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Dorsoventral patterning of the vertebrate neural tube is conserved in a protochordate

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SUMMARY

The notochord and dorsal ectoderm induce dorsoventral compartmentalization of the vertebrate neural tube through the differential regulation of genes such as *HNF-3β*, *Pax3*, *Pax6* and *snail*. Here we analyze the expression of *HNF-3β* and *snail* homologues in the ascidian, *Ciona intestinalis*, a member of the subphylum Urochordata, the earliest branch in the chordate phylum. A combination of in situ hybridization and promoter fusion analyses was used to demonstrate that the *Ciona HNF-3β* homologue is expressed in the ventralmost ependymal cells of the neural tube, while the *Ciona snail* homologue is expressed at the junction between the invaginating neuroepithelium and dorsal ectoderm, similar to the patterns seen in vertebrates. These findings provide evidence that dorsoventral compartmentalization of the chordate neural tube is not an innovation of the vertebrates. We propose that precursors of the floor plate and neural crest were present in a common ancestor of both vertebrates and ascidians.

Key words: *Ciona*, notochord, dorsoventral patterning, neural tube, vertebrate, protochordate, *HNF-3β*, *snail*

INTRODUCTION

Kowalevsky (1866) first recognized that ascidians represent a critical evolutionary link between invertebrates and vertebrates. Darwin embraced this idea proposing that the common ancestor of ascidians and vertebrates was an animal very similar to the present-day ascidian tadpole (Darwin, 1871). Ascidians belong to the subphylum Urochordata, which is widely believed to represent the sister group to the cephalochordate/vertebrate clade (Schaeffer, 1987; Turbeville et al., 1994; Wada and Satoh, 1994). They therefore represent the most distant living relatives of the vertebrates. In addition, ascidians are very simple chordates that possess a small genome (only 5% the size of the human genome) and uncomplicated embryonic lineages (Satoh, 1994). In the present study, we analyze gene expression patterns in the ascidian to investigate the evolutionary origins of the vertebrate neural tube.

The two features of the ascidian tadpole that most clearly demonstrate their affinity to vertebrates are the notochord and dorsal neural tube. The notochord is a stiff, rod-like skeletal structure that is used for locomotion (Cloney, 1964). It consists of large vacuolated cells exhibiting the basic 'chordoid' histology typical of vertebrate notochords (Cloney, 1964, 1990; Mancuso and Dolcemascolo, 1977). Recent molecular studies are consistent with the notion that the ascidian notochord is indeed homologous to the vertebrate notochord. For example, the vertebrate *Brachyury* gene encodes a sequence-specific transcriptional activator that is essential for the terminal differentiation of the dorsalmost, axial mesoderm into notochord (reviewed by Herrmann, 1995). The *Brachyury* genes of two distantly related ascidians, *Halocynthia roretzi* and *Ciona intestinalis*, are both expressed in notochord precursor cells at the 64-cell stage of embryogenesis, which coincides with the time of clonal restriction (Yasuo and Satoh, 1994; Corbo et al., 1997).

The CNS of the ascidian tadpole is composed of just ~330 cells (Nicol and Meinertzhagen, 1991; summarized in Fig. 1). It consists of an anterior cerebral vesicle containing sensory structures and motor neurons, and a caudal neural tube extending along the length of the tail (Crowther and Whittaker, 1992). The simple, invariant embryonic cleavage patterns have permitted extensive lineage tracings, thereby facilitating the analysis of gene expression patterns during neurogenesis (Nishida and Satoh, 1983, 1985; Nishida, 1987; Nicol and Meinertzhagen, 1988a,b, 1991).

In cross-section, the *Ciona* neural tube consists of just four ependymal cells that surround a central canal (Nicol and Meinertzhagen, 1988a,b; Fig. 1C,D). Only three of these cells arise from the invaginating neural plate, the ventralmost ependymal cell as well as the two lateral cells (Fig. 1 A,D). The dorsalmost cell of the mature spinal cord is recruited from the dorsal ectoderm during closure of the neural tube (Nicol and Meinertzhagen, 1988a,b).

The simplicity of the *Ciona* CNS raises questions regarding the extent to which it has diverged from the vertebrate CNS. For example, the ependymal cells of the *Ciona* 'spinal cord' do not project axonal processes; so the only axons in the tail derive from neurons contained within the cerebral vesicle (see Fig. 1D). In an effort to compare the ascidian and vertebrate

CNS, we have analyzed the expression of regulatory genes implicated in the dorsoventral patterning of the vertebrate neural tube.

The vertebrate spinal cord exhibits extensive dorsoventral compartmentalization, which is initiated by two non-neural tissues, the notochord and the dorsal ectoderm (Tanabe and Jessell, 1996). The specification of ventral cell types, especially the floorplate and motor neurons, is controlled by signals emanating from the underlying notochord. The notochord is a source of Sonic hedgehog (Shh), which triggers the ventralmost regions of the neural tube to express floor plate markers, including the winged-helix transcription factor, *HNF-3β* (Sasaki and Hogan, 1994; Ruiz i Altaba et al., 1995a,b).

The notochord also triggers the repression of various regulatory genes, such as *Pax3*, *Pax6* and *Pax7*, in ventral regions of the neural tube (Goulding et al., 1993; Liem et al., 1995). For example, *Pax3* is initially expressed at all mediolateral positions in the caudal neural plate (Goulding et al., 1993; Tanabe and Jessell, 1996), but is progressively repressed in the presumptive floorplate. This repression ultimately restricts its expression to the dorsal half of the neural tube.

Dorsal ectoderm, through the expression of bone morphogenetic proteins (BMPs), activates the expression of a number of genes in the lateral border of the neural plate including a homologue of the *Drosophila* gene *snail* (Dickinson, et al, 1995). The *Pax* genes and *snail* homologues expressed in dorsal regions of the neural tube appear to be important for the development of dorsal tissues such as the neural crest (Epstein et al., 1991; Mansouri et al., 1996; Nieto et al., 1994). Thus, signals emanating from the notochord and dorsal ectoderm lead to the dorsoventral compartmentalization of the vertebrate neural tube by inducing the localized expression of a number of regulatory genes such as *HNF-3β*, *Pax3*, *Pax6* and *snail*.

To determine whether similar gene networks govern the compartmentalization of the ascidian neural tube, we have characterized *Ciona* homologues of *HNF-3β* (*fork head*) and *snail*. The *Ciona fork head* gene (*Ci-fkh*) is initially expressed in a broad, dynamic pattern that encompasses the presumptive CNS, notochord and gut. Expression becomes progressively restricted to notochord precursor cells during gastrulation. It is subsequently reactivated in the ventralmost ependymal cell of the neural tube and the endodermal strand underlying the notochord during neurulation.

The *Ciona snail* (*Ci-sna*) gene is specifically expressed in muscle and trunk mesenchyme precursors, as well as cells of the lateral neural plate border. *Ci-sna* expression is lost from the descendants of these cells by the onset of neurulation. However, staining was visualized in the neural tube through the analysis of *Ci-sna*/GFP (green fluorescent protein) and β-galactosidase fusion genes. These studies establish that *Ci-fkh* and *Ci-sna* are expressed in the ventral and lateral ependymal cells, respectively, of the CNS. These patterns are comparable to those seen in vertebrates, suggesting that dorsoventral compartmentalization of the neural tube arose prior to the divergence of ascidians and vertebrates.

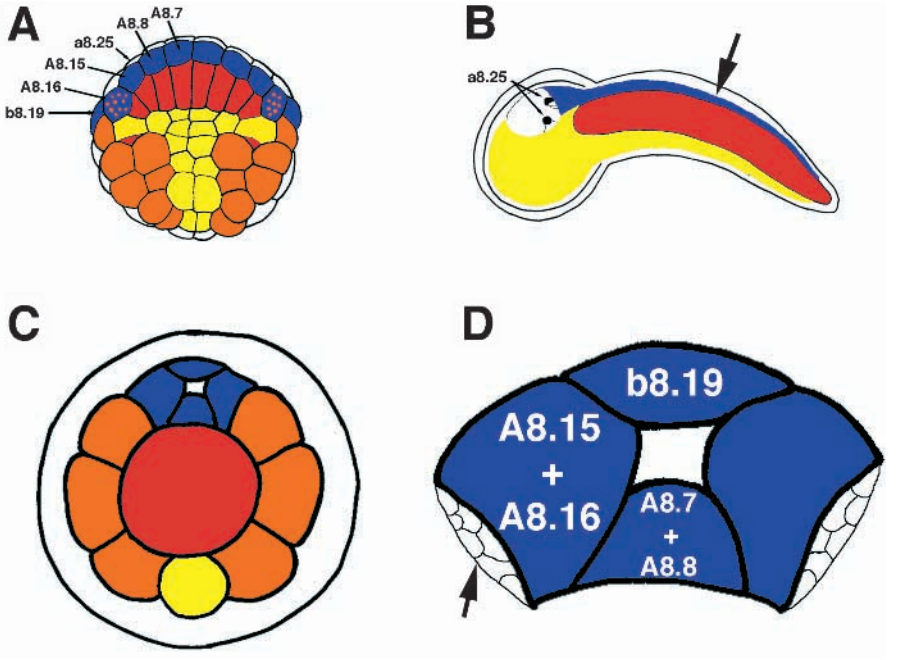

Fig. 1. Summary of tissue lineages in *Ciona intestinalis*. (A) Diagram of a 110-cell embryo, 5 hours postfertilization, viewed from the vegetal pole. The blastomeres that give rise to the tadpole CNS, notochord and gut are colored blue, red and yellow, respectively. The lateralmost cells colored yellow give rise to 'trunk lateral cells' (Satoh, 1994). Muscle/trunk mesenchyme precursors are in orange. The A8.16 blastomeres are colored blue and stippled in orange since they give rise to both muscle cells and ependymal cells of the CNS. Note that ascidians are bilaterally symmetrical, and so only the left-sided blastomeres are labeled. (B) A side view of a mid-tailbud-stage embryo, ~12 hrs postfertilization. This mid-sagittal plane of focus shows the dorsal CNS, the central notochord and ventral endodermal strand (gut) that runs along the length of the tail. Note that the same color coding is used here as in A. In anterior trunk regions, the ventral gut lies just below the cerebral vesicle (unfilled open circle) which contains two sensory structures, the statocyte and ocellus (in black), which derive from a8.25. (C) Diagram of a cross-section through the tail region of a mid-tailbud-stage embryo (the plane of sectioning is indicated by the arrow in B). The large, central notochord (red) is surrounded by the dorsal spinal cord (blue), ventral endodermal strand (yellow) and lateral muscles (orange). (D) A magnified view of the four ependymal cells that constitute the spinal cord in cross-section. Each of these cells arises from distinct progenitors at the 110-cell stage (see A). In particular, the ventralmost ependymal cell arises from the A8.7 and A8.8 blastomere pairs, while the lateral ependymal cells arise from the A8.15 and A8.16 blastomere pairs. The dorsalmost ependymal cell arises from the b8.19 blastomere pair of the 110-cell-stage embryo. The arrow indicates the ventrolateral axon bundles that emerge from the cerebral vesicle and extend throughout the tail to innervate the tail muscles (Crowther and Whittaker, 1992).

MATERIALS AND METHODS

Animal husbandry

Adult *Ciona intestinalis* were collected from several locations in San Diego and Los Angeles counties. Further details concerning the maintenance and rearing of the animals can be found in Corbo et al. (1997).

Isolation of *Ci-sna* and *Ci-fkh*

In order to isolate a *snail* homologue from *Ciona* a 650 bp *Bam*HI-*Nde*I fragment from the *Drosophila snail* gene was labeled by random priming and used to screen a *Ciona intestinalis* genomic DNA library. Out of approximately four genome equivalents, three positive clones were isolated. The cross-hybridizing portion of clone #1 was subcloned and sequenced to confirm its homology to *Drosophila snail*. This fragment was then used to screen a gastrula-stage *Ciona* cDNA library (kindly provided by Drs Tom Meedel and Jamie Lee). Several positive clones were isolated, the longest of which being approximately 1500 bp. Nested deletions of both strands of this clone were generated by exonuclease III digestion and sequenced by standard dideoxy chain termination methods (Sambrook et al., 1989). This cDNA did not appear to be full length, since it identified a 2 kb RNA in northern blot assays (data not shown). Therefore 5′ RACE (rapid amplification of cDNA ends; Clontech) was used to isolate the remainder of the cDNA. Briefly, poly(A)$^+$ RNA was made from late gastrulae (6 hours) and used to generate a RACE cDNA library. This library was then used as a template in a PCR reaction using the 5′ primer supplied by the manufacturer and the following 3′ primer which was based on the sequence of the 1500 bp cDNA previously isolated: 5′-CGAAATTGATCGCATGTCGTTAAG-3′. This reaction generated a product of approximately 1.1 kb, which was subsequently sequenced on both strands.

The cDNA library from which the original 1500 bp clone was isolated was made from animals from the east coast of the United States. The RACE cDNA library, however, was made from animals collected in Californian waters. In comparing sequences derived from east coast DNA and west coast DNA (derived from a genomic DNA library), there were various base pair changes, which usually did not result in any differences in amino acid composition. Such sequence heterogeneity among wild populations is common and has been observed among different individuals of the sea urchin species, *Strongylocentrotus purpuratus*, collected from the same southern Californian locale (Britten et al., 1978). In addition to such population heterogeneity, the original *Ci-sna* RACE product isolated had a single point mutation in its open reading frame, which resulted in a stop codon. Upon reisolation of the RACE product by PCR, this mutation was absent. Apparently this mutation was generated during the original round of PCR by *Taq* polymerase which is known to introduce mutations at low frequency. The putative *Ci-sna* protein sequence presented in Fig. 2A is based solely on the cDNA sequence.

In order to isolate a *Ciona fork head* homolog, a 350 bp PCR product containing the entire winged-helix domain from the *Xenopus Pintallavis* gene (Ruiz i Altaba and Jessell, 1992) was labeled by random priming and used to screen a *Ciona* genomic DNA library. Several positive clones were isolated, subcloned and sequenced. These genomic clones fell into two classes, which appear to represent two different winged-helix genes. The two genes differ within the winged-helix domain by twelve amino acids. Upon screening a gastrula-stage cDNA library with the *Xenopus Pintallavis* probe, four positive clones were isolated, all of which corresponded to the first class of genomic clones. Since the second class of winged-helix clones isolated was somewhat less conserved with respect to the *HNF-3β* subfamily than the first, we chose to concentrate on the first class. The longest cDNA was about 2 kb in length. This clone was estimated not to be full-length based on the observation that it detects a species of approximately 2.4 kb on a northern blot (data not shown). 5′ RACE was performed as described above for *Ci-sna* to isolate the 5′ end of the cDNA (3′ RACE primer: 5′-CTTGGTACTTTAACGAAG-CAATCG-3′). This reaction generated a PCR product of approximately 650 bp, which was subcloned and sequenced on both strands. Note that most of the *Ci-fkh* protein sequence is derived from the cDNA sequences (see legend of Fig. 2B).

In situ hybridization

In situ hybridization assays were performed with digoxigenin-labeled antisense RNA probes as described in Corbo et al. (1997). The *Ci-sna* in situs were carried out using an antisense probe derived from the *Ci-sna* RACE product. The *Ci-fkh* in situs were carried out using a probe from the *Ci-fkh* cDNA.

Ci-sna injection constructs and electroporation

Fusion genes were prepared with a genomic DNA fragment containing 3.3 kb upstream and 1.3 kb downstream of the *Ci-sna* cDNA start site. This fragment was fused to either β-galactosidase in a vector known as 72-1.27 (Corbo et al., 1997) or an enhanced form of green fluorescent protein (eGFP; see Corbo et al., 1997). The constructs were then electroporated into fertilized eggs exactly as described in Corbo et al. (1997).

RESULTS

Detailed lineages have been determined for each of the basic tissues in the ascidian tadpole, including the central nervous system (CNS), notochord, muscles and gut (Nishida and Satoh, 1983, 1985; Nishida, 1987; Satoh, 1994; summarized in Fig. 1). A cross-section through the tail of the tadpole (Fig. 1B,C) reveals the conserved organization of the chordate body plan. The ‘spinal cord’ (blue) resides just dorsal to the large notochord (red), while the gut (yellow) and tail muscles (orange) are ventral and lateral to the notochord, respectively. Each of the ependymal cells that enclose the lumen of the neural tube can be traced to distinct blastomeres in the 110-cell gastrula (Fig. 1D, compare with 1A).

HNF-3β and *snail* are among the earliest known molecular markers for floor plate and neural crest, respectively (Ruiz i Altaba and Jessell, 1992; Sasaki and Hogan, 1994; Ruiz i Altaba et al., 1995a; Dickinson et al., 1995; Thisse et al., 1995). To determine whether the ascidian CNS exhibits dorsoventral patterning similar to that in vertebrates, we isolated homologs of *HNF-3β* and *snail* from *Ciona intestinalis*.

Isolation of *Ci-fkh* and *Ci-sna*

The *Drosophila snail* (*sna*) gene encodes a regulatory protein that contains five putative zinc fingers (Boulay et al., 1987). It is expressed in the presumptive mesoderm in early embryos and then is reactivated in neurons of the CNS as they delaminate from the neuroectoderm (Alberga et al., 1991; Kosman et al., 1991; Leptin, 1991). *Ci-sna* was isolated from a *Ciona* genomic DNA library using the conserved zinc-finger domain of the *Drosophila snail* gene as a probe. Genomic clones were subsequently used to screen a cDNA library that was prepared with mRNA from gastrulating embryos (see Materials and methods). The largest cDNA that was isolated is 1500 bp in length, which is smaller than the predominant mRNA species identified in northern blot assays (data not shown). A PCR-based RACE protocol was used to obtain the remaining 5′ coding sequences not present in the cDNA.

The putative *Ci-sna* protein is composed of 584 amino acid residues and shares substantial homology with other members

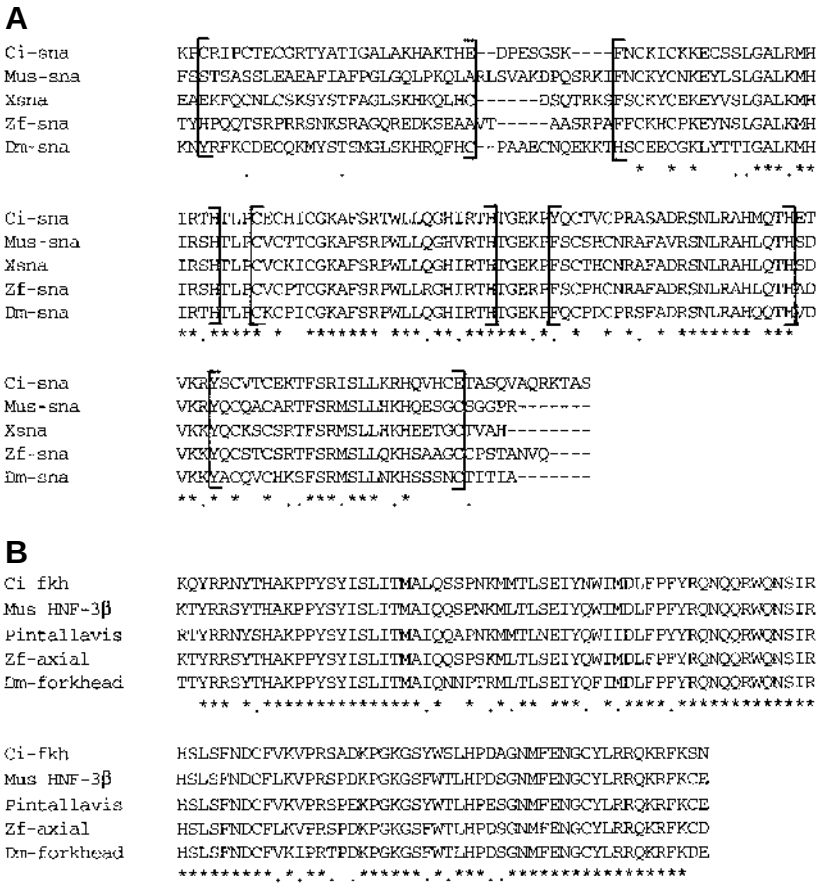

Fig. 2. Amino acid comparisons of *Ci-sna* and *Ci-fkh*. (A) Comparison of the zinc finger sequences of *Ci-sna* with the corresponding regions of the fly and vertebrate members of the *snail* gene family. *Ci-sna* contains five C-terminal zinc finger sequences (demarcated by brackets), similar to the situation seen for the *Xenopus Xsna* gene and *Drosophila snail*. The sequences shown in this comparison are as follows: *Mus-sna* (mouse *snail*; Nieto et al., 1992); *Xsna* (*Xenopus snail*; Sargent and Bennett, 1990); *Zf-sna* (zebrafish *snail*; Hammerschmidt and Nusslein-Vohard, 1993); *Dm-sna* (*Drosophila snail*; Boulay et al., 1987). (B) Comparison of the *Ci-fkh* winged-helix with the corresponding regions of the fly and vertebrate *fork head* genes. The sequences shown in the comparison are as follows: *Mus* HNF-3β (mouse *HNF-3β*; Kaestner et al., 1994; *Pintallavis* (*Xenopus fork head*; Ruiz i Altaba and Jessell, 1992); Zf-*axial* (zebrafish *fork head*; Strahle et al., 1993); Dm-*fork head* (*Drosophila fork head*; Weigel et al., 1989). The Genbank accession numbers for *Ci-sna* is AF002987 and for *Ci-frch* is AF002988.

of the *snail* family. In particular the C-terminal 153 amino acid residues include five putative zinc fingers that are homologous to the corresponding zinc fingers of the *Drosophila* protein (Fig. 2A). *Ci-sna* appears to represent a bona fide *snail* homolog based on several criteria. Most notably, it contains five zinc fingers, similar to the situation observed for the *Drosophila* and *Xenopus* sequences. In contrast, *snail* genes isolated from zebrafish and mouse lack the first of these putative zinc-finger motifs. Moreover, like other members of the family, *Ci-sna* contains particularly extensive homology in the second, third and fourth fingers (Fig. 2A). Although we cannot exclude the possibility that there are additional members of the *snail* gene family in the *Ciona* genome, the high degree of sequence conservation places *Ci-sna* firmly within the core group of *snail* genes involved in mesoderm and neural crest patterning.

The *Drosophila fork head* (*fkh*) gene encodes a DNA-binding protein that has been implicated in the differentiation of anterior and posterior regions of the gut (Weigel et al., 1989). DNA binding is mediated by a highly conserved region of the fork head protein, the winged-helix domain, which is composed of ~110 amino acid residues (Kaufmann et al., 1995). Vertebrate *fkh* genes, such as *Pintallavis* in *Xenopus* and *HNF-3β* in mice, are important for differentiation of the notochord, endoderm and floor plate (Sasaki and Hogan, 1994; Ang and Rossant, 1994; Weinstein et al., 1994; Ruiz i Altaba, 1995a,b).

The winged-helix coding region from *Pintallavis* was used to screen both a *Ciona* genomic DNA library and a gastrula-stage *Ciona* cDNA library. As in the case of *Ci-sna*, none of the resulting *Ci-fkh* cDNAs were sufficiently long to represent a full-length transcript. PCR-based RACE assays were performed to isolate the remaining 5′ coding region.

The *Ci-fkh* protein coding region is composed of 587 codons, and includes a highly conserved putative winged-helix domain spanning amino acid residues 121 to 230. The *Ci-fkh* winged-helix is closely related to other members of the *fork head*/*HNF-3β* family (Fig. 2B). The five members of the family that are compared share 84 invariant residues across the length of the domain. Most of the remaining residues are also conserved. The strong conservation of the *Ci-fkh* winged-helix domain (~90% identity with mouse *HNF-3β*) suggests that it is a legitimate member of the *fkh*/*HNF-3β* gene subfamily.

Ci-sna and *Ci-fkh* expression patterns

In situ hybridization assays were done to determine the patterns of *Ci-sna* and *Ci-fkh* expression during *Ciona* embryogenesis. Various *Ci-sna* and *Ci-fkh* hybridization probes were used, including probes derived from both cDNAs and RACE products (see Materials and Methods). Digoxigenin-labeled antisense RNA probes were used in these studies, as described by Corbo et al. (1997).

Ci-sna is expressed in precursors of the tail muscles and neural plate border cells at the 110-cell stage (Fig. 3A; dark-blue shading in Fig. 3C). Specifically, staining is seen in blastomere A8.15, which gives rise to cells in the posterior region of the neural plate border, and in A8.16, which generates both neural plate border cells and posterior tail muscles (arrows Fig.

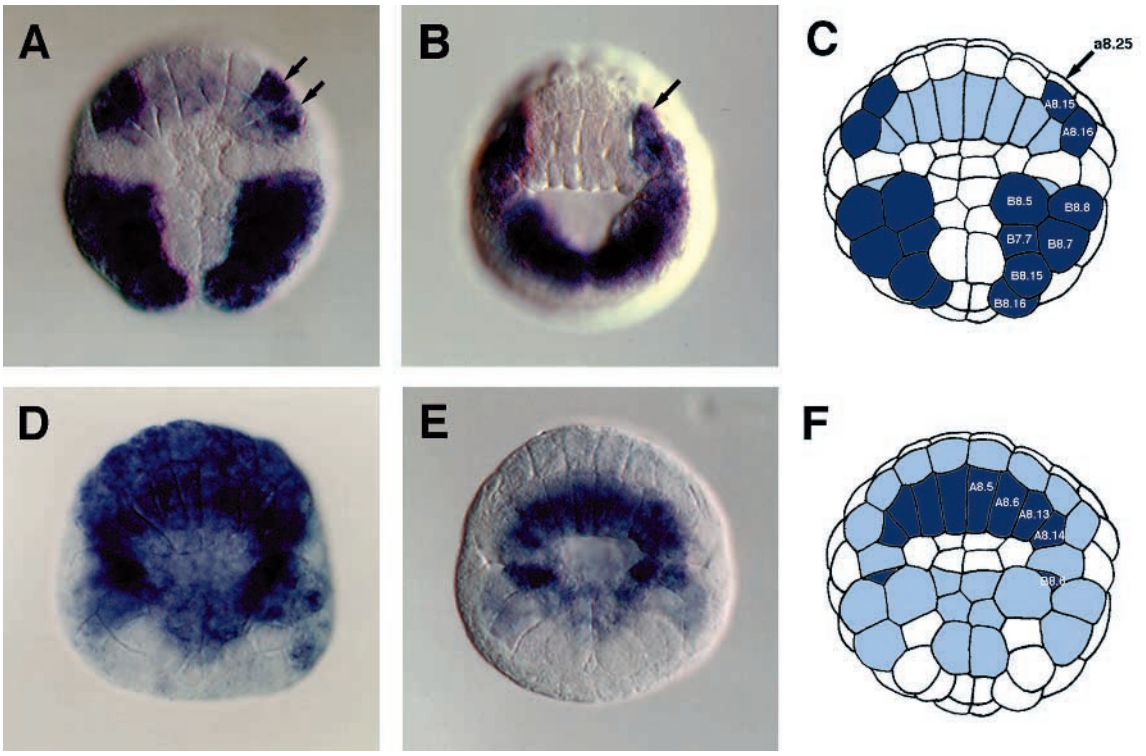

Fig. 3. In situ localization of *Ci-sna* and *Ci-fkh* transcripts in gastrulating embryos. Whole-mount embryos were hybridized with digoxigenin-labeled antisense RNA probes, and the signal was detected by histochemical staining with alkaline phosphatase (see Corbo et al., 1997 for technical details). (A,B,D,E) Vegetal views of stained embryos, with anterior up. (A) 110-cell-stage embryo stained with a *Ci-sna* antisense RNA probe. Intense expression is observed in muscle/trunk mesenchyme precursors (strong staining in the posterior of the embryo) and in the progenitors of the lateral ependymal cells, A8.15 and A8.16 (arrows). Weaker staining is observed in the primary lineage notochord precursor cells (posterior and medial to the strongly stained CNS precursors indicated by the arrows). (B) Similar to A except that the embryo is 1 hour older and is at an angle that exposes more of the presumptive neural plate. Staining persists in the muscle and CNS precursors. New staining has appeared in muscle precursors that lie between the posterior and anterior patches of staining seen in A as well as in the a8.25 blastomere pair (arrow). Staining has become undetectable in the notochord precursors. (C) Schematic representation of the hybridization pattern seen in A. The dark-blue shading indicates sites of strong expression in the CNS and muscles. The lighter blue indicates weaker staining in the primary (arc of eight cells) and secondary notochord lineages. (D) 110-cell embryo stained with a *Ci-fkh* antisense RNA probe. A broad staining pattern is observed, which includes precursors of the notochord, CNS (both the ventral and lateral ependymal cells), gut (mainly endodermal strand) and some muscles. (E) Same as D, except that the embryo is 1 hour older and undergoing gastrulation. Staining is lost from the CNS blastomeres, is reduced in the muscle and endodermal strand precursors and is restricted mainly to the notochord precursor cells. (F) Schematic representation of the *Ci-fkh*-staining pattern shown in D. The dark blue corresponds to the notochord lineages, while the lighter blue shading denotes the precursors of the CNS, gut and some muscles.

3A; Nicol and Meinertzhagen, 1988a,b; Satoh, 1994). Staining in the muscle and CNS precursors persists during gastrulation (Fig. 3B). In addition, a new site of expression is detected in the a8.25 blastomere (arrow in Fig. 3B,C), which gives rise to a dorsal portion of the cerebral vesicle and to the pigmented statocyte and ocellus sensory cells (Nishida and Satoh, 1989; Satoh, 1994; see Fig. 1B). By the onset of neurulation, *Ci-sna* transcripts are lost from the CNS but persist in tail muscles until the early tailbud stage (data not shown).

Prior to gastrulation, *Ci-fkh* is expressed in notochord precursors (Fig. 3D; dark-blue shading in Fig. 3F), as well as in the CNS, gut and some muscle precursors (light-blue shading in Fig. 3F, compare with Fig. 1A). This rather broad expression pattern is rapidly refined by late gastrulation (Fig. 3E), so that staining is largely lost from the CNS, gut and muscle, and restricted to notochord precursors.

Ci-fkh expression is reactivated in the CNS and gut during neurulation (Fig. 4A,B). In the CNS, this de novo expression (arrow Fig. 4A) appears to be restricted to the ventralmost region of the invaginating neuroepithelium immediately adjacent to the notochord (Fig. 4B, dark-blue shading in Fig. 4C). *Ci-fkh* expression persists in tailbud embryos (Fig. 4D). An optical cross-section through the tail of such an embryo reveals intense staining in the ventralmost ependymal cell of the spinal cord, as well as the endodermal strand of the gut (Fig. 4E; dark-blue shading in Fig. 4F). This late staining pattern is consistent with the possibility that the notochord induces *Ci-fkh* expression in the CNS and gut, similar to the situation encountered in vertebrates (Ruiz i Altaba et al., 1995a,b).

C-sna is expressed in the lateral ependymal cells of the neural tube

The preceding in situ hybridization assays suggest that *Ci-sna* and *Ci-fkh* are expressed in complementary patterns within the neural tube. However, due to the transience of the *Ci-sna* CNS

pattern, restricted expression in the neural plate border is inferred from the staining seen in precursor cells during gastrulation (see Fig. 3A,C). Fusion genes were analyzed in order to visualize the restricted *Ci-sna* expression pattern within the neural tube.

We have recently shown that electroporation gives efficient incorporation of exogenous DNA in *Ciona* embryos (Corbo et al., 1997). This procedure permits the rapid identification of *cis*-regulatory elements that mediate tissue-specific patterns of gene expression. A 3.3 kb sequence from the *Ci-sna* 5′ regulatory region (Fig. 5A) directs the expression of a β-galactosidase reporter gene in a pattern that is virtually identical to the endogenous gene (Fig. 5B). Staining is detected in the tail muscles, trunk mesenchyme, progenitors of the adult muscles and the CNS, including both the cerebral vesicle and spinal cord.

The *Ci-sna/lacZ* fusion gene serves as an effective lineage tracer since β-galactosidase perdures long after the endogenous transcript becomes undetectable in the CNS. However, spinal cord expression is obscured (Fig. 5B) by the intense staining in the tail muscles. An earlier tail bud stage provides better resolution of the CNS staining pattern (Fig. 5C). Expression is detected in two rows of ependymal cells in the spinal cord (arrows). These cells are the descendants of the two blastomere pairs, A8.15 and A8.16, which transiently express *Ci-sna* during gastrulation (see Figs 1D, 3A).

Green fluorescent protein (GFP) (Fig. 5D-F) permitted the detailed visualization of neural tube closure in living embryos. The neurula shown in Fig. 5E has completed about 60% of neural tube closure, which occurs from posterior to anterior in ascidians (Satoh, 1994). The cells anterior (to the left) of the arrow correspond to progenitors of the cerebral vesicle that have not yet undergone medial convergence. These anterior cells reside in the neural folds in a position analogous to *snail*-expressing cells in vertebrates (Dickinson et al., 1995; Mayor et al., 1995; Thisse et al., 1995), and correspond to the descendants of the a8.25 blastomere seen in gastrulating embryos (arrows, Fig. 3B,C). The cells posterior to the arrow correspond to the descendants of A8.15 and A8.16 (arrows, Fig. 3A), which have converged at the midline to form the lateral ependymal cells of the spinal cord. The completion of this process leads to the closure of the cerebral vesicle (Fig. 5F). The GFP reporter permits detailed resolution of individual ependymal cells, which will elongate along the anteroposterior axis during extension of the tail.

DISCUSSION

The notochord and dorsal ectoderm induce dorsoventral compartmentalization of the vertebrate neural tube through the differential regulation of genes such as *HNF-3β*, *Pax3*, *Pax6* and *snail*. *HNF-3β* is expressed in the ventralmost region of the vertebrate neural tube, which forms the floor plate (Ruiz i Altaba, 1995a,b). *Pax6* expression is progressively restricted to lateral regions, while *Pax3* and *Pax7* are restricted to the dorsal half of the neural tube (Goulding et al., 1993; Tanabe and Jessell, 1996). Members of the *snail* family of transcription factors are expressed in cells of the lateral border of the neural plate, which form the neural crest and dorsal roof of the neural tube (Nieto et al., 1994).

We have presented evidence that dorsoventral patterning of the vertebrate neural tube is conserved in the ascidian, *Ciona intestinalis*. *Ci-fkh* and *Ci-sna* exhibit patterns of expression in the neural tube that are quite similar to those seen in vertebrates, as summarized in Fig. 6. In vertebrates (Fig. 6A),

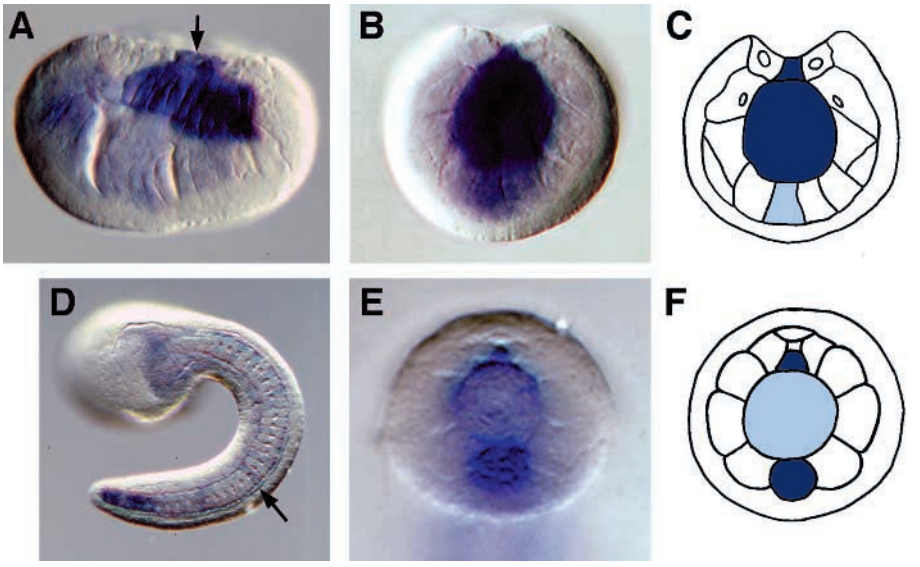

Fig. 4. Reactivation of *Ci-fkh* in the gut and CNS. Whole-mount embryos were hybridized with a digoxigenin-labeled *Ci-fkh* antisense RNA probe. (A) Lateral view of a neurulating embryo stained for *Ci-fkh*. Intense expression is observed in the notochord. Weaker staining is also detected in the underlying endoderm. The arrow indicates reactivation of the *Ci-fkh* gene in the invaginating neural plate. (B) Optical cross-section of a neurulating embryo stained for *Ci-fkh*. The level of the section is indicated by the arrow in A. Specific staining is observed in the ventralmost region of the neuroepithelium, and not in the lateral ependymal cells. Equally strong staining is also seen in the adjacent notochord. Weaker staining is present in the ventral endoderm. (C) Schematic representation of the staining pattern shown in B. Intense staining in the large, central notochord and in the ventralmost ependymal cell is indicated by dark-blue shading. Weak staining in the ventral endoderm is indicated by light-blue shading. (D) Lateral view of a 12 hour tailbud-stage embryo stained for *Ci-fkh*. The notochord is composed of a central column of cells that extend along the length of the tail. Stronger staining is observed in the secondary lineage notochord cells at the posterior tip of the tail, as compared with the primary notochord cells in more anterior regions. Staining is visible in cells dorsal (arrow) and ventral to the notochord, which is weaker than in E since this embryo is somewhat older. (E) Optical cross-section through the tail region of a mid-tailbud embryo stained for *Ci-fkh*. Strong staining is detected in the ventral endodermal strand and in the ventralmost ependymal cell of the spinal cord. Weaker staining is observed in the central notochord. (F) Schematic representation of the staining pattern shown in E. The dark-blue shading corresponds to staining in the ventral endodermal strand and the ventralmost ependymal cell of the spinal cord. Weak notochord staining is indicated by light-blue shading.

the notochord (red) induces the ventralmost regions of the invaginating neural plate to express *HNF-3β* (light blue; Placzek et al., 1993; Roelink et al., 1995). Recent studies in the chick (Dickinson et al., 1995) suggest that the dorsal ectoderm induces the neighboring cells of the neural plate to express *Slug* (a *snail* homolog) and form the precursors of the neural crest (dark blue). Virtually identical topological patterns of *Ci-fkh* and *Ci-sna* expression are observed in the *Ciona* neurula (summarized in Fig. 6B). This striking conservation provides evidence that homologous mechanisms are employed for at least the initial stages of neural patterning.

Evolutionary origins of the floor plate

It is possible that *Ci-fkh* has a conserved function in the specification of an ascidian 'floor plate'. The floor plate plays a key role in axonal guidance during vertebrate neurogenesis (Colamarino and Tessier-Lavigne, 1995). In particular, in the zebrafish CNS, the floor plate is required to prevent aberrant crossing-over of long, bilateral axonal fascicles known as the median longitudinal fascicles (MLF) (Hatta, 1992). In fact, the MLF axonal bundles directly contact the lateral portions of cells immediately adjacent to the midline floor plate cells. An analogous situation exists in the ascidian CNS where the ventrolateral axonal bundles are in direct contact with the ependymal cells on either side of the midline 'floor plate' cell (see Crowther and Whittaker, 1992; Fig. 1D). Perhaps the ascidian 'floor plate' has a function similar to that in vertebrates in guiding these axonal bundles into the tail or in restricting their course to only one side of the spinal cord.

Despite the cellular simplicity of the ascidian CNS, it appears to possess a fair degree of molecular sophistication. Indeed, expression of *Ci-fkh* in a single midline cell in the ascidian larval tail may represent the ancestral condition of the floor plate. Although the floor plate spans 15-20 cells in cross-section in warm-blooded vertebrates (Colamarino and Tessier-Lavigne, 1995), in zebrafish the floor plate is represented by a single midline cell (Hatta, 1992). It is possible that the floor plate in birds and mammals represents a derived condition and that the situation found in ascidians and fish is more primitive.

Origins of dorsal lineages

Recently, Wada and colleagues (1996) reported the cloning of a homolog of *Pax3* and *Pax7*, known as *HrPax-37*, from the ascidian, *Halocynthia roretzi*. This gene is expressed in cells destined to give rise to dorsal portions of the neural tube (a8.25, b8.19 and b8.17). The *Ci-sna* and *HrPax37* expression patterns overlap, but are not identical. Both genes are expressed in the

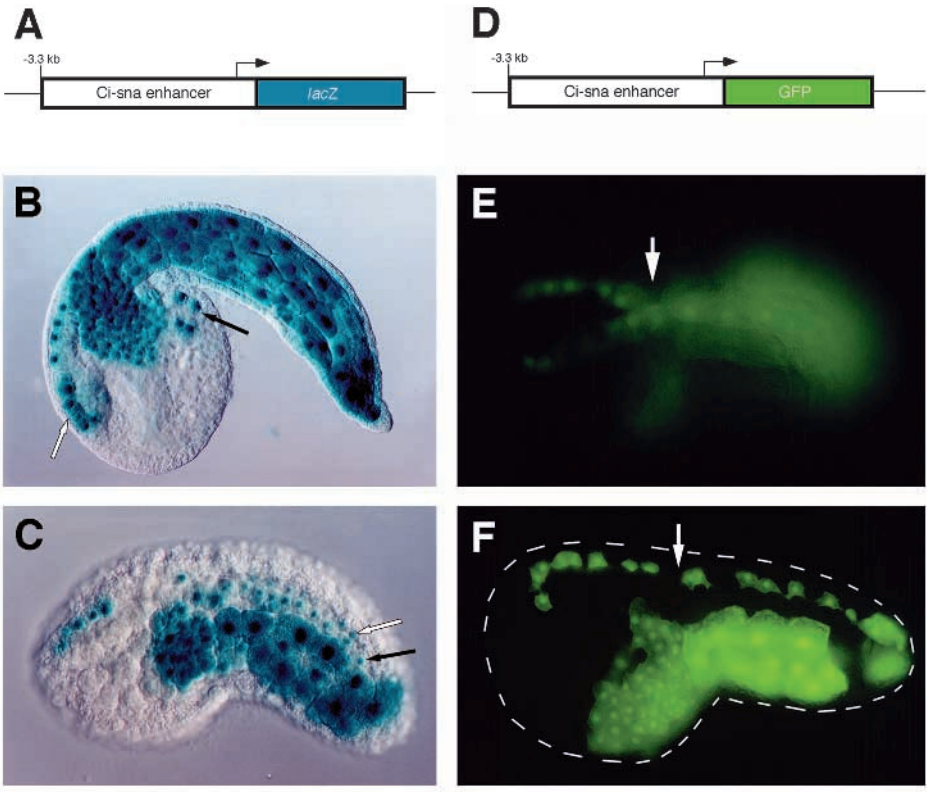

Fig. 5. Expression of *Ci-sna* fusion genes in electroporated embryos. *Ci-sna* fusion genes were introduced via electroporation into fertilized single-cell embryos (see MATERIALS AND METHODS). The embryos were then allowed to develop for 8 to 12 hours prior to visualization of either the *lacZ* (B,C) or GFP (E,F) reporter. Anterior is to the left and dorsal is up. (A) Diagram of the *Ci-sna*/*lac*Z fusion gene. A 4.6 kb genomic DNA fragment from the *Ci-sna* gene containing 3.3 kb upstream and 1.3 kb downstream of the putative transcriptional start site was fused to the *lac*Z coding sequence. (B) β-galactosidase staining in a mid-tailbud-stage embryo. This embryo shows expression in all the muscles of the tail and in trunk mesenchyme cells (small cells immediately anterior to the muscles). The white arrow indicates staining in the dorsal portion of the cerebral vesicle in descendants of a8.25. The black arrow indicates four B7.5-derived 'trunk ventral cells' which are the progenitors of adult muscle cells (Kusakabe et al., 1995). (C) β-galactosidase staining in an early tailbud-stage embryo. This embryo, as indicated by the arrows, shows expression in two rows of A8.15 and A8.16 derived ependymal cells in the spinal cord. The row indicated by the white arrow appears fainter since it is out of the plane of focus. (D) Diagram of the *Ci-sna*/GFP fusion gene. Same construct as in A except GFP replaces *lac*Z. (E) GFP expression in a living neurula carrying the *Ci-sna* fusion gene shown in D. Most of the intense fluorescence that is out of the plane of focus in the posterior of the embryo corresponds to expression in the tail muscles. To the right of the arrow lie cells that have converged at the dorsal midline and which derive from A8.15 and A8.16. Fluorescing cells that lie anterior to the arrow correspond to progenitors of the cerebral vesicle derived from a8.25 that have not yet converged at the dorsal midline. (F) GFP expression in an 11 hour embryo that contains the *Ci-sna* transgene shown in D. Intense fluorescence is detected in a group of large, ventral muscle cells of the tail and immediately anterior to these cells in a group of smaller trunk mesenchyme cells. Fluorescence can also be seen in a single row of dorsal cells that run along the length of the tail and into the trunk. The cells to the right of the arrow correspond to the lateral ependymal cells. Those cells to the left constitute a portion of the cerebral vesicle. Only a single row of cells is detected in this embryo on account of stabilization of the reporter construct in only half of the blastomeres.

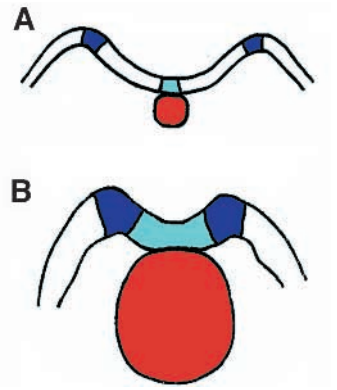

Fig. 6. Summary of dorsoventral patterning in the vertebrate (A) and *Ciona* (B) neuroepithelium. The diagrams represent an early stage during formation of the neural tube. The central red structure corresponds to the notochord, while the light-blue and dark-blue structures depict sites of *HNF-3β* and *snail* expression, respectively. (A) In vertebrates the notochord is a source for Sonic hedgehog, which induces the overlying neuroepithelium to express *HNF-3β* and form the floor plate (Roelink et al., 1994). The lateralmost margin of the neuroepithelium is induced by the adjacent dorsal ectoderm to express *snail* and form the progenitors of the neural crest (Dickinson et al., 1995). (B) In *Ciona* a similar pattern of *Ci-fkh* and *Ci-sna* is observed in the ventralmost ependymal cell and cells of the neural plate border, respectively. Unlike in vertebrates, the expression domains of *Ci-fkh* and *Ci-sna* are immediately apposed.

progenitors of the dorsal cerebral vesicle (a8.25). However, *HrPax37* does not appear to be expressed in the lateral ependymal cells of the caudal neural tube, whereas *Ci-sna* expression extends along the entire length of the tail in these cells. In addition, *HrPax37* is expressed in the progenitors of the dorsalmost ependymal cells of the neural tube, which arise from the b8.19 blastomere; *Ci-sna* is not expressed in this lineage.

A homolog of *Pax6* was recently isolated from another ascidian, *Phallusia mammillata* (Glardon et al., 1997). The gene appears to be expressed in the lateral border of the neural plate in many of the same cells that express *Ci-sna*. Future studies should permit a detailed comparison of the *Ci-sna*, *HrPax37* and *PPax6* expression patterns in an effort to determine the extent of dorsoventral patterning in the ascidian neural tube.

Although ascidians lack neural crest cells, it is intriguing that *Ci-sna* is expressed in the precursors of the pigmented statocyte and ocellus (blastomere a8.25), since pigmented melanocytes are one of the principal cell types derived from neural crest (Bennett, 1993).

Induction of dorsoventral compartmentalization

It is unclear whether similar mechanisms are used for establishing the localized expression of *HNF-3β*, *snail* and *Pax* genes in the vertebrate and ascidian CNS. At least one BMP homolog has been shown to exist in ascidians (Miya et al., 1996). It is transiently expressed in the precursors of the CNS and in the dorsal ectoderm immediately adjacent to the neural tube during closure. Perhaps this BMP helps induce the expression of *Pax* or *snail* genes in dorsal regions of the neural tube.

There is currently no evidence that the ascidian notochord induces *HNF-3β* expression in ventral ependymal cells via a homologue of Sonic hedgehog. In fact, we have recently characterized a divergent *hedgehog* gene from *Ciona* (J. Corbo, A. Di Gregorio, and M. Levine, unpublished data). Preliminary studies suggest that it is not expressed in the notochord. This observation raises the possibility that different mechanisms lead to the localized expression of *HNF-3β* in the vertebrate floorplate and ascidian ventral midline.

Origins of the vertebrate hypochord

The ascidian endodermal strand bears some resemblance to the hypochord of fish and amphibians. The hypochord is a rod-shaped cord of cells derived from endoderm that lies immediately ventral to the notochord (Yan et al., 1995). Although the evolutionary origins of the hypochord have been debated for over a century (see, for example, Willey, 1899), it appears to function simply as an axial supportive structure similar to the notochord.

Certain genes in zebrafish show a pattern of expression in the hypochord similar to that of *Ci-fkh* in the endodermal strand. For example, a type II collagen gene, *col2a1*, is first expressed in the notochord (Yan et al., 1995). Later, it is activated in the floorplate and, still later, in the underlying hypochord. This pattern of expression is very similar to that seen for *Ci-fkh* in the endodermal strand.

Origins of the vertebrate CNS

The remarkable simplicity of the ascidian larva, and its nervous system in particular, has led some authors to suggest that ascidians represent a highly simplified clade within the chordate phylum (e.g., Jollie, 1973). Under this assumption, the common ancestor of both tunicates and cephalochordates/vertebrates was an organism possessing a more sophisticated central nervous system, which went on to be further elaborated in cephalochordates/vertebrates and subsequently reduced in tunicates. Indeed, Darwin proposed that the ascidians represent a retrograde form of the last common ancestor (Darwin, 1871). Our demonstration of complex patterns of gene expression in the *Ciona* neural tube is consistent with the notion that this common ancestor possessed a fairly sophisticated CNS.

In contrast, the theory of the origin of vertebrates from a tunicate-like ancestor via neoteny (Garstang, 1928; Berrill, 1955) suggests that the transitory nature of the ascidian larval nervous system and its simple structure are rudimentary and not vestigial features. Although our studies are compatible with either theory, we favor Darwin's view that ascidians are retrograde forms of a more sophisticated chordate ancestor. Regardless of which model is correct, our data provide strong evidence that the neural tubes of ascidians and vertebrates are truly homologous.

These considerations raise the issue of the origin of the lateral regions of the vertebrate spinal cord. These cells do not exist in the *Ciona* neural tube. Does their absence imply a primitive condition or a simplification within the clade giving rise to the modern-day ascidians? We propose that the lateral lineages are a relatively recent innovation of the vertebrate CNS. Perhaps these cells arose from inductive signals emanating from ventral midline cells that express *HNF-3β* and dorsolateral cells that express *snail* and *Pax* regulatory factors.

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