *Xenopus* Slug and *Xenopus* Snail. Both of these Snail homologs are expressed in neural crest cells, and were originally thought to have largely redundant functions. However, recent work has suggested that they may, in fact, have overlapping but non-identical roles in neural crest development (Aybar et al., 2003). It is possible that amphioxus Snail retains the combined functionality of both genes and is thus a more potent inducer of some targets than *Xenopus* Slug. This assertion is testable by comparing the abilities of *Xenopus* Snail and Slug overexpression to induce particular markers. A specific prediction would be that *Xenopus* Snail, like amphioxus Snail, is more effective at upregulating FoxD3, Sox9, and Twist than *Xenopus* Slug.

Another observed difference between amphioxus Snail and *Xenopus* Slug function is that amphioxus Snail seems able to repress terminal neural differentiation as assayed by β-tubulin expression. In my hands, both Wnt alone, and Wnt/Slug caused some baseline level of neural differentiation in cultured animal caps. Wnt combined with amphioxus Snail injection, however, prevented this differentiation. As a result Wnt/amphioxus Snail injected animals caps have about 7-fold less β-tubulin than either

Wnt-injected or Wnt/Slug injected caps. This result is intriguing as it suggests that either amphioxus Snail has gained the ability to repress terminal neural differentiation, or *Xenopus* Slug has lost this function. If this latter scenario is correct, the ability to repress neural differentiation may have been lost by *Xenopus* Slug, but maintained in *Xenopus* Snail. Further experiments comparing *Xenopus* Slug and *Xenopus* Snail would address this potential subfunctionalization.

Still another possible scenario is the evolution of an 'attenuation' domain in vertebrate Snail genes that make their repressive activities conditional on interactions with other factors. Amphioxus Snail, lacking this domain, is thus always maximally 'on,' and represses every possible target, while *Xenopus* Slug is a more selective, performing its role in neural crest cells without effecting neural differentiation. Evolution of a conditional repressor from a constitutive one is thought to have driven diversification of the arthropod body plan (Ronshaugen et al., 2002). Provocatively, a block of residues conserved in vertebrate, but not amphioxus, Snail genes is located adjacent to the N-terminal SNAG repressor domain (Fig. 2). It would be interesting to see if mutating this domain would cause *Xenopus* Slug to behave less selectively, or if engineering amphioxus Snail to contain this region would attenuate its broad repressive activities.

Regardless of the evolutionary implications, it will be necessary to further characterize exactly how amphioxus Snail is effecting neural differentiation. β-tubulin is a marker for a variety of differentiated neurons, making it important to establish if amphioxus Snail is interfering with CNS formation, or blocking differentiation of neural crest-derived neurons. Assaying expression of the early CNS marker Sox2 would help clarify this issue.

CONCLUSIONS AND FUTURE DIRECTIONS

Taken together, the data in this study suggest amphioxus Snail is more functional, in some respects, than *Xenopus* Slug. *Xenopus* Slug is not only a less potent inducer of certain neural crest markers, but is also unable to repress neural differentiation. This is surprising, since vertebrate Snail/Slug genes appear to have completely novel roles in neural crest development, and might be expected to be more functional than amphioxus Snail. It will be interesting to see if the apparent reduced functionality of *Xenopus* Slug relative to amphioxus Snail reflects a partitioning of ancestral functions between vertebrate Snail and Slug proteins, or the evolution of a 'smarter' Snail protein with the ability to attenuate its repressive activity.

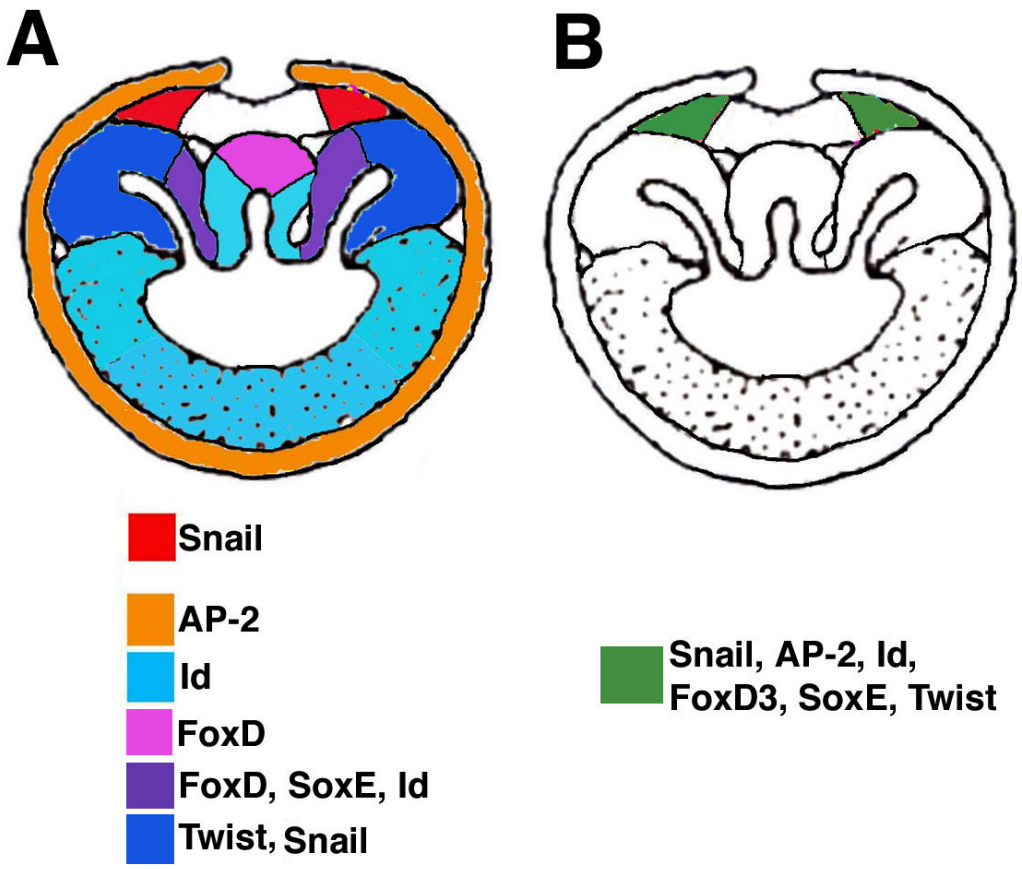


Figure 1. Cooption of various neural crest markers to the neural plate border early in vertebrate evolution. (A) In amphioxus, the genes FoxD, SoxE, AP-2, Id, and Twist are expressed in various non-neural tissues. (B) Early in the vertebrate lineage , all of these factors were recruited to cells at the neural plate border.

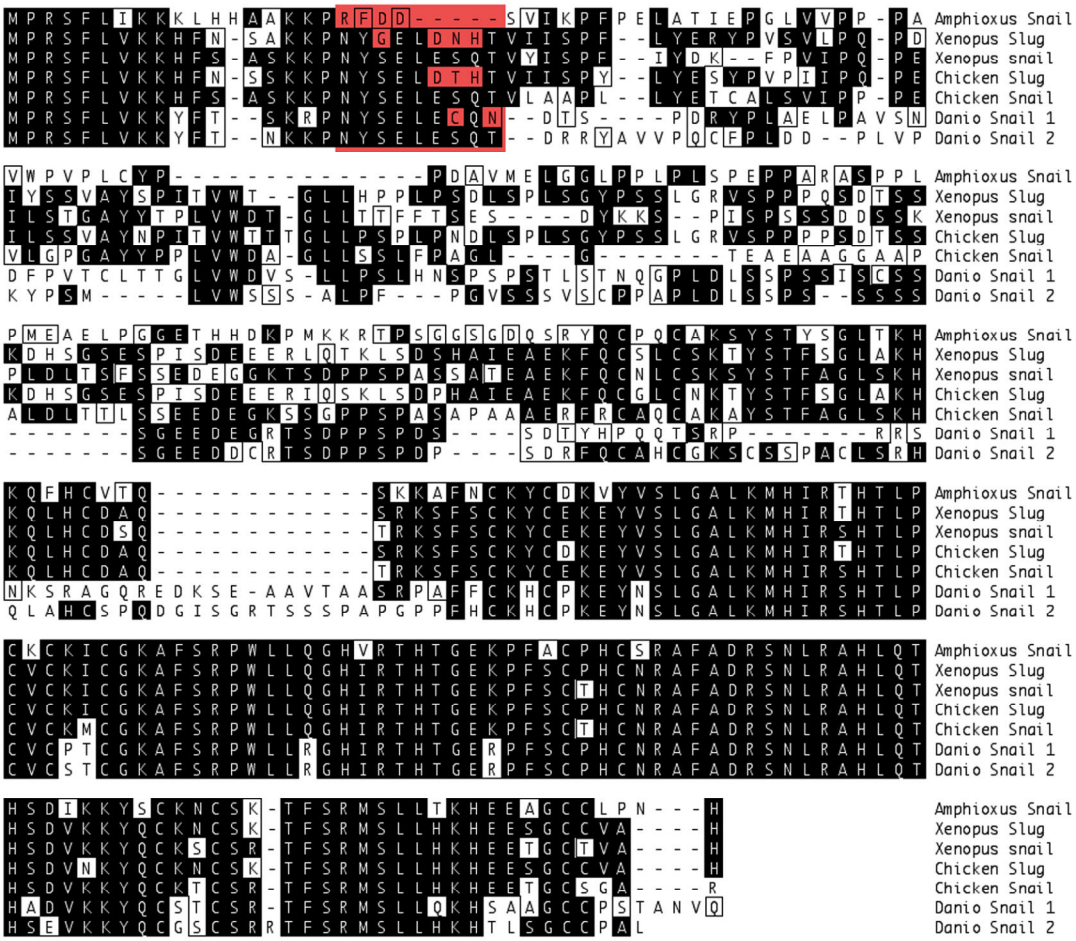


Figure 2. Alignment of vertebrate and amphioxus Snail proteins. High conservation is seen in the C-terminal DNA binding region and the very N-terminal SNAG repressor domain. Highlighted in red is a region adjacent to the SNAG domain that is unique to vertebrate Snail genes. This domain may confer regulatory properties upon these proteins not possessed by amphioxus Snail.

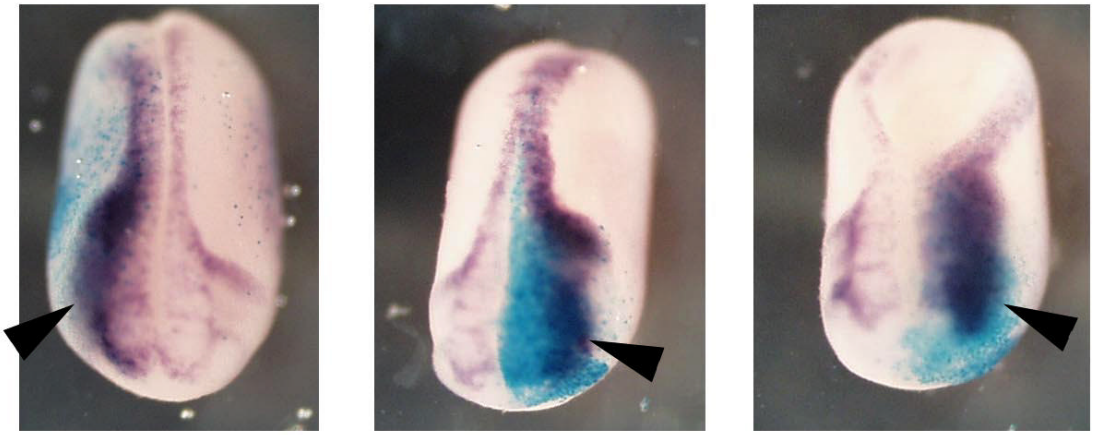


Figure 3. Ectopic amphioxus Snail expands Slug expression. Injection of 500pg amphioxus Snail into one blastomere at the 2-cell stage causes an increase in Slug expression on the injected side (arrowheads).

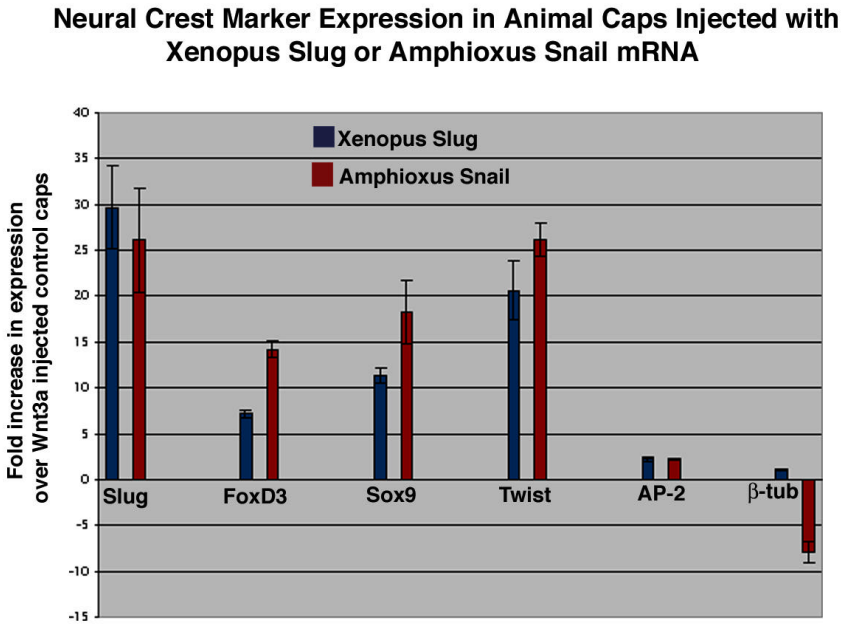


Figure 4. Quantitation of mRNA expression levels in Wnt-sensitized animal caps injected with amphioxus Snail or *Xenopus* Slug transcripts. Animal caps were injected with 500pg amphioxus Snail or 500pg *Xenopus* Slug mRNA together with 100pg Wnt3a and cultured to stage 17. Quantities of target transcripts were determined by quantitative real-time PCR and are expressed on the chart as fold differences in expression relative to control animal caps injected with Wnt3a only. Error bars represent ±1 standard deviation from the mean of three replicates.

Chapter 6:

Towards Unraveling the Evolution of Vertebrate Snail Gene *Cis*-Regulation

ABSTRACT

In amphioxus and vertebrate neurulae, Snail genes are expressed at the neural plate border. As neurulation procedes, however, vertebrate Snail genes mark pre- and migratory neural crest while amphioxus Snail expression expands throughout the neural tube. To elucidate the evolutionary origins of neural crest-specific Snail gene regulation, I have begun to characterize the *cis*-regulatory DNA of amphioxus and chicken Snail homologs . 60 kilobases of amphioxus Snail and 14 kilobases of chicken Slug genomic DNA were assayed for the presence of neural regulatory elements by transient transfection of chicken, zebrafish, frog, mouse, and amphioxus embryos. No specific neural modules were identified, though the amphioxus Snail basal promoter drove non-specific reporter expression in virtually all cells. These preliminary results constitute a foundation for further *cis*-regulatory analyses of these genes.

INTRODUCTION

Snail genes are necessary for both neural crest induction and migration (LaBonne and Bronner-Fraser, 2000). Consistent with this dual role, vertebrate Snail homologs are expressed early at the neural plate border, and later in migrating neural crest cells (Locascio et al., 2002; Sefton et al., 1998). In chordates lacking neural crest cells, Snail expression is similarly observed at the neural plate border, but is then rapidly downregulated, as in Ciona (Corbo et al., 1997), or turned on throughout the neural tube, as in amphioxus (Langeland et al., 1997) (Fig. 1). These expression patterns imply that

regulatory modules controlling early deployment at the neural plate border are conserved among chordates while sequences involved with later neural expression have diverged. Of particular interest are those elements driving Snail expression in the neural crest, as neural crest cells have no obvious homologs in amphioxus or ascidian embryos.

In an effort to reconstruct the evolution of neural crest-specific Snail gene regulation in vertebrates, I have begun to characterize the *cis*-regulatory DNA of amphioxus and chicken Snail homologs . Amphioxus Snail and chicken Slug genomic DNA was isolated, partially sequenced, and assayed for regulatory activity in amphioxus, zebrafish, chicken, mouse, and Xenopus. Out of 60 kilobases of amphioxus Snail genomic DNA, no tissue specific enhancers were found. However, a small fragment of DNA 5' of the start site was sufficient to direct non-specific expression of a reporter gene in various tissues. 14 kilobases of chicken Slug genomic DNA was not able to direct reporter expression when electroporated into chick. Sequence analyses revealed the location of probable basal promoters and potential transcription factor binding sites. These results and their relevance to future investigations are discussed.

MATERIALS AND METHODS

Isolation of genomic DNA and reporter vector construction

The 5' end of the amphioxus Snail cDNA was used to screen a commercially available arrayed amphioxus genomic cosmid library (RZPD, Germany). Positive reacting clones were identified and ordered from the supplier. Clones were grown and cosmids digested with EcoRI then analyzed by Southern blot. A 5 kilobase EcoRI

fragment containing the start codon was identified and partially sequenced. A portion of this fragment including 1700 basepairs 5' of the start site, the first exon, the first intron, and the very 5' of exon 2, was cloned into the vector pSP72.1.27. The last exon was ligated in frame with lac Z. Additional Spe I and Eco RI fragments from the cosmids were cloned upstream of this insert to generate several reporter constructs.

Chicken Slug genomic fragments were isolated by screening a conventional lambda genomic library with radiolabeled chicken Slug cDNA. Seven clones were identified and digested with XhoI. A 4 kilobase XhoI fragment containing the start codon was sequenced. Part of this fragment containing 2,100 basepairs 5' of the start codon, the first exon, the first intron, and the beginning of the second exon was cloned in frame with the lac Z gene of pSP72.1.27. A second reporter construct was made by inserting an additional 11 kilobase XhoI fragment upstream of the original insert.

Transient transfection of zebrafish, chick, frog, and amphioxus

For injection into zebrafish, reporter DNA was purified by phenol/chloroform extraction/ ethanol precipitation and resuspended in water to a concentration of between 40 and 100ng/uL. Phenol red was added as a tracer dye. Fertilized eggs were injected at the 1-2 cell stage and embryos were incubated at 28°C for between 12 and 48 hrs, collected, fixed, and stained for lac Z activity.

Reporter constructs were introduced into the chicken neural tube by electroporation. Reporter DNA was resuspended in water to a concentration of between .5 and 1 ug/uL, with 1% Fast Green added as a tracer dye. The neural plates and neural tubes of 2-6 somite embryos were covered or filled with the DNA solution and

electroporated by application of 1-2 15-25 volt pulses lasting 50 ms each. Embryos were cultured for 12-48 hours, fixed and stained for lac Z activity.

Xenopus embryos were injected with 7-9 nL of reporter DNA at 25-50 ng/uL and allowed to develop at 16°C to late neurula stages then fixed and stained for lac Z activity.

Unfertilized amphioxus oocytes were injected with 25-100pg reporter DNA in 5 nL water using a pulled glass needle, then fertilized and incubated to mid-neurula stages (about 15 hours) using Texas Red as a tracer dye. Embryos were then fixed and stained for lac Z activity.

Sequence analyses

Amphioxus Snail and chicken Slug genomic sequences searched for basal promoters using Berkeley Fly Genome Project Promoter Predictor (http://www.fruitfly.org/seq_tools/promoter.html) and for selected transcription factor binding sites suing MATINSPECTOR v 2.2.

RESULTS

Genomic DNA isolation and reporter plasmid construction

Amphioxus and chicken genomic libraries were screened and positive clones were partially sequenced. For amphiouxs Snail, 4 cosmid clones were isolated, each containing between 20 and 50 kilobases of sequence. For chicken Slug, 7 lambda phage clones were identified with inserts of between 9 and 20 kilobases. Fragments from each

gene were isolated which contained about 2 kilobases 5' of the translational start site, the first and second exons, and the first intron (Fig. 2). These pieces of DNA were cloned into the pSP72.1.27 reporter vector so that the first 7 amino acids of exon 2 were fused in frame with the lac Z gene. This reporter design was modeled after the Ciona Snail reporter constructs used successfully to identify mesoderm specific enhancers in Ciona Snail genomic DNA (Erives et al., 1998). Additional pieces of genomic DNA were subcloned 5' of these inserts. In the case of chicken Slug, a single 11 kilobase fragment was added to the initial 3 kilobase insert to generate a second, larger, construct. For amphioxus Snail, an additional 2.5 kilobases of 5' genomic DNA were added to the original insert and then a shot-gun strategy was employed to scan the remaining cosmid DNA for regulatory elements. This method (outlined in Fig. 3) was used to scan about 60 kilobases of genomic DNA for fragments with the capacity to attenuate the activity of the amphioxus Snail basal promoter.

Expression of reporter constructs in zebrafish, Xenopus, chicken and amphioxus

Reporter constructs were tested for activity in several vertebrate embryos. The initial amphioxus Snail reporter constructs consisting of 1700 and 4200 basepairs 5' of the start site were sufficient to direct non-specific expression of lac Z when electroporated into the chicken neural tube (Fig. 4A, arrow) and ectoderm (Fig. 4A, arrowhead). Similar non-specific activity was seen when the constructs were injected into zebrafish (Fig. 5B), Xenopus (Fig. 4C), and mouse embryos (Miguel Manzanares, personal communication). To test if this lack of tissue-specificity was due to the inability of the reporter to be properly regulated in vertebrate cells, the larger construct was tested

in amphioxus (Jr Kai Yu, unpublished results). Comparable dysregulation was observed (Fig. 4D). Using the shot-gun strategy summarized in Fig. 3, I assayed the remainder of the amphioxus Snail cosmid DNA for the ability to attenuate the non-specific activity of the minimal construct by electorporation into chicken. Like the original construct, these shot-gun constructs directed non-specific expression of lac Z in the neural tube and ectoderm.

Two chicken Slug constructs encompassing 14 kilobases of Slug genomic DNA were tested for activity by electroporation into chick and injection into zebrafish embryos. In chick, less than 20 lac Z expressing cells were observed with either construct, and most of these were ectodermal (data not shown). In zebrafish, only scattered muscle and yolk sac cells expressed the reporter gene (data not shown).

DISCUSSION

A total of 14 kilobases of chicken Slug genomic DNA and 60 kilobases of amphioxus Snail DNA were assayed for the presence of neural-specific regulatory elements. In neither case were any such elements found. However, a small region surrounding the translation start site of amphioxus Snail was able to direct expression of the reporter gene in a variety of tissue and cell types, suggesting the presence of an active basal promoter. Analysis of this fragment reveals a probable promoter sequence located within 300 basepairs of the translational start site (Fig. 5). Similar analysis of the minimal chicken Slug reporter construct also revealed a likely promoter near the translational start site, although this construct was only nominally active. Interestingly, the genomic sequence immediately upstream of the amphioxus Snail promoter contains numerous Sp1

binding sites, whereas the homologous region of chicken Slug DNA has none (Fig. 5). Sp1 consensus binding sties are often found near promoters where they act to enhance transcription , and may explain the observed difference in activity between the two minimal constructs.

Taken together, these results imply differing mechanisms of Snail/Slug regulation in amphioxus and chicken. For amphioxus Snail, repressive modules which attenuate the activity of the strong basal promoter may dominate its regulatory apparatus. In chicken, Slug expression may be driven largely by positive regulatory interactions between enhancers and the weak Slug basal promoter. Whether this is a general difference between amphioxus and vertebrate Snail gene regulation will be resolved as more vertebrate Snail promoters are described.

Regardless of the mechanism, these findings have implications for further *cis*-regulatory analyses of these two genes. Since the amphioxus Snail promoter is moderately active, it serves as a good starting point for finding regulatory modules which can enhance or restrict its activity. Thus, applying the shot-gun strategy used in this study to a larger segment of DNA, such as a BAC insert, may yet prove fruitful. In contrast, the weak basal promoter of chicken Slug may not respond well to isolated enhancers in the context of an artificial reporter construct, and may thus need to be replaced with a more active basal promoter for further screening.

CONCLUSIONS AND FUTURE DIRECTIONS

Future analyses of chicken Slug and amphioxus Snail regulation will be facilitated by several recent technical advances. Arrayed amphioxus and chicken BAC libraries are now commercially available and will allow greater regions of genomic DNA to be quickly surveyed for *cis*-regulatory elements. In addition, techniques for broader and earlier electroporation of chicken embryos have developed to the point where rapid *cis*-regulatory analysis is feasible. The first example of such an analysis leading to the isolation of novel enhancers was just recently published (Uchikawa et al., 2003). Finally, a technique for reliably introducing and expressing reporter constructs in amphioxus oocytes has been developed (Jr Kai Yu, personal communication) making possible the verification of putative amphioxus regulatory elements in amphioxus embryos.

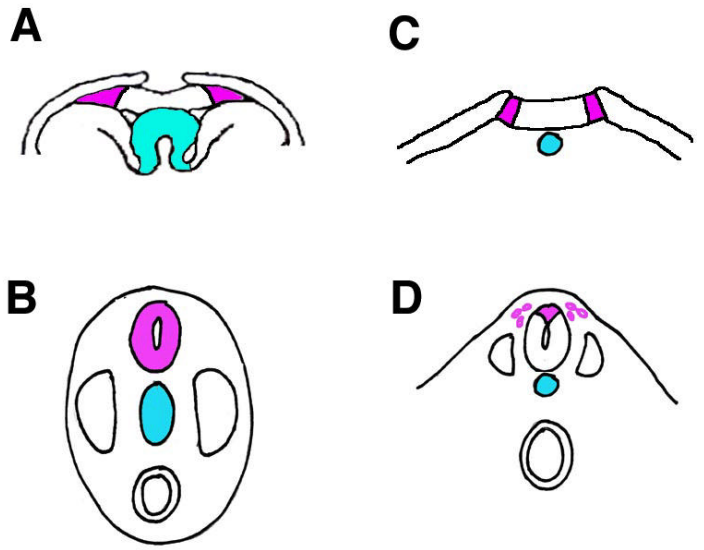


Figure 1. Expression of Snail homologs in amphioxus and vertebrate embryos. At open neural plate stages amphioxus Snail (A) and the vertebrate Snail homologs, Snail and Slug, (C) are expressed at the neural plate border (pink). After neural tube closure, amphioxus Snail expression expands throughout the dorsoventral aspect of the neural tube (B) while vertebrate Snail genes mark the dorsal neural tube and neural crest (D). The notochord is colored blue for reference.

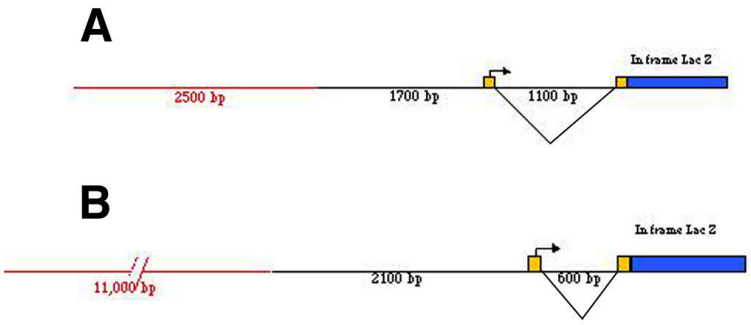


Figure 2. Amphioxus Snail and chicken Slug reporter constructs. (A) The initial amphioxus reporter construct was made by cloning 1700 basepairs of 5' genomic DNA, the first exon, the first intron, and the beginning of the second exon in frame with lac Z. The second construct included 2500 additional basepairs of 5' sequence (red). (B) The first chicken Slug construct was made by cloning 2100 basepairs of 5' genomic DNA, the first exon, the first intron, and the beginning of the second exon in frame with lac Z. The second was generated by adding 11 kilobases of 5' genomic sequence (in red).

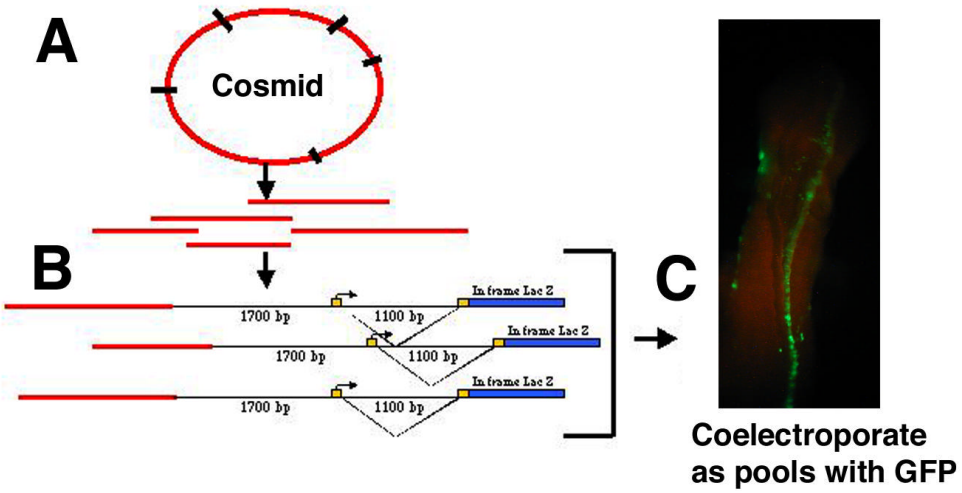


Figure 3. Schematic diagram of the shotgun strategy used to screen amphioxus Snail cosmids for regulatory elements. (A) Cosmids are digested with Spe I. (B) The resulting 2-5 kilobase fragments are ligated into the smaller of the two original reporter constructs. (C)The resulting shot-gun constructs are co-electroporated as pools of 4-5 into the neural plate and neural tube. A GFP plasmid is included as an electroporation control.

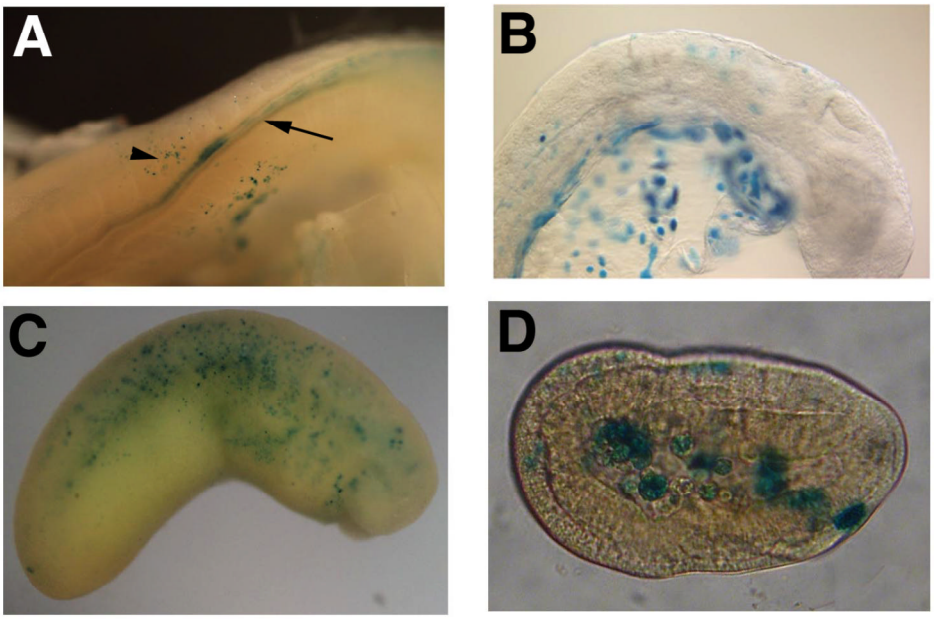


Figure 4. Amphioxus Snail reporter expression in chicken, zebrafish, frog and amphioxus. (A) When electroporated into the chicken neural tube (arrow) and adjacent ectoderm (arrowhead), the constructs show non-specific activity in both tissues. (B) Non-specific expression in zebrafish 36 hours after injection. Non-specific expression in Xenopus embryo (C) and 15 hour amphioxus neurula. (D) Amphioxus injections by Jr Kai Yu.

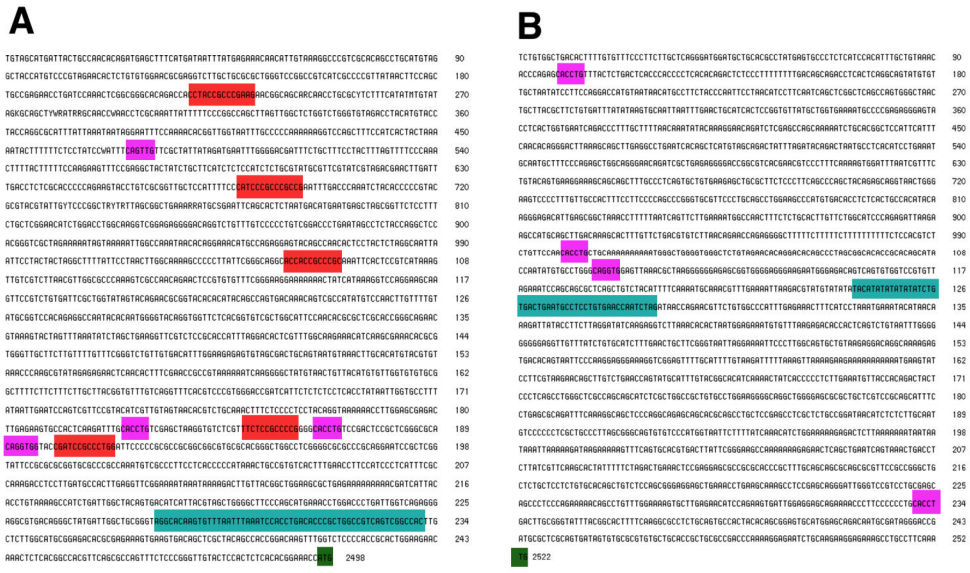


Figure 5. Sequence analysis of amphioxus Snail and chicken Slug 5' genomic DNA. (A) 2500 base pairs of amphioxus Snail genomic DNA upstream of the translational start site (green) were scanned for the presence of likely promoters (aqua blue), Sp1 binding sides (red), and E-boxes (pink). (B) 2500 base pairs of chicken slug genomic DNA similarly analyzed. Note that the region surrounding the putative amphioxus Snail promoter has 5 Sp1 binding sites, while the homologous region of chicken Slug DNA has none.

Chapter 7:

CONCLUSIONS

What is known about the neural crest gene regulatory network

It is widely accepted that the appearance of neural crest cells was a defining event in vertebrate evolution. This assertion reflects the fact that neural crest cells give rise to adult structures that define the vertebrate clade, including the peripheral ganglia, pharyngeal arch skeletomusculature, and cranium. Accordingly, definitive neural crest cells have been described in the most basal extant vertebrates, the agnathans, but not in their closest living invertebrate relatives, the cephalochordates.

Gene expression patterns and functional comparisons suggest that the genetic interactions driving neural crest formation are conserved among vertebrates. However, the mechanistic data at the core of the neural crest gene network has been derived chiefly from molecular manipulation of a single vertebrate, *Xenopus*. As a result, experimental analyses of neural crest gene regulatory relationships are largely restricted to gain or loss of function studies in *Xenopus* using injected mRNA and antisense oligonucleotides. Further limiting a comprehensive dissection of the neural crest regulatory network is the fact that *Xenopus*, as most other vertebrates, is poorly suited for *cis*-regulatory analyses due to the dysregulated and mosaic expression of injected reporter constructs and the difficulty of making transgenics. Thus, the gene regulatory network operating in neural crest cells has only been described at a rudimentary level largely in a single species refractory to *cis*-regulatory analyses. Practically, this means that for most "neural crest genes" it is simply known that perturbing expression or function in frog has a predictable effect on the expression of certain other factors. Whether this interdependency involves direct binding of a transcription factor to an enhancer, or works through any number of intermediate genes, is usually unknown.

Wnts, Fgfs, and various levels of BMP signaling induce epidermal, neural plate, neural crest gene expression

Regardless, what is known about the neural crest gene network in vertebrates constitutes a framework from which to begin examining the regulatory evolution of neural crest cells, and will be reviewed here. The bulk of this information is summarized in Tables 1-4. The neural crest regulatory state is likely initiated by BMP, Wnt, and Fgf signals from underlying mesoderm and adjacent non-neural ectoderm (Table 1). These signals also function simultaneously to segregate neural from non-neural ectoderm during neural induction. Low levels of BMP are necessary and sufficient for the expression of Sox2 and Zicr-1 genes in the neural plate. Sox2 and Zicr-1 in turn activate neural differentiation genes such as N-CAM and N-tubulin, likely through the neurogenic bHLH transcription factors Neurogenin, Neuro-D, and achaete-scute (Table 2). In non-neural ectoderm, high levels of BMP signal activate the epidermal program via Msx1, Dlx3/5, and AP-2. Msx1, an immediate early target of BMP2/4 signaling,is sufficient to activate keratin expression and repress the neural markers Zic3 and N-CAM (Table 2). Dlx3 and 5 similarly repress neural factors such as Sox2, while ectodermal AP-2 activates epidermal keratin expression by direct binding of the keratin promoter (Table 2).

At the neural plate border intermediate levels of BMP signal are necessary for the expression of the neural crest markers Snail/Slug, AP-2, Sox9 and FoxD3 (Table 1). In addition to an attenuated BMP signal, input from the Wnt pathway also appears required for expression of Snail/Slug, AP-2, and FoxD3, as well as virtually every other described neural crest/neural plate border marker. Less evidence exists demonstrating the role of

Fgf signaling in neural crest induction except that it seems necessary for Snail/slug expression.

It is unknown if influence of BMP, Wnt and Fgf pathways on neural crest marker expression is direct or controlled by other factors. Evidence presented below suggests that Zic factors and Pax3/7 may mediate the effects of BMPs and Wnts on neural crest marker expression. However, data from *Xenopus* suggests that Slug expression in neural crest may be partly dependent on a Tcf/Lef binding site in the Slug promoter (demonstrating direct regulation of Slug expression by Wnts), though this finding has proven difficult to replicate (Carole LaBonne, personal communication).

"Neural plate border specifiers" may mediate the influence of Wnts and BMPs on "neural crest specifiers"

Concurrent with, or shortly following, the initial signaling events that establish the neural plate border is expression of a small set of transcription factors genes designated here as "neural plate border specifiers". This group of genes includes Zic factors, Pax3/7, and Msx2. Of these, Pax3 and Msx2 have been shown to be downstream of Wnt signaling. Zic factors are activated in the neural plate and neural plate border in response to BMP repression. These genes display features that distinguish them from other neural plate border markers, and suggest they (particularly Zic and Pax3/7) may mediate the influence of Wnts and BMPs on neural crest specifiers like Slug/Snail. For one, they are expressed quite early in neural plate border cells and precede most neural crest markers. Secondly, they do not generally mark migrating neural crest cells (the exceptions to this are the expression of Msx2 a small subset of cranial neural crest, and

the expression of Pax3 in post-migratory neural crest derived neurons). Thirdly, they also function in a broad range of neural plate border-derived cell types including roof plate and Rohon-Beard cells. Finally, experimental evidence shows that ectopic Zic5 or Zicr-1 alone is sufficient to activate expression of Slug/Snail, FoxD3, and Twist (in naïve ectoderm and in vivo) and Pax3/7 is required for the expression of Sox10, Slug/Snail, FoxD3and cRet in mouse and chick (Table 3). Thus, the timing and breadth of neural plate border specifier expression as well as their demonstrated regulatory capacities, suggest they act upstream of neural crest specifiers— and downstream of Wnt and BMP signals.

Neural crest specifiers occupy a distinct position in the neural crest gene network

After the onset of neural plate border specifier expression, a suite of genes, including Slug/Snail, AP-2, FoxD3, Sox10, and Sox9, is activated at the neural plate border. These "neural crest specifiers" display functional and regulatory characteristics suggestive of a distinct position in the neural crest regulatory network downstream of Wnt/BMP/Fgf inductive signals and the "neural plate border specifiers" Zic and Pax3/7 (Table 1, 2). All neural crest specifiers are expressed initially at the neural plate border and persist in a substantial portion of neural crest cells throughout migration. Furthermore, all of these factors can repress Sox2 expression in the neural plate and most require a Wnt signal for activation (the dependence of Sox10 expression on Wnts is unexamined). Finally, Zic factors and Pax3/7 are variously sufficient and necessary for the expression of Slug/Snail, FoxD3 and Sox10 (Table 3).

Neural crest specifiers cross-regulate

Every neural crest specifier factor examined thus far also appears necessary and sufficient (in vivo) for the expression of the other factors (Table 4). Thus, injection of a Sox10 morpholino causes loss of Sox9, Slug/Snail and FoxD3, while Sox10 overexpression expands the expression domain of Slug and Sox9. Similarly, ectopic Slug transcripts activate expression of AP-2, FoxD3, Sox9, Sox10 and FoxD3, while dominant negative Slug suppresses expression of Sox10 and FoxD3, etc. The interdependence of Slug/Snail, AP-2, FoxD3, Sox10, and Sox9 expression makes assigning hierarchical relationships to these factors risky. Despite this, arguments have been made which alternately place AP-2, Slug/Snail, and FoxD3 upstream of all the other factors. Two pieces of evidence suggest AP-2 occupies a unique place amongst above the other neural crest specifiers. First, AP-2 is earlier and more broadly deployed at the neural plate border than any of the other neural crest specifiers. Second, AP-2 upregulation in Wnt-sensitized ectoderm occurs with minimal BMP inhibition, approximately 30-fold less than is need for Slug or Sox9. Evidence for Snail and FoxD3 as “neural crest master regulators” is basically the observation that both genes, when misexpressed at high enough levels in naïve ectoderm, are capable of inducing the expression of some of the other neural crest specifiers. Neither Slug, AP-2, Sox9 or Sox10 seem to have this ability and can only induce neural crest in Wnt or BMP sensitized ectoderom. Ultimately, it may be meaningless to try and assign simple linear relationships to these factors as they not only activate each other but regulate genes putatively upstream in the neural crest regulatory cascade. FoxD3 is capable of inducing Zicr1 and neural markers, while Sox9 is required for continued expression of the neural plate border specifier Pax3 (Table 4).

Slug/Snail genes have a central role in neural crest cells

While not likely at the top of a neural crest regulatory hierarchy, Slug/Snail does appear to have a central role among the other neural crest specifiers. Unlike most of the other factors, Slug/Snail genes are transcriptional repressors. Thus, they necessarily activate expression of the other crest specifiers through another as yet unidentified repressor. This role as an anti-repressor suggests that the main function of Slug/Snail may be the maintenance of the other neural crest specifiers, rather than controlling lower-level effector genes. Direct regulation of these effectors may thus be accomplished by the remaining "activating" specifiers or their downstream targets. It will be interesting to find out what the main targets of AP-2, FoxD3, Sox9 and Sox10 activation are in pre- and migratory neural crest as these genes are likely key mediators of the neural crest cell phenotype.

Twist and Id

Two other transcriptional regulators are coexpressed with the neural crest specifiers but likely lie outside their tightly interdependent network. Twist, a bHLH transcriptional regulator, and Id HLH transcriptional inhibitors, are expressed in pre- and migratory neural crest cells. Twist is activated by many of the neural crest specifier genes, but has not been shown to cross-regulate with them. Rather, Twist appears necessary for the differentiation of specific crest-derived pharyngeal arch structures and thus likely operates between neural crest specifiers and differentiation effector genes. The regulatory relationship of Id genes to any other neural crest gene is unknown. It is

suspected that the function of Id at the neural plate border and in neural crest cells may be suppression of pro-neural bHLHs and/or regulation of Twist.

Neural crest specifiers down-regulate in post-migratory neural crest

After neural crest cells have reached their destinations and begun to differentiate, expression of AP-2, Slug/Snail, FoxD3, and Id is lost. What triggers this cessation of neural crest gene expression, multipotency, and migratory capacity is unknown. It is possible that downregulation of Slug/Snail allows repression of the other neural crest specifiers. Loss of the neural crest specifier expression then results in the abolishment of migratory ability and multipotency. Similarly, loss of Id expression may release bHLH transcription factors from inhibition, permitting differentiation into neural and mesodermal-type derivatives.

The neural crest specifiers Sox9 and Sox10 have later roles in neural crest differentiation

Though the genetic switches that cause neural crest cells to stop migrating and lose their multipotency are unknown, the molecular mechanisms actively driving differentiation of post-migratory neural crest are slightly less mysterious. It has been shown that Sox9 and Sox10, unlike the other neural crest specifiers, persist in post-migratory neural crest and directly regulate differentiation. Sox9 directly and positively regulates the expression of collagen in neural crest derived cartilage while Sox10 and Pax3 cooperate to turn on cRet in enteric neurons by binding cRet enhancer elements (Table 4). Sox10 also activates a battery of genes involved in pigment formation in

melanogenic neural crest including Trp2, Mitf, ckit (Table 4). Interestingly, expression of Sox9 during and after migration is restricted to cranial neural crest, while Sox10 is maintained in trunk crest. This difference in later SoxE gene expression may partly explain the differing potential of cranial and trunk neural crest to generate mesodermal-type derivatives.

Overview of the neural crest gene network

The genetic interactions reviewed above and in Tables 1-4 are synthesized diagramatically in Figure 1A. The neural crest regulatory state is initiated by Wnt and attenuated BMP signals, and requires Fgf. These inductive signals activate "neural plate border specifiers" (Zic, Pax3/7, and Msx2) as well as "neural crest specifiers" (Slug/Snail, FoxD3, Sox9, Sox10, and AP-2). Zic factors are sufficient for the expression of Slug/Snail and FoxD3 while Pax3/7 is required for Slug/Snail and Sox10expression— suggesting neural plate border specifiers act upstream of neural crest specifiers. The neural crest specifiers cross- and auto-regulate until neural crest cells begin differentiating. Little is known about the downstream targets of neural crest specifiers, or the mechanisms that quash their expression and cue crest cells to stop migrating and lose their multipotency. The neural crest specifiers Sox9, and Sox10 have later roles in post-migratory neural crest where they are direct regulators of cartilage, melanocyte, and neural differentiation.

Gene expression in amphioxus implies genetic cascades in the epidermis and neural plate are conserved with vertebrates

At the gene regulatory level, neural crest evolution is the sum of the molecular alterations that drove the gradual assembly of the neural crest gene network outlined above. In this thesis, I have begun to define some of these novel regulatory interactions using amphioxus as a living approximation of the vertebrate ancestor. Amphioxus is particularly well suited for this task because— despite its own long evolutionary history— it likely retains many features shared with the ancestral pre-vertebrate chordate. The core findings of this thesis are included in Figure 1, in which the network of gene regulatory interactions present at the vertebrate neural plate border (Figure 1A) is compared to a hypothetical regulatory network inferred from amphioxus embryonic gene expression patterns (Figure 1B). Except for Fgfs, the expression pattern of every signaling molecule and transcription factor depicted in vertebrate network diagram have been described in amphioxus. Genes and interactions elucidated in this thesis are indicated with asterisks in Figure 1B. It should be noted that except for the ability of amphioxus Snail and amphi Neurogenin to interact with appropriate downstream targets in vertebrate assay systems, none of the relationships in Figure 1B have been tested by perturbation. Thus, the network is necessarily hypothetical, though broadly supported by homologous vertebrate gene expression data. Recent development of a technique for injecting anti-sense morpholinos (Jr Kai Yu, personal communication) should allow future experimental verification of key nodes in the proposed amphioxus ectodermal gene network.

Overall, the major genetic regulatory and signaling pathways of vertebrate neural induction appear to be utilized in amphioxus. Consistent with conserved roles in patterning the early ectoderm, amphioxus BMPs and Wnts are expressed in a pattern

similar to their vertebrate homologs (Panopoulou et al., 1998; Schubert et al., 2001). In the vertebrate epidermal lineage, high levels of BMP signaling induce expression of Dlx, AP-2 and Msx1. These genes act directly upstream of epidermal effectors such as keratin. Consistent with conservation of this genetic cascade, BMP2/4, Dlx, and AP-2 are all expressed in presumptive epidermis of amphioxus (Holland et al., 1996; Meulemans and Bronner-Fraser, 2002; Panopoulou et al., 1998). In the developing vertebrate neural plate, low levels of BMP signal induce expression of SoxB and proneural bHLH genes. These, in turn, activate a battery of neural differentiation and patterning genes including neuro-tubulins, Islet, and Hu/Elav. Homologs of all these factors have been described in amphioxus and are temporally and spatially deployed in a manner identical to their vertebrate counterparts (Holland et al., 2000; Jackman et al., 1997; Satoh et al., 2001; Yasui et al., 1998a).

Amphioxus and vertebrates utilize some genes differently at the neural plate border, highlighting potential evolutionary novelties

In cells at the vertebrate neural plate border, intermediate levels of BMP signal, together with Wnts and Fgfs, induce expression of early patterning genes such as Pax3/7, Msx2, and Zic. All three of these factors are present at the amphioxus neural plate border, suggesting conservation of these initial steps of neural plate border specification (Gostling and Shimeld, 2003; Holland et al., 1999; Sharman et al., 1999). As neurulation procedes in vertebrates these neural plate border specifiers (Pax3/7 and Zic) and early inductive signals (BMP, Wnt), activate a suite of transcriptional regulators which specify the neural crest fate (AP-2, Slug/Snail, FoxD3, Sox9, Sox10, Twist, Id). Amphioxus

appears to lack this network of interacting transcription factors. Thus, while early induction signals likely activate expression of the neural plate border specifiers Pax3/7, Msx, and Zic; expression of the neural crest specifiers AP-2, Sox9, Sox10, FoxD3 (Yu et al., 2002), Id, and Twist (Yasui et al., 1998b) is not observed at the amphioxus neural plate border. These differences suggest regulation of neural crest specifiers (besides Slug/Snail) by neural plate border specifiers (Pax3/7 and Zic) and early inductive signals (Wnt, BMP) is a vertebrate novelty. Furthermore, while functional studies in this thesis suggest that amphioxus Snail represses the same unknown repressor as its vertebrate homologs; this repression necesarily does not effect expression of neural crest genes in the amphioxus embryo— as none are coexpressed with Snail at the neural plate border. Thus, the relationship between Snail and its immediate downstream target appears conserved, while the interactions between this unknown repressor and neural crest genes is unique to vertebrate embryos. Taken together, these studies provide a rich observational foundation upon which tesTable hypotheses regarding the gene regulatory evolution of the chordate neural plate border can be constructed.

Testing conserved and divergent elements of the amphioxus and vertebrate neural plate border gene networks

A critical node amenable to empirical anaylsis is the response of amphioxus neural crest gene homologs to Wnt and BMP inductive signals. In vertebrate embryos perturbation of Wnt and BMP signaling leads to the appropriate expansion or collapse of the neural plate border/neural crest domain as assayed by marker gene expression. Thus, inhibition of BMP signaling using chordin or ectopic Wnt causes an increase of Pax3/7,

Slug, AP-2, and FoxD3 expression, while inhibition of Wnt signaling using dominant negative Wnts or GSK reduces the expression of Slug, FoxD3, AP-2 and Sox9. The hypothetical network described above predicts a vertebrate-like response to inductive signal perturbation for amphioxus factors such as Pax3/7, Msx, Zic and Snail. Specifically, one would expect that ectopic expression of Wnts and chordins in amphioxus embryos would expand expression of these markers, while dominant negative Wnt or GSK would reduce expression. Homologs of other neural crest specifiers, which are not expressed at the amphioxus neural plate border (AP-2, FoxD3, SoxE, Twist) should not be effected by such manipulations. The results would support the assertion that the responsiveness of neural crest specifiers to Wnt and BMP signals is a vertebrate novelty associated with neural crest evolution.

The amphioxus network model could also be tested at lower levels. Zic factors are sufficient to induce expression of FoxD3 and Snail/Slug in frog, and Pax3/7 genes are required for Slug/Snail , FoxD3, and Sox10 expression. These “neural plate border specifiers” may be key mediators of the Wnt/BMP induced expression of the “neural crest specifier” genes. In amphioxus, only Snail is coexpressed with Zic and Pax3/7 at the neural plate border (Langeland et al., 1997)— suggesting this relationship between neural plate border specifiers and neural crest specifiers is unique to vertebrates. If this is indeed the case, suppression of amphioxus Pax3/7 expression with morpholinos should interfere with amphioxus Snail expression, but not effect amphioxus SoxE or FoxD. Similarly, ectopic expression of amphioxus Zic may be expected to expand the Snail expression domain but not effect FoxD; demonstrating that the interaction of Zic and FoxD at the neural plate border is unique to vertebrates. Alternately, Zic overexpression

could expand FoxD and Snail expression in presomitic mesoderm, as all three factors are coexpressed in these cells. Conservation of this relationship would suggest that a network of factors including Snail, FoxD, and Zic was coopted to the neural plate border from the presomitic mesoderm during the course of neural crest evolution.

AP-2 genes are required for both epidermis formation and neural crest induction in vertebrate embryos. Perturbation of AP-2 activity in frog using antisense oligonucleotides results in the inhibition of Dlx5, Msx1, and epidermal keratin as well as the repression of Sox9 and Slug in neural crest cells (Luo et al., 2003; Luo et al., 2002). The expression of amphioxus AP-2 in non-neural ectoderm but not in neural plate border cells suggests a conserved role for AP-2 in epidermis and a novel role for AP-2 in neural crest. Both of these assertions are testable using morpholinos to inhibit AP-2 translation in amphioxus embryos. The expected outcome would be a vertebrate-like reduction in Distalless and keratin expression, and unaltered (or expanded) expression of Snail and SoxE; both of which mark the neural plate in amphioxus and are not coexpressed with AP-2.

Differences at the lowest levels of the vertebrate and amphioxus neural plate border network models imply specific evolutionary changes altered the ability of these cells to differentiate. Functional definition of these changes can be accomplished by comparing the results of similar loss- and gain- of-function experiments in amphioxus and vertebrate embryos. Some interactions of particular evolutionary interest are the direct and positive regulation of neural crest differentiation genes by SoxE family members. In migrating trunk neural crest Sox10 and Pax3 cooperatively bind the a cRet enhancer to activate its expression in developing enteric neurons. In chondrogenic

cranial neural crest, Sox9 drives expression of collagen by direct binding of enhancer elements. Amphioxus SoxE is not expressed at the neural plate border. However, expression of amphioxus SoxE in the pharyngeal gill bars and CNS implies that cooption of SoxE genes by neural crest cells conferred upon them novel chondrogenic and/or neural properties. This scenario predicts that regulatory relationships between SoxE genes, collagen, and cRet are conserved in amphioxus chondrogenic and neural cell types. Thus, as in vertebrates, morpholino-mediated suppression of SoxE activity should disrupt collagen expression and gill bar formation in amphioxus. Furthermore, functionally relevant SoxE binding sites should be present in the amphioxus collagen promoter. Another testable prediction is that amphioxus cRet is expressed in the neural tube and that this expression in SoxE-dependent. Again, morpholino knock-down should interfere with cRet expression, and the amphioxus cRet promoter should contain functional SoxE binding sites.

Deeper comparisons of amphioxus and vertebrate neural plate border gene networks beyond simple gain-of-function and loss-of-function perturbations will become realistic as more is known about the *cis*-regulation of vertebrate neural crest genes. Critical questions to be answered are; 1) What are the specific *cis*-regulatory DNAs governing the relationships between early inductive signals and vertebrate neural plate border genes? 2) Are these elements conserved in amphioxus? 3) What are the elements controlling the response of neural crest specifiers to the inductive signals and neural plate border specifiers? 4) Which of these are conserved or absent in amphioxus? 5) What *cis* regulatory DNAs mediate the cross-regulation of the neural crest specifiers? 6) Which are conserved and absent in amphioxus? 7) What are the downstream targets of the

neural crest specifiers, and the *cis*-regulatory DNAs mediating their influence? 8) Are these regulatory relationships and *cis*-acting DNAs conserved in amphioxus?

The unique ontogeny, morphology, behavior, and gene expression profile of neural crest cells demonstrate that they are a true vertebrate novelty. Critical to understanding the evolutionary origins of these cells is elucidation of related developmental mechanisms operating in closely related invertebrate chordates such as amphioxus. Essential first steps towards this end are thorough and thoughtful descriptions of salient gene expression patterns. As techniques for manipulating amphioxus embryos evolve, such descriptive work will provide an indispensible foundation for rigorous empirical analyses.

Signaling Pathway →	**Downstream Targets** (grouped according to relationship with sig. pathway)				
	Necessary	Sufficient naïve ect.	Sufficient in embryo	Direct regulatory interaction	Ref.
BMP (low)	SoxB, Zicr-1,Zic3	SoxB, Zicr-1, Zic3	SoxB, Zicr-1, Zic3	?	(Mizuseki et al., 1998; Nakata et al., 1997)
BMP (mid)	Slug/Snail AP-2 Sox9 FoxD3	No for Slug	?	?	(LaBonne and Bronner-Fraser, 1998; Luo et al., 2003; Sasai et al., 2001)
BMP (high)	Msx1 Dlx AP-2(low)	Msx1 Dlx AP2(low)	Msx1 Dlx AP2(low)	Msx1	(Feledy et al., 1999)
Fgf	Slug	No	Msx1	?	(LaBonne and Bronner-Fraser, 1998; Streit and Stern, 1999)
Wnt	Slug AP-2 Pax3 FoxD3 Slug/Snail Msx2 Sox9 Twist	No for all neural crest genes	Slug AP-2 Pax3 FoxD3 Slug/Snail Msx2 Sox9 Twist	Slug	(Bang et al., 1999; Garcia-Castro et al., 2002; LaBonne and Bronner-Fraser, 1998; Luo et al., 2003; Saint-Jeannet et al., 1997; Sasai et al., 2001; Vallin et al., 2001)

Table 1. Catalog of signaling pathways (leftmost column) and their proven downstream targets (middle 4 columns) in vertebrate embryonic ectoderm. A signal is deemed necessary for expression of a given target if its perturbation disrupts target expression in the manipulated embryo. Similarly, a signaling pathway is sufficient if excess signal causes ectopic esxpression of the target in the embryo. "Sufficient naïve ect." is a more rigorous definition of sufficiency and indicates that a signal can induce target expression in an isolated, and putatively naïve, ectodermal explant— in this case a cultured *Xenopus* animal cap.

Trans. Factors →	**Downstream Targets** (grouped according to relationship with trans. factor)				
	Necessary	Sufficient naïve ect.	Sufficient in embryo	Direct regulatory interaction	Ref.
SoxB	N-CAM N-tub neurogenin	No	No	?	(Kishi et al., 2000)
Pro-neural bHLHs	Neurofilament N-tubulin N-CAM	Neurofilament N-tubulin N-CAM	Neurofilament N-tubulin N-CAM	?	(Ferreiro et al., 1994; Korzh and Strahle, 2002; Lee et al., 1995; Perez et al., 1999)
Dlx3/5	Repression of: Sox2 Zic3 N-tubulin	?	Repression of: Sox2 Zic3 N-tubulin	?	(Feledy et al., 1999; McLarren et al., 2003; Yang et al., 1998)
Msx-1	?	keratin	keratin Repression of: Zic3 N-CAM	?	(Feledy et al., 1999; Suzuki et al., 1997)

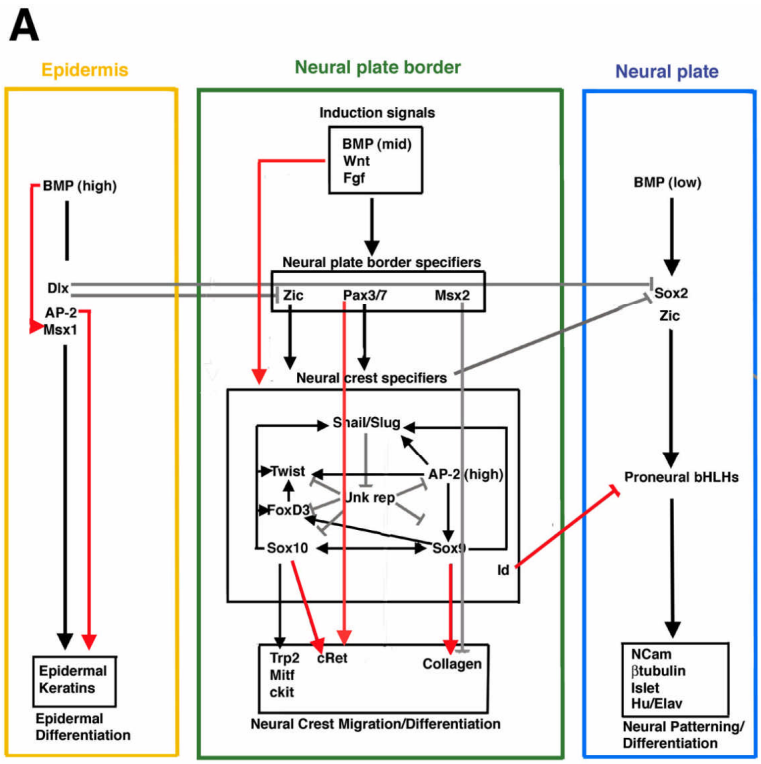
Table 2. Catalog of major transcriptional regulators (leftmost column) and their proven downstream targets (middle 4 columns) in the vertebrate neural plate and epidermis. A factor is deemed necessary for expression of a given target if its perturbation disrupts target expression in the manipulated embryo. Similarly, factor is sufficient if excess signal causes ectopic esxpression of the target in the embryo. "Sufficient naïve ect." is a more rigorous definition of sufficiency and indicates that a factor can induce target expression in an isolated, and putatively naïve, ectodermal explant— in this case a cultured *Xenopus* animal cap.

Trans. Factors →	**Downstream Targets** (grouped according to relationship with trans. factor)				
	Necessary	Sufficient naïve ect.	Sufficient in embryo	Direct regulatory interaction	Ref.
Pax3/7	Sox10 Slug/Snail FoxD3 cRet	?	?	cRet	(Dottori et al., 2001; Lang and Epstein, 2003) (M.Garcia-Castro, P.C.)
Zic5/Zicr-1 /Zic3	?	Slug/Snail FoxD3 Twist N-CAM N-tub Neurogenin Neuro-D	Slug/Snail FoxD3 Twist N-CAM N-tub Neurogenin Neuro-D	?	(Brewster et al., 1998; Mizuseki et al., 1998; Nakata et al., 2000; Sasai et al., 2001)
Msx-2	?	?	Inhibits collagen expression	?	(Takahashi et al., 2001)

Table 3. Catalog of "neural plate border specifiers" (leftmost column) and their proven downstream targets (middle 4 columns) in vertebrate neurectoderm. A factor is deemed necessary for expression of a given target if its perturbation disrupts target expression in the manipulated embryo. Similarly, factor is sufficient if excess signal causes ectopic esxpression of the target in the embryo. "Sufficient naïve ect." is a more rigorous definition of sufficiency and indicates that a factor can induce target expression in an isolated, and putatively naïve, ectodermal explant— in this case a cultured *Xenopus* animal cap.

Trans. Factors →	**Downstream Targets** (grouped according to relationship with trans. factor)				
	Necessary	Sufficient naïve ect.	Sufficient in embryo	Direct regulatory interaction	Ref.
Slug/Snail	Twist Sox10 FoxD3	Twist Zic5	Twist Sox10 Sox9 Zic5/Zicr-1 AP-2 FoxD3	No, via anti-repression	(Aybar et al., 2003) (Aoki et al., 2003; del Barrio and Nieto, 2002; Sasai et al., 2001)
AP-2 **AP-2(low)**	Slug Sox9 Sox2 (repression) keratin	?	Slug Sox9 Sox2 (repression) keratin	 keratin	(Luo et al., 2003; Luo et al., 2002) (Snape et al., 1991)
FoxD3	Slug Twist	Slug Twist Zic2 Zicr-1 Sox2 NCAM Neurogenin	Slug AP-2 Sox2 (repression)	?	(Sasai et al., 2001)
Sox9	Twist Snail Msx-2 Pax3 FoxD3 Sox10	No	No for neural crest markers Sox2 (repression)	TypeII Collagen (activation)	(Ng et al., 1997; Spokony et al., 2002)
Sox10	Slug/Snail Sox9 FoxD3 Twist cRet Mitf Trp cKit	Trp	Slug Sox9 Trp Sox2 (repression)	cRet (activation)	(Honore et al., 2003; Kim et al., 2003; Lang and Epstein, 2003; Potterf et al., 2000)
Twist	1st arch bone/muscle	?	?	?	(Soo et al., 2002)
Id	?	?	?	myogenic/ proneural bHLH (repression)	(Jogi et al., 2002; Langlands et al., 1997)

Table 4. Catalog of "neural crest specifiers" (leftmost column) and their downstream targets (middle 4 columns) in neural crest cells. A factor is deemed necessary for expression of a given target if its perturbation disrupts target expression in the manipulated embryo. Similarly, factor is sufficient if excess signal causes ectopic esxpression of the target in the embryo. "Sufficient naïve ect." is a more rigorous definition of sufficiency and indicates that a factor can induce target expression in an isolated, and putatively naïve, ectodermal explant— in this case a cultured *Xenopus* animal cap.


A
Epidermis
Neural plate border
Neural plate
Induction signals
BMP (mid)
Wnt
Fgf
BMP (high)
BMP (low)
Neural plate border specifiers
Dlx
Zic
Pax3/7
Msx2
Sox2
Zic
AP-2
Msx1
Neural crest specifiers
Snail/Slug
Twist
AP-2 (high)
Unk rep
FoxD3
Sox10
Sox9
Id
Proneural bHLHs
Trp2
Mitf
ckit
cRet
Collagen
Epidermal
Keratins
Epidermal
Differentiation
Neural Crest Migration/Differentiation
NCam
βtubulin
Islet
Hu/Elav
Neural Patterning/
Differentiation

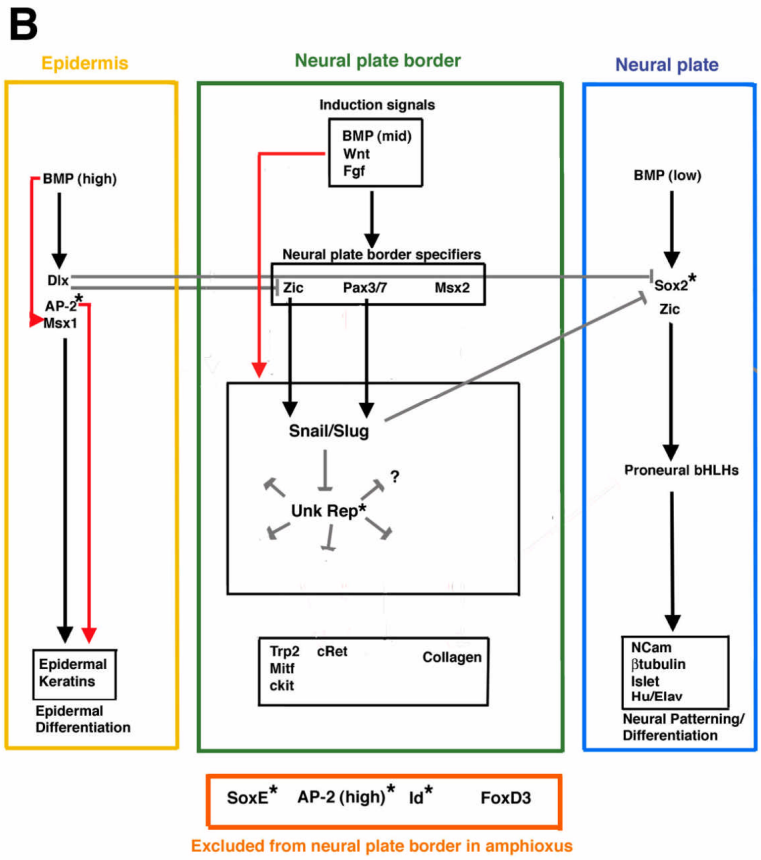

B
Epidermis
Neural plate border
Neural plate
Induction signals
BMP (mid)
Wnt
Fgf
BMP (high)
BMP (low)
Neural plate border specifiers
Dlx
Zic
Pax3/7
Msx2
Sox2*
Zic
AP-2*
Msx1
Snail/Slug
Unk Rep*
?
Proneural bHLHs
Trp2
Mitf
ckit
cRet
Collagen
Epidermal
Keratins
Epidermal
Differentiation
NCam
βtubulin
Islet
Hu/Elav
Neural Patterning/
Differentiation
SoxE*
AP-2 (high)*
Id*
FoxD3
Excluded from neural plate border in amphioxus

Figure 1. Comparison of putative gene regulatory and signaling interactions operating at the neural plate border of vertebrates and amphioxus. Red arrows indicate proven direct regulatory interactions. Black arrows are genetic interactions suggested by gain and loss of function analyses largely in *Xenopus*. Gray lines indicate repression. (A) In vertebrates, dorsal ectoderm is segregated into presumptive epidermal, neural crest, and neural plate domains by distinct, but interacting genetic cascades. The epidermal fate is specified early by high levels of BMP signaling which act through a battery of transcription factors to turn on epidermis-specific effector genes such as keratin. In the neural plate, low levels of BMPs, as well as inductive signals from underlying mesoderm, lead to the expression of Zic and Sox group B genes, proneural bHLH transcription factors, and neural-specific effectors. At the in the neural plate border, intermediate levels of BMPs, as well as Wnt, and Fgf signals induce expression of neural plate border and neural crest cell specifiers. Gene regulatory cross-talk between neural crest genes maintains their expression until migration and differentiation, when neural crest effector genes are expressed. (B) The *hypothetical* network operating at the neural plate border of amphioxus based on embryonic gene expression data. Except for Fgfs, the expression pattern of every signaling molecule and transcription factor depicted in vertebrate network diagram have been described in amphioxus. While the essential structure of the epidermal and neural regulatory cascades appears conserved in amphioxus and vertebrates, only the earliest steps in neural plate border pathway appear conserved between the two subphyla. Amphioxus lacks a vertebrate-like neural plate border gene network below the level of Snail repression. Asterisks indicate contributions of this thesis work to the amphioxus network model.

Appendix 1:

Independent Duplication and Subfunctionalization of Amphioxus SoxB Genes

ABSTRACT

In a screen for SoxE genes, I isolated two novel SoxB genes from amphioxus, amphioxus SoxB1, and amphioxus SoxB2. Phylogenetic analysis including Drosophila and vertebrate SoxB sequences groups each gene within their respective SoxB subfamilies at high confidence values. Furthermore, like their vertebrate homologs, amphioxus SoxB1 and SoxB2 are expressed throughout the embryonic CNS and foregut. However, unlike vertebrate SoxB genes, neither amphioxus gene is expressed in the PNS— suggesting deployment of SoxB genes in peripheral sensory elements is a vertebrate innovation related to the evolution of epidermal placodes. Finally, I show that the combined expression profiles of amphioxus SoxB1 and AmphiSox1/2/3 (Holland et al., 2000) mimics the composite expression of the vertebrate SoxB1 paralogs— indicating that duplication and subfunctionalization of SoxB1 genes occurred independently in both lineages.

INTRODUCTION

In vertebrate embryos, the presumptive nervous system is partitioned from the non-neural ectoderm around the time of gastrulation. During this period, signals from the underlying mesoderm and adjoining ectoderm induce expression of neural-specific genes in a broad domain of dorsal ectoderm (reviewed by (Bally-Cuif and Hammerschmidt, 2003)). One of the earliest genes expressed in response to these inductive signals is

Sox2 (Mizuseki et al., 1998), a Sox group B1 transcription factor (Bowles et al., 2000). Sox2 is necessary for the formation of neural derivatives in the Xenopus embryo (Kishi et al., 2000) and is sufficient to neuralize naïve ectoderm in the presence of FGF signals (Mizuseki et al., 1998). Expression of Sox2 orthologs and paralogs in the neural plate and neural tube of various vertebrates suggest a highly conserved role for SoxB1 genes in CNS formation (Penzel et al., 1997; Rex et al., 1997a; Uwanogho et al., 1995; Wood and Episkopou, 1999). This conservation extends beyond the vertebrates as an amphioxus SoxB1 gene, AmphiSox1/2/3, marks the neural plate(Holland et al., 2000), and the Drosophila SoxB1 homolog, SoxNeuro, is necessary for CNS formation (Buescher et al., 2002). In vertebrates, SoxB1 genes also play a role in the formation of some PNS elements. The Sox2 paralog, Sox3, is expressed in sensory placodes (Abu-Elmagd et al., 2001; Groves and Bronner-Fraser, 2000; Ishii et al., 2001) and is sufficient to induce ectopic placodes in non-neural ectoderm (Koster et al., 2000). Furthermore, all three SoxB1 paralogs (Sox1, 2 and 3) function during lens induction in the chicken (Kamachi et al., 1998). Outside neural tissues, vertebrate SoxB1 genes are also expressed in the anterior gut, though their precise function in this tissue is unknown (Chalmers et al., 2000; Ishii et al., 1998).

Like most HMG-box transcription factors, SoxB1 proteins act as transcriptional activators. Evidence from trans-activation assays suggests this positive regulatory influence is attenuated by the repressing activity of the closely related SoxB2 genes, Sox14 and Sox21 (Uchikawa et al., 1999). Consistent with this, SoxB2 genes are coexpressed with SoxB1 genes in many neural tissues (Rex et al., 1997b; Rimini et al., 1999; Uchikawa et al., 1999). It is likely that a balance between the activating and

repressing activities of the different SoxB family members controls their ultimate effects during development.

In a screen for SoxE genes, I isolated two novel SoxB family members from amphioxus. Amphioxus SoxB1 groups with vertebrate and Drosophila SoxB1 homologs and is more similar to these genes than the previously described AmphiSox1/2/3. Amphioxus SoxB2 groups within the SoxB2 clade. Like vertebrate SoxB genes, both amphioxus SoxB1 and SoxB2 are expressed in the developing CNS. Like vertebrate Sox2, amphioxus SoxB1 is also expressed in the foregut, a domain where AmphiSox1/2/3 is absent; implying duplication and subfunctionalization of SoxB1 genes in amphioxus. Finally, unlike vertebrate SoxB1 genes, amphioxus SoxB1 neural expression does not extend outside the CNS, suggesting SoxB1 cooption was an important step in the evolution of sensory placodes.

MATERIALS AND METHODS

Amphioxus Collection

Amphioxus adults (*Branchiostoma floridae*) were collected from Tampa Bay, Florida and electrostimulated to induce gamete release. Eggs were fertilized, and embryos were cultured and fixed per the methods of Holland et al. (Holland et al., 1996).

Isolation of amphioxus SoxB genes

The following completely degenerate primers were designed against the conserved HMG box of all known vertebrate Sox group E proteins: SoxE5'2:AAGCCBCAYGTIAARMGNCCIATGAA, SoxE3'2:TAITCIGGGTRRTCYTTYTTRTGYTG.

An approximately 220 bp PCR fragment was amplified from a diluted amphioxus Lambda Zap II embryonic cDNA library kindly provided by Jim Langeland. Nine fragments were sequenced and one was found to code for a putative amphioxus Sox group B2 gene. This fragment was then used to screen the plated library at low stringency (2XSSC/.1%SDS at 40˚C) for full length cDNAs. Fourteen phagemid clones were isolated, excised, partially sequenced, and found to encode two SoxB genes. The largest cDNAs of each were completely sequenced from both ends.

Phylogenetic analysis

Full-length cDNAs were translated and their conceptual protein products were aligned to published Drosophila and vertebrate Sox group B sequences. A bootstrapped Neighbor-Joining tree (Saitou and Nei, 1987) was then constructed using the ClustalX program (Thompson et al., 1997). The related Sox group F gene, Human Sox17, was included as an outgroup.

In situ Hybridization

In situ hybridizations were as described previously (Meulemans and Bronner-Fraser, 2002). Riboprobes were made against the entire transcript.

RESULTS

Isolation of amphioxus SoxB genes

Completely degenerate primers were designed to recognize all published vertebrate Sox group E gene sequences. Out of 9 resulting PCR fragments, 8 were identical and corresponded to the same Sox group E protein. The remaining fragment showed high sequence similarity to vertebrate Sox group B2 genes. This fragment was used to screen the same library for full length clones at low stringency. Two Sox group B genes were identified, one corresponding to the original SoxB2 fragment and the other to a novel SoxB1 gene. Full coding sequences were translated, aligned, and used to construct a phylogenetic tree with vertebrate, Drosophila and amphioxus SoxB protein sequences. Both amphioxus SoxB genes, named amphioxus SoxB1 and amphioxus SoxB2, grouped within their respective SoxB subfamilies at high bootstrap values (Fig. 1). The previously described AmphiSox1/2/3 falls as an outgroup to Drosophila, amphioxus, and vertebrate SoxB1 genes, suggesting it arose by duplication of an ancestral amphioxus SoxB1 gene and diverged rapidly.

Embryonic and larval expression of amphioxus SoxB1

At the earliest stage examined, the 9-hour early neurula, amphioxus SoxB1 transcripts are detected in a patch of dorsal ectoderm near the blastopore (Fig. 2A, arrow), and in some ventral anterior mesendoderm cells (Fig. 2A, arrowhead). As the ectoderm closes over the neural plate at 12 hours, a new patch of expression is seen in the anterior neural plate (Fig. 2B, arrow), while staining in the posterior neural plate (Fig. 2B, arrowhead) and ventral mesendoderm (Fig. 2C, arrow) persist. In the 18-hour larva, neurulation is almost complete and SoxB1 expression has expanded throughout the neural tube (Fig. 2D, double arrowheads, Fig. 2G, arrow). Expression in the anterior gut has also increased (Fig. 2D, single arrowhead, Fig. 2G arrowhead). In 24-hour larvae expression in the anterior gut and neural tube is much the same as at 18 hours (Fig. 2E). Sometime between 24 and 48 hours, SoxB1 expression is extinguished throughout most of the larva (data not shown). At 3 days, transcripts appear again around edge of the mouth (Fig. 2F, arrow).

Embryonic expression of amphioxus SoxB2

Amphioxus SoxB2 expression is observed at 9 hours in a small patch of dorsal ectoderm near the blastopore (Fig. 3A, B, arrows). At 12 hours, this neurectodermal expression has expanded rostrally and includes the posterior half of the neural plate (Fig. 3C, D, arrows). From 12 to 15 hours, expression of SoxB2 expands throughout the neural tube (Fig. 3E, arrow) while weaker staining appears in the gut (Fig. 3E,

arrowhead). An optical cross section shows strong expression in the neural tube (Fig. 3F, arrow) and the weaker gut staining (Fig. 3F, arrowhead). Similar expression is seen in 18-hour larvae (not shown). By 24 hours, detectable expression of amphioxus SoxB2 has ceased.

DISCUSSION

SoxB genes in amphioxus

In a low-stringency screen for SoxE genes, I isolated two amphioxus SoxB genes, amphioxus SoxB1 and amphioxus SoxB2. Together with their vertebrate homologs, both form well-supported clades with their Drosophila orthologs acting as outgroups. Unexpectedly, the previously reported AmphiSox1/2/3 (Holland et al., 2000) is not included within the amphioxus plus vertebrate clade and falls outside of a clade including amphioxus, Drosophila, and vertebrate SoxB1 genes. Despite this, AmphiSox1/2/3 is likely a SoxB1 gene as it shows highest similarity to SoxB1s when human Sox17 is used as an outgroup. Thus, AmphiSox1/2/3 probably represents a divergent SoxB1 generated by an amphioxus-specific duplication event. Several examples of similar duplications in amphioxus have been reported (reviewed by Minguillon et al., 2002).

Conserved and divergent expression of SoxB genes in amphioxus and vertebrates

SoxB genes are required for earliest steps of CNS formation in vertebrates (Kishi et al., 2000) as well as Drosophila (Buescher et al., 2002; Overton et al., 2002; Soriano and Russell, 1998), suggesting an ancient role for these factors in neurectoderm

specification. Consistent with this, amphioxus SoxB genes are expressed in the embryonic CNS from late gastrula/early neurula stages. In vertebrates, SoxB1 and SoxB2 genes are also deployed in foregut endoderm from early in development (Chalmers et al., 2000; Ishii et al., 1998; Uchikawa et al., 1999; Wood and Episkopou, 1999). Similar expression of both amphioxus SoxB1 and SoxB2 is seen in the foregut, though amphioxus SoxB2 transcripts are also detected in the mid- and hind- gut.

Unlike amphioxus SoxB genes, however, vertebrate SoxB1 genes are expressed in the PNS where they mark the nascent sensory placodes (Abu-Elmagd et al., 2001; Groves and Bronner-Fraser, 2000; Ishii et al., 2001). None of the three described amphioxus SoxB genes are expressed in the PNS; which is thought to have no vertebrate-like placodal tissue. However, expression of Distalless, Msx, and Id gene homologs suggests that some areas of amphioxus dorso-anterior ectoderm may represent the evolutionary precursor to vertebrate placodes (Holland et al., 1996; Sharman et al., 1999) chapter 3 of this thesis). If this is the case, SoxB genes may have been recruited later to the PNS during the evolution of definitive placodes.

Differences in AmphiSox1/2/3 and amphioxus SoxB1 expression demonstrate subfunctionalization of amphioxus-specific paralogs

Based on phylogenetic analysis, both AmphiSox1/2/3 and amphioxus SoxB1 diverged from vertebrate SoxB1 genes before the duplications that generated the three vertebrate paralogs. However, unlike many amphioxus genes, neither exhibits the complete composite expression pattern of its vertebrate homologs. AmphiSox1/2/3 is expressed early throughout the neurectoderm but is downregulated before the end of

neurulation (Holland et al., 2000). In addition, it is never expressed in the foregut endoderm. By contrast, early neurectoderm expression of amphioxus SoxB1 is restricted to small caudal and rostral patches. It is only towards the end of neurulation that amphioxus SoxB1 expands throughout the CNS. Furthermore, like vertebrate SoxB1 genes, amphioxus SoxB1 is expressed in the foregut endoderm. Thus, AmphiSox1/2/3 appears to fulfill the earliest function of SoxB1genes in neurectoderm specification while amphioxus SoxB1 assumes later functions in the neural tube and foregut. This partitioning of SoxB1 functionality may explain the divergence of AmphiSox1/2/3 from other SoxB1 homologs. Domains required for the later neural and endodermal functions may have been conserved in amphioxus SoxB1, but lost in AmphiSox1/2/3, resulting in a more divergent sequence. A similar partitioning of early and late functions between two amphioxus-specific paralogs is seen in MRF genes (Schubert et al., 2003).

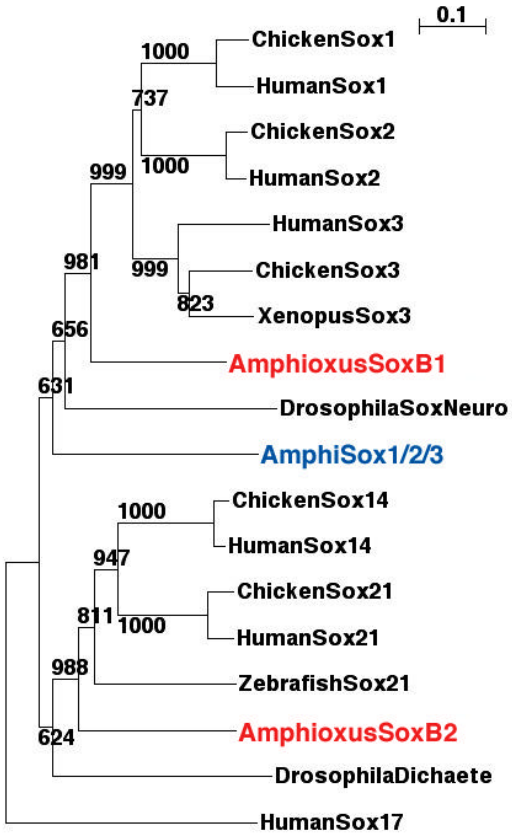


Figure 1. Phylogenetic tree of SoxE proteins created using the Neighbor-Joining method with human Sox17 as the outgroup. Numbers at branch bases are confidence values derived from 1000 bootstrap resamplings of the alignment data. Sequence distance is indicated at the top right as substitutions per base. Amphioxus SoxB1 and amphioxus SoxB2 clearly fall with their respective SoxB subfamilies. In this phylogeny AmphiSox1/2/3 (Holland et al., 2000) falls outside the clade including vertebrate, amphioxus and Drosophila SoxB1 genes, but still shows more affinity for Sox group B1 than Sox group B2. Thus, AmphiSox1/2/3 likely represents a divergent SoxB1 gene.

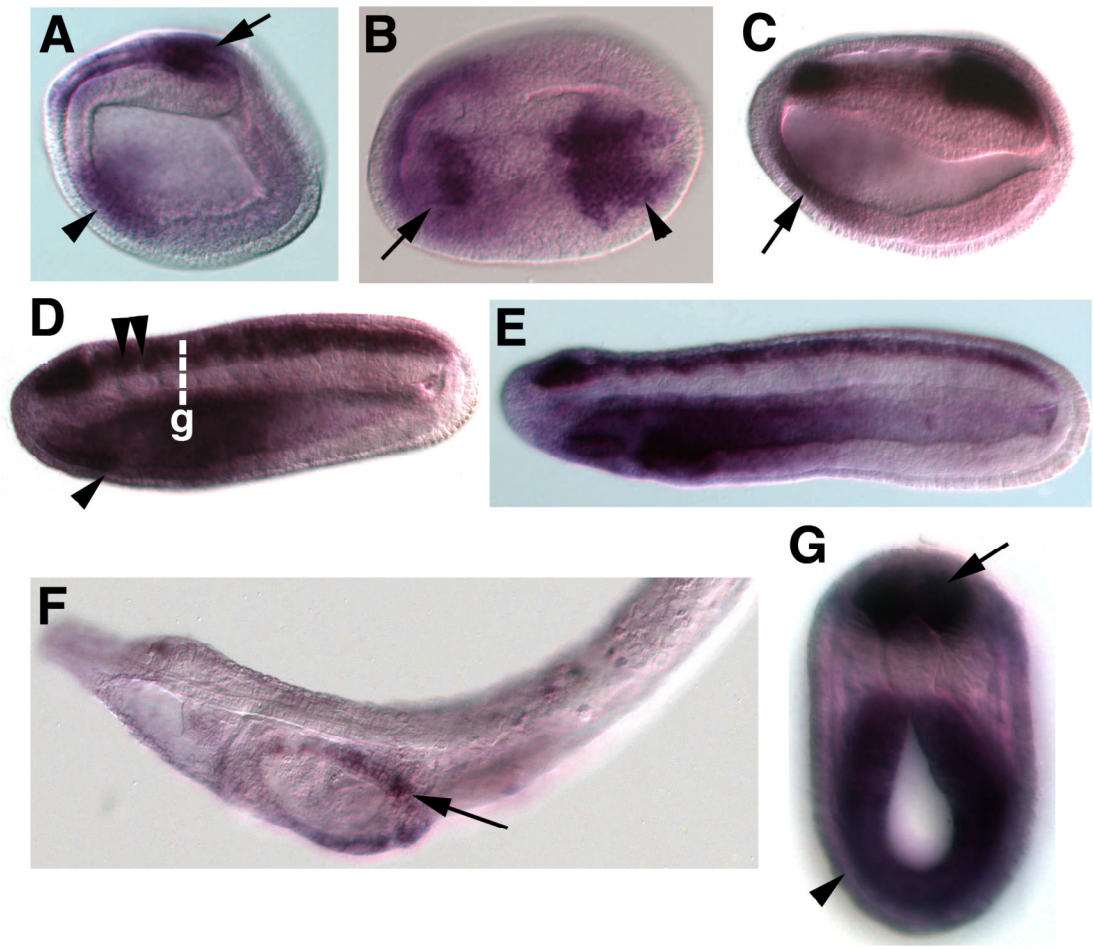


Figure 2. Embryonic and larval expression of amphioxus SoxB1. Unless otherwise indicated, anterior is to the left. (A) Side view of 9-hour early neurula. SoxB1 expression is seen in a patch of neurectoderm near the blastopore (arrow) and in the anterior mesendoderm (arrowhead). (B) Dorsal view of 12-hour neurula, expression in restricted areas of the rostral (arrow) and caudal (arrowhead) neural plate. (C) Side view of 12-hour neurula. SoxB1 transcripts persist in the anterior mesendoderm (arrow). (D) Side view of 18-hour late neurula. SoxB1 expression has expanded throughout the entire neural tube (double arrowheads) and foregut (arrowhead). (E) Side view of 24-hour late neurula. Expression in the neural tube and foregut persists. (F) Side view of a 3-day larva. A new domain of SoxB1 expression appears around the mouth (arrow) while expression in the foregut and neural tube has ceased. (G) Optical cross section through a bissected 18-hour neurula at the level of g in D. Strong expression is seen throughout the dorsoventral extent of the neural tube (arrow) and foregut (arrowhead).

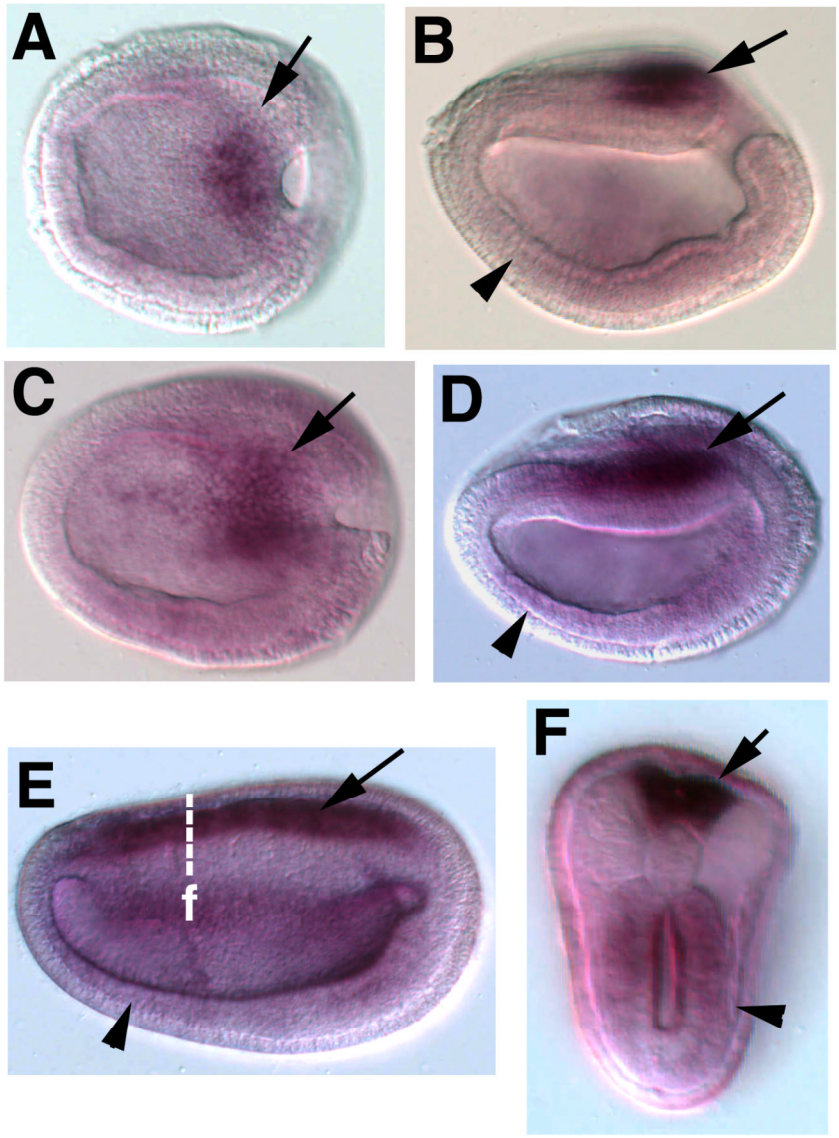


Figure 3. Embryonic expression of amphioxus SoxB2. Where applicable, anterior is to the left. (A) Dorsal view of 9-hour early neurula. SoxB2 transcripts are seen in a small region of neurectoderm bordering the blastopore (arrow). (B) Side view of 9-hour neurula. SoxB2 transcripts are seen in the caudal-most neurectoderm (arrow) and throughout the mesendoderm (arrowhead). (C) Dorsal view of 12-hour neurula. SoxB2 expression has begun to expand into the anterior neural plate (arrow). (D) Side view of 12-hour neurula. Expression of SoxB2 in the neural plate as it is covered by ectoderm (arrow). Weak endodermal expression is also observed (arrowhead). (E) Side view of 15-hour neurula. High levels of SoxB2 transcripts are detected throughout the neural

tube (arrow). Lower levels are seen in the gut (arrowhead). (F) Optical cross section through a bissected 15-hour neurula at the level of f in E. Strong SoxB2 signal is observed in the neural tube (arrow), while lower levels persist in the gut (arrowhead).

Appendix 2:

List of Publications

Epperlein, H. H., Meulemans, D., Bronner-Fraser, M., Steinbeisser, H., and Selleck, M. A. J. (2000). Analysis of cranial neural crest migratory pathways in axolotl using cell markers and transplantation. *Development* **127,** 2751-2761.

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Meulemans, D., McCauley, D., Bronner-Fraser, M. (2003) Id expression in amphioxus and lamprey highlights the role of gene co-option during neural crest evolution. Accepted with revisions to *Developmental Biology*

Cerny, R., Meulemans, D., Berger, J., Wilsch-Bräuninger, M., Kurth, T., Bronner-Fraser, M., and Epperlein, H. H., (2003). Cranial neural crest migration and pharyngeal arch morphogenesis in axolotl. Submitted.

Appendix 3:

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