Development and application of novel cloning strategies for analysis of genes controlling embryo development

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Submitted for partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Adelaide, Australia

January 2004

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January 2004

Richard Tamme

ACKNOWLEDGEMENTS

First and foremost, I am immensely grateful to my dear supervisor, Dr Michael Lardelli, for his excellent guidance, patience, support and understanding as well as teaching me the importance of control experiments. I promise that the 'PhD student belt-of-luck' I inherited from Michael will be passed on to my first postgraduate student (provided that I can trick someone into doing that, of course) once the time is ripe. I hope that this belt will become valuable in a few generations time.

I am also thankful to all the past and present members of Dr Lardelli group - Esther Camp, Casper Groth, Christine Hepperle, Dan Kortschak, Rosa McCarty, Kathryn Mills, Svanhild Nornes, Barry Rainbird, Ben Tucker, Simon Wells and everyone at the Discipline of Genetics for providing an intellectually stimulating environment which was also friendly and relaxed.

During my years as a postgraduate student, Adelaide University supported my studies by awarding me the International Postgraduate Research Scholarship. Thus, I am doubly indebted to my alma mater, both for providing me with enlightenment and means of sustenance.

I am also grateful to my current employer, Dr Tõnis Timmusk for allowing me to go on leave to complete the thesis and Dr Kaia Palm for helpful comments and proofreading. I am also obliged to Dr Priit Kogerman for providing access to the relevant electronic journals.

Completion of this thesis would not have been possible without the continuous support and love from my dear family, my parents Julia and Mihkel and my brother Otto, and Egle, my Tibuke.

CONTENTS

'Development and Application of Novel Cloning Strategies for Analysis of Genes Controlling Embryo Development.'

Richard Tamme -PhD Thesis, The University of Adelaide 2004

ABSTRACT

Initially, we aimed to identify novel genes regulating vertebrate neurogenesis and somitogenesis by screening cDNAs derived from gastrulation/neurulation stage zebrafish embryos for clones revealing corresponding genes with expression patterns suggestive of roles in these processes. The lack of suitable cDNA libraries prompted us to devise a simplified method for producing randomly-primed, directionally cloned cDNA libraries from small amounts of embryonic tissue. To achieve this, several techniques were combined, including cDNA synthesis on a solid carrier, random priming of 1st cDNA strand synthesis, non-specific priming of 2nd cDNA strand synthesis and amplification of initially small amounts of cDNAs by suppression-PCR.

A pilot-scale *in situ* screen using a cDNA library produced by the above method identified a gene, *spadetail*, that is expressed in presomitic mesoderm and in unidentified, apparently irregularly distributed cells of the spinal cord. *spt* functions in mesodermal development, yet its role in neural tissue remains unknown. Analysis of the *spadetail*-expressing neural cells' gene co-expression profile and dorsoventral location implied that they are Dorsal Longitudinal Ascending interneurons. Quantitative analysis of these cells' rostrocaudal distribution showed that there is a tendency to higher cell numbers in rostral spinal segments. The observation that *spadetail*-expressing neurons are frequently juxtaposed to somitic cells expressing *spadetail* at low levels suggests that the distribution of *spadetail*-expressing neurons may be 'inefficiently' patterned by *spadetail*-expressing somitic cells or that the expression of *spadetail* in both tissues is induced by a common positional cue.

The strategy for non-specific priming was then extended to develop a simple technique for cloning unknown DNA sequences flanking known DNA. An initial non-specific PCR amplification was performed with a single primer that binds specifically within known sequence and non-specifically in the unknown DNA region. In a second reaction, the sequences of interest were amplified from the primary reaction mixture (that also contains undesired sequences) with nested PCR using a primer that had been extended further downstream from the primer used in the initial PCR. This enabled isolation of a 0.5 kb region of amphioxus *Notch* cDNA, that, in turn, contributed to the subsequent analysis of the evolution of vertebrate *Notch* genes.

List of publications and declaration of contributions to each publication

I. Tamme, R., Mills, K., Rainbird, B., Nornes, S., Lardelli, M. (2001)
Simple, directional cDNA cloning for in situ transcript hybridization screens. *Biotechniques* 31(4): 938-42, 944, 946.

Richard Tamme was responsible for:

- isolation of mRNA from zebrafish embryos and cDNA library preparation;
- characterisation of insert sizes of 2,600 cDNA clones;
- sequence analysis of 66 randomly chosen cDNA clones;
- conducting a trial *in situ* hybridisation screen with three cDNA clones;
- cloning and sequencing one gene (*spadetail*) identified by the trial *in situ* screen.

Richard Tamme

II. Tamme, R., Wells, S., Conran SG., Lardelli, M. (2002)
The identity and distribution of neural cells expressing the mesodermal determinant *spadetail*. *BMC Dev Biol.*, 2(1): 9.

Richard Tamme was responsible for:

- cloning of fragments of *huC, islet2* and *valentino* cDNAs for use as templates for riboprobe synthesis;
- double *in situ* staining of 24 hpf embryos for simultaneous visualisation of the expression of *spadetail* and neural marker genes;
- staining of 24 hpf embryos for *spadetail* expression for extended periods;
- counting of *spadetail*-expressing neural cells at each somite level;
- collection and statistical analysis of data for testing for the presence of left-right bias in the distribution of *spadetail*-expressing neurons;
- performing the bulk of the statistical analysis of the *spadetail*-expressing neurons' rostrocaudal distribution.

Richard Tamme

III. Tamme, R., Camp, E., Kortschak, D., Lardelli, M. (2000)
Nonspecific, nested suppression PCR method for isolation of unknown flanking DNA. *Biotechniques* 28(5): 895-9, 902.

E. Camp and **R. Tamme** contributed equally to this work. **Richard Tamme** was responsible for:

- reverse transcription of amphioxus RNA with an *AmphiNotch*-specific primer;

- performing non-specifically primed suppression PCR (NSPS-PCR) on reverse transcribed amphioxus cDNA;

- performing Southern analysis to select the correct NSPS-PCR products for reamplification with nested suppression PCR;

- using the non-specific, nested suppression PCR method to clone unknown DNA flanking a region of known *AmphiNotch* sequence.

Richard Tamme

IV. Holland LZ, Rached LA, Tamme R, Holland ND, Kortschak D, Inoko H, Shiina T, Burgtorf C, Lardelli M. (2001)
Charatcerization and developmental expression of the amphioxus homolog of *Notch* (*AmphiNotch*): evolutionary conservation of multiple expression domains in amphioxus. *Dev Biol.* 232(2): 493-507.

Richard Tamme was responsible for:

- cloning and sequencing of cDNA sequences corresponding to EGF repeats 4-8 by NSPS-PCR (see above);

- cloning and sequencing of cDNA sequences corresponding to EGF repeats 8-34 by RT-PCR with gene-specific primers;

- sequencing of most of the intracellular domain encoding sequences, including the ankyrin repeat and the 3' regions.

Richard Tamme

LITERATURE REVIEW

My thesis project began with development of a whole mount *in situ* hybridisation-based screening strategy for identifying novel genes involved in vertebrate development. As our main focus was on neural development, I first present an overview of the genetic mechanisms controlling the early stages of this process.

One of the genes identified in our pilot *in situ* transcript hybridisation screen (described in Paper I) was *spadetail*, a member of the T-box gene family. We began an investigation of the role of this gene in CNS development in Paper II. Thus, an overview of this gene family is also presented.

Two novel cloning techniques were developed in the course of my thesis project. Firstly, development of an *in situ* screen required the invention of a novel method for cDNA library synthesis (described in Paper I). Therefore, a brief review of both the major genetic strategies currently deployed for identifying novel developmental control genes as well as the main stages of cDNA library construction is given. Secondly, our cDNA library construction method was extended to develop a novel technique for isolating unknown DNA sequences flanking known DNA (Paper III). Thus, I briefly describe the most commonly used methods used for cloning such DNA sequences.

As zebrafish was used to conduct the *in situ* screen, the embryological and genetic characteristics of this model system are overviewed in the subsequent section. Finally, I also review the use of amphioxus as a model system for studying vertebrate evolution since our unknown flanking DNA cloning technique was used to obtain part of the sequence of the *AmphiNotch* gene. This information was then used in phylogenetic comparison of various vertebrate *Notch* genes to their amphioxus counterpart (Paper IV).

1. Early development of the vertebrate CNS

My brain is my second favourite organ. - Woody Allen

The complexity of the vertebrate central nervous system partially stems from its structure: it consists of an enormous number of diverse neuronal cell types (e.g. the human brain contains over 10^{11} neurons), which are all properly positioned. Furthermore, each neuron has a thousand or more reliable synaptic connections with other neurons. This complex tissue architecture enables the central nervous system to perform its function - integration of sensory and motor functions.

The formation of the vertebrate central nervous system can be divided into five successive well-defined stages:

1) neural induction – allocation of a subset of ectodermal cells as progenitors of the entire nervous system via an inductive process: the dorsal mesoderm instructs its overlying ectoderm to form a columnar layer of neuroepithelial cells, the neural plate.

2) formation of the neural tube and neural crest – the neural plate folds up along its lateral margins, the folds then fuse to form a hollow tube which segregates from the surface; the dorsalmost cells of the neural folds, neural crest cells (precursors of the peripheral nervous system), detach from neighbouring cells and disseminate (during the separation of the neural tube) to various regions of the embryo, eventually differentiating into diverse cell types (including sensory ganglia of the spinal and cranial nerves and pigment cells).

3) regionalisation and differentiation of the neural tube (occurs simultaneously on three levels):
- at morphological level, the neural tube expands in the anterior to become subdivided into the forebrain, midbrain, hindbrain whereas the narrower posterior part forms the spinal cord;
- at tissue level, the cells within the neural tube rearrange to form the various functional regions of the CNS;

- at cellular level, neuroepithelial cells differentiate into the vast array of neuronal and glial cell types appropriate for their positions along both antero-posterior and dorso-ventral axes.

4) formation of synaptic connections and neural circuits - developing neurons extend cellular projections, neurites, which traverse long distances to connect appropriately to their target cells.

5) elimination of supernumerary neurons and neuronal connections - neuronal apoptosis is thought to play a role in sculpting the developing CNS and regulating its cell number.

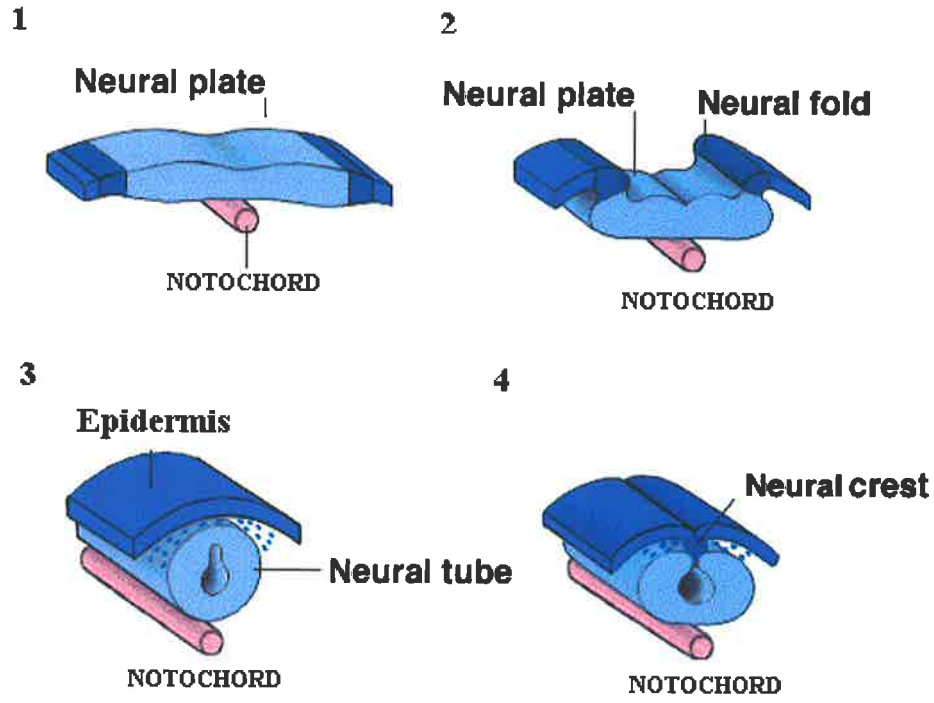

Figure 1. Schematic representation of neural tube formation. The image represents a cross-section of a neurulation-stage amphibian embryo at the prospective spinal cord level. Dorsal is up. Apart from the notochord, no other mesodermal structures (e.g. somites) are shown. (Future) epidermis is shown in blue, neural tube/neural plate in pale blue and the (prospective) neural crest in dark blue. **1.** Neural plate is induced from ectoderm during neural induction by the underlying mesoderm. **2.** The edges of the neural plate fold and the neural plate 'sinks' below the surface of the ectoderm. **3.** The 'folds' of the neural plate have fused to form a neural tube. At their junction, the 'folds' give rise to neural crest. **4.** Neural crest cells disperse throughout the embryo giving rise to various neural (e.g. dorsal root ganglia) and non-neural tissues (e.g. cartilage). Reproduced from Phelps (1998) with permission.

1.1. Mechanisms of neural induction

1.1.1. Spemann's organiser and BMP antagonists as putative neural inducers in *Xenopus*

The vertebrate nervous system forms from ectodermal cells in response to inductive signals from Spemann's organiser. This developmental process, termed neural induction, was discovered by Spemann and Mangold (1924, see ref. Gilbert 2000). Subsequently, Hensen's node (i.e. the tip of the primitive streak; Gilbert 2000) in amniotes and the embryonic shield (Oppenheimer 1936, cited in Streit and Stern 1999) in teleosts were identified as functional homologues of Spemann's organiser. While these studies showed that the organiser is sufficient for ectopic neural tissue formation, they did not address its necessity for neural induction (i.e. whether neural tissue forms in the absence of the organiser). Also, the molecular nature of the neural inducing signals emanating from the organiser remained elusive due to limitations imposed by existing techniques.

Two technological breakthroughs of the early 1990s, the deployment of definitive neural markers and expression cloning, were instrumental in the identification of multiple putative neural inducers in *Xenopus*. All of these candidate neural inducers, Noggin (Lamb et al., 1993), Chordin (Sasai et al., 1994), Follistatin (Hemmati-Brivanlou et al., 1994), Cerberus (Bouwmeester et al., 1996) and Xnr3 (Hansen et al., 1997) are secreted factors expressed in the organiser at the gastrula stage, consistent with a role in neural induction. They meet the main criterion for a neural inducer – the ability to induce neural tissue in the absence of other tissues (especially the mesoderm). They also share the ability to inhibit signalling by bone morphogenetic proteins (BMPs). The BMPs are required for promoting epidermal fate during gastrulation, when ectodermal cells choose between neural and epidermal fates (Wilson and Hemmati-Brivanlou, 1995). At the same time, *in vitro* experiments demonstrated that undifferentiated ectodermal cells were able to initiate neural marker gene expression in the absence of candidate neural inducers. Taken together, these results suggested that epidermis is an induced fate whereas neural tissue is an embryonic default fate, which is uncovered by neural inducers operating by antagonising epidermal inducers (Harland, 2000).

1.1.2. Requirement for BMP antagonists in neural induction in other vertebrates

To determine whether the *Xenopus* candidate neural inducers are required for normal neural development in other vertebrates, the phenotypes of mutants were examined in mice and zebrafish. Interestingly, null mutant mice for *noggin* (McMahon et al., 1998), *follistatin* (Matzuk et al., 1995) and *cerberus* (Simpson et al., 1999) form a fairly normal neural plate and display neural patterning abnormalities only at later stages. This lack of phenotypic effects cannot be explained simply by redundancy - a neural plate forms even in a mouse embryo double mutant for *noggin* and *chordin* (Bachiller et al., 2000).

In zebrafish, *dino* (or *chordino*) is the only known mutant with a reduced neural plate phenotype caused by mutated BMP antagonists (Hammerschmidt et al., 1996; Schulte-Merker et al., 1997). However, because *dino* also has a 'ventralised' phenotype - defects in dorsal mesoderm (which includes the organiser) - its neural phenotype may be indirect, resulting rather from deficits in the neural-inducing tissue than a nonfunctional neural inducer. Mutations in genes encoding BMPs (*swirl*, Kishimoto et al., 1997; *snailhouse*, Dick et al., 2000) or BMP signal transducers (*somitabun*, Hild et al., 1999) yield opposing, dorsalised phenotypes with excess neural plate and dorsal mesoderm. These data from mutant phenotypes and expression analysis suggest that regulation of BMP signaling is important for neural and mesodermal development whereas the molecular mechanisms of neural induction may differ among various vertebrate species. Taken together, it is likely that multiple, partially overlapping mechanisms operate during neural induction and suppression of BMPs is but one of these.

1.1.3. Genes linking neural induction and neurogenesis

Neural induction leads to the expression of positive and negative regulators (proneural and neurogenic genes, respectively) of neural determination. Homologues of the *Drosophila* proneural genes *achaete-scute* and *atonal* genes encoding bHLH proteins are expressed in the neurectoderm during early vertebrate development (reviewed in

Anderson and Jan, 1997 and Bertrand et al., 2002). The expression of proneural genes (vertebrate homologues of *Drosophila* genes *achate-scute* and *atonal*) precedes the activation of *Notch/Delta*-related neurogenic genes, which mediate lateral inhibition, a process negatively regulating the determination of neurons. However, relatively little is known about the molecular factors linking extracellularly acting candidate neural inducers to the onset of proneural/neurogenic gene expression, the process controlling the generation of neurons (reviewed in Sasai, 1998 and Sasai, 2001).

The molecules mediating neural induction include proteins encoded by the *Zic* (zinc-finger transcription factors related to *Drosophila odd-paired*) and *Sox* (Sry-related transcription factors) gene families (Nakata et al., 1997 and 1998; Kuo et al., 1998; Mizuseki et al., 1998a and 1998b; Brewster et al., 1998), and *Geminin* (Kroll et al., 1998). Overexpression of *Zic*-related genes *Zicr1* and *Xzic-3* can neuralise the naïve ectoderm, indicating that these genes may act downstream of chordin and BMPs (Mizuseki et al., 1998a). *Zicr1* and *Xzic3* can upregulate the expression of the proneural gene *Xenopus neurogenin* (*Xngnr1*) (Nakata et al., 1997; Mizuseki et al., 1998a). In turn, the expression of *Xngnr1* in three bilateral longitudinal stripes of the neural plate defines the domains where neurogenesis occurs (reviewed in Chitnis, 1999).

Sox-related genes *SoxD* and *Sox2* are positively regulated by *chordin* (a candidate neural inducer) and *Zicr1*. *SoxD* functions in mediating the induction of anterior neural structures (Sasai, 1998), whereas *Sox2* does not induce neural fate on its own, but rather, in conjunction with additional signals, it acts as a competence modifier of unspecified ectoderm. Thus, FGF (which has no neuralising effect by itself) can trigger development of posterior neural development in *Sox2*-injected animal caps (Sasai, 1998).

Xiro1-3 genes (*Xenopus* homologues of the fly *Iroquois* complex) are all expressed in the medial-intermediate region of neural plate; their expression is dependent on both neural inducers and posteriorising molecules (e.g. FGF) (Bellefroid et al., 1998; Gomez-Skarmeta et al., 1998). Overexpression of *Xiro* mRNAs causes the expansion of the *Sox2/3* expressing region of the neural plate. Interestingly, *Xiro* genes do not activate *neurogenin*, instead, they suppress this gene and activate another proneural

gene, *XASH-3* which is thought to be involved in dividing the neurectoderm into neural plate and neural crest forming territories (Morgan and Sargent, 1997).

In conclusion, according to the 'default' model of neural induction, inhibition of BMPs leads to activation of Sox and Zic-related transcription factors; these genes, in turn, regulate proneural and neurogenic genes, the positive and negative regulators of neuronal determination, respectively.

1.2. Genetic control of neurogenesis

The vertebrate nervous system contains an enormous array of diverse classes of neurons and glial cells. Moreover, all these neuronal classes have defined patterns of three-dimensional distribution, implying that spatio-temporal orchestration of neurogenesis is under very precise genetic control.

1.2.1. Acquisition of neural cell fate is achieved by the interplay between extrinsic and intrinsic cell fate regulators

Vertebrate neurogenesis occurs over a protracted time period, from the stage of neurulation until adulthood. Different classes of neuronal progenitors become determined, or acquire their fates, at different developmental stages (reviewed in Wolpert et al., 1999). The fate of the first neuronal progenitors is determined towards the end of gastrulation whereas other progenitor cells remains plastic and establish their fates at later stages (Gilbert, 2000). Determination of cell fate (including neural cell fate) is a stepwise process involving progressive restrictions in the developmental potential (i.e. the range of available cell fates) of initially multipotential neural progenitor cells (reviewed in Edlund and Jessell, 1999). Thus, at each cell fate restriction 'point', progenitor cells are faced with a choice between alternative fates.

The outcome of this decision-making depends on several factors: the cell's developmental stage (e.g. initially BMPs are required for epidermal development whereas at later stages, they are involved in the establishment of certain neuronal cell fates; reviewed in Bally-Cuif and Hammerschmidt, 2003), cell's developmental history/lineage (that is manifest as the nature of regulatory factors or states of gene expression inherited from a given cell's ancestor), and regulatory interactions between cells (e.g. lateral inhibition and community effects).

Two kinds of regulators, which form interacting regulatory networks, are thought to act in the progressive acquisition of cell fate: extrinsic (extracellular), which are present in the local environment of progenitor cells, and intrinsic, functioning inside the progenitor cell, usually downstream of the extrinsic regulators (reviewed in Harris and Hartenstein, 1999). Extrinsic regulators function as extracellular signalling molecules; they are more important for the initial stages of the progressive neural cell fate determination. Later, when progenitor cells have become progressively more independent from outside signals, intrinsic regulators become crucial (Edlund and Jessell, 1999). The intrinsic factors are often transcriptional activators/repressors or regulators thereof that are either expressed in a cell or inherited from its ancestor (Harris and Hartenstein, 1999).

One example of the interplay between extrinsic and intrinsic neural determinative factors is the specification of ventral cell types along the dorsoventral axis of the developing spinal cord. The three main cell types arise at distinct positions in the cord: motoneurons develop ventrally, interneurons in the medial part and sensory neurons dorsally (reviewed in Briscoe and Ericson, 2001). This establishment of regional identity is achieved by the action of extrinsic patterning signals emanating from tissues adjacent to the neural tube, the floor plate and roof plate (the most ventral and dorsal parts of the neural tube, respectively) as well as the notochord (located ventrally to the neural tube). Sonic hedgehog (Shh), a notochord-derived secreted morphogen, induces the development of floor plate and various ventral neuronal cell type progenitors (precursors of certain classes of motor neurons and interneurons) from undetermined spinal cord cells in a graded fashion. Thus, highest concentrations of Shh induce both floorplate and motoneurons (Roelink et al., 1995), whereas lower concentrations induce interneurons (Ericson et al., 1997). This is accomplished by

concentration-dependent activation or repression of distinct transcription factors (i.e. cell-intrinsic regulators) in the undetermined cells. Subsequent cross-inhibitory interactions between these transcription factors refine their expression domains. The expression of cell-type specific transcription factors and their unique combinations, in turn, defines the domains of progenitors of distinct neuronal cell types (Briscoe and Ericson, 2001).

1.2.2. Proneural and neuorogenic genes – positive and negative regulators of neural cell fate

In both vertebrates and invertebrates, neurogenesis occurs within neurogenic neuroepithelium (neurectoderm) where neurons are born as isolated cells. This process requires an intermediate stage: first, a proliferating neural progenitor cell, also known as neuroblast, is generated, that will, in turn, produce both neurons and glia, the two main cell types of the nervous system. Thus, subsequent to the designation of an embryo's neurogenic region (i.e. as a result of neural induction in vertebrates), undifferentiated cells of the neuroepithelium are faced with a choice between proliferating neural progenitor (in vertebrates) or epithelial (in invertebrates) and neural cell fates. While many neuroepithelial cells have the potential to give rise to neurons, only a few do so at any given time. Such pattern of neuronal development is regulated by cell-cell interactions known as lateral inhibition whereby nascent neurons inhibit their neighbours from adapting the same fate (reviewed in Wolpert et al., 1999). The best-studied model system for the analysis of the genetic mechanisms of lateral inhibition and decision-making during neural fate acquisition is the development of neuroblasts and sensory organ precursors (SOPs), which are neural precursors in the *Drosophila* CNS and PNS, respectively (reviewed in Bertrand et al., 2002). For example, in the fly peripheral nervous system, the even segregation pattern of SOPs (each giving rise to one sensory organ) underlies the eventual even spacing of bristles, a form of external sensory organ (reviewed in Campos-Ortega, 1995). However, at each site of SOP segregation, several neighbouring cells, known as a proneual cluster, have the potential to acquire neural fate, yet only one does so eventually. As the cells of a proneural cluster thus 'compete' for neural fate they

constitute an equivalence group (Campos-Ortega, 1995). Proneural clusters are defined by the expression of proneural proteins (i.e. positive regulators of neural fate that are activated by globally-acting mediators of positional information such as *wingless*), members of the *achate-scute* and *atonal* gene families that encode bHLH (basic helix-loop-helix) DNA-binding proteins (reviewed in Brunet and Ghysen, 1999). Since cells of a proneural cluster 'compete' with each other for neural fate via lateral inhibitory cell-cell interactions (mediated by the function of neurogenic genes, see below), eventually only one cell becomes committed to neural fate. This cell 'escapes' lateral inhibition delivered by neighbouring cells, and thus becomes a neuroblast/sensory organ precursor cell (reviewed in Simpson, 1997). As it inhibits the neighbouring cells from becoming neural precursors, these cells will develop into epidermal cells (i.e. they adopt the alternative fate). Subsequently, a committed neuroblast/SOP detaches from epithelium and generates daughter cells, which, in turn, differentiate into the various cell types (neuronal and glia-like) comprising ganglia/sensory organs (Campos-Ortega, 1995).

In invertebrate and (apparently) vertebrate neurogenesis, lateral inhibition is mediated by the function of the evolutionarily conserved neurogenic genes. These are negative regulators of neural fate as loss-of-function mutations in these genes lead to an increase in neuronal numbers (reviewed in Wolpert et al., 1999). The activity of neurogenic genes leads to down-regulation of the expression of proneural genes in the cells receiving inhibition (reviewed in Simpson, 1997, see also below). The neurogenic genes include the ligand-encoding *Delta* and the receptor-encoding *Notch* (that are used for delivering and receiving inhibition, respectively) as well as *Suppressor-of-Hairless* (encodes a co-activator of Notch), *deltex*, *neuralised* (reviewed in Justice and Jan, 2002; see also below). Stochastic differences in the activity of proneural/neurogenic genes are thought to result in different 'strengths' of lateral inhibition and thus lead to competition among the cells of a proneural cluster. Eventually, only a single cell eventually 'escapes' lateral inhibition and becomes a neural or neuronal precursor (reviewed in Wolpert et al., 1999).

The genetic mechanisms regulating neural cell fate determination in vertebrates are less well understood. However, the observation that homologues of the proneural genes *achaete-scute* and *atonal* (i.e. *ASH* genes, homologues of AS-C genes, and *ATH*

genes, homologues of *atonal*; reviewed in Lee et al., 1997) as well as neurogenic genes *Delta* and *Notch* exist in vertebrate genomes and are expressed during neurogenesis implies that this developmental process in vertebrates is probably based on mechanisms similar to those operating in flies (reviewed in Lewis, 1996; Blader et al., 1997). However, multiple vertebrate homologues exist for each fly proneural and neurogenic gene, and these have both complementary and overlapping expression patterns (reviewed in Bertrand et al., 2002). The increase in the number of genes regulating neurogenesis in vertebrates relative to arthropods is consistent with the considerably more complex range of neuronal cell types seen in the former, implying that the extra genes are required for the formation of vertebrate-specific neuronal cell types (reviewed in Hassan and Bellen, 2000; Bertrand et al., 2002; see also sections 1.2.4 - 'Evolution of vertebrate *Notch* genes' and 7 - 'Amphioxus as a model organism in evolutionary developmental biology' below).

Vertebrate proneural genes can be divided into two families on the basis of sequence homology to *Drosophila* counterparts. Thus, one family is similar to the *Drosophila achaete-scute* (*as-sc*) genes (i.e. the *Mash* subfamily), whereas the other family (including *neurogenins*, the *NeuroD*-like, and the *ATH* subfamily) is related to *atonal* (reviewed in Kintner, 2002). These genes have multiple functions during neurogenesis and appear to form a regulatory cascade that functions in both acquisition of a generic neuronal fate and specific neuronal subtype identities and differentiation (Bertrand et al., 2002). It is thought that proneural bHLH genes activate other bHLH genes that govern neuronal differentiation (Kintner, 2002). The proneural role of several neural bHLH proteins has been demonstrated both by gain- and loss-of-function experiments. For instance, overexpression of *Xash-3*, one of the *Xenopus* homologues of *achaete-scute* genes, results in the formation of increased numbers of neural progenitor cells at the expense of epidermal and neural crest cells (Ferreiro et al., 1994). Likewise, *Xenopus* ectoderm can be converted to neurons by overexpression of *NeuroD*, another bHLH proneural gene (Lee et al., 1995). Elimination of *math-5*, one of the mouse homologues of the *Drosophila atonal* gene, leads to an 80% reduction in the number of retinal ganglion cells (Wang et al., 2001).

Different vertebrate bHLH proneural genes are expressed in specific subsets of neuronal progenitor cell types and are thus required for the development of specific

neuronal subtypes. However, these genes also have partially redundant functions. Deletion of *mash-1*, a mouse *as-sc* homologue that is expressed in both in the CNS and PNS, eliminates olfactory sensory neurons and the peripheral neurons of the autonomic nervous system whereas the CNS itself appears normal (Guillemot et al., 1993). Proneural proteins also contribute to neuronal cell fate acquisition by promoting cell cycle exit (Farah et al., 2000).

As in invertebrate neurogenesis, the numbers of neural progenitors cells 'allowed' to embark on differentiation and the decisions between alternative neural cell fates in vertebrates are regulated by the action of neurogenic genes (reviewed in Lewis, 1996). For instance, overexpression of a dominant negative version of *Delta* in zebrafish embryos leads to the expansion of primary motoneurons at the expense of later-developing secondary neurons (Appel and Eisen, 1998; Haddon et al., 1998). Thus, as in invertebrate proneural clusters, expression of *Delta* in prospective primary neurons leads to inhibition of this fate in surrounding cells (Appel and Eisen, 1998; Appel et al., 2001). Also, the absence of Delta-Notch signalling leads to the increase in the number of interneurons expressing *lim1*, *lim2* and Pax2 and to the reduction in the number of interneurons expressing neurotransmitter GABA (Appel et al., 2001). In addition, gain-of-function experiments in other vertebrate species have revealed that Delta-Notch signalling has an instructive role in the promotion of astrocyte and oligodendrocyte fates (Wang et al., 1998; reviewed in Gaiano and Fishell, 2002).

1.2.3. Mechanisms of Delta-Notch signalling

Lateral inhibition is mediated by the function of neurogenic genes. The inhibitory signal is delivered by the protein product of *Delta* and received by the receptor encoded by the *Notch* gene. Both genes encode for large transmembrane glycoproteins. Following Delta's engagement with Notch, the intracellular domain (NICD) of the receptor is cleaved by a proteolytic processing event known as regulated intramembrane proteolysis (RIP) that is mediated by presenilins and the gamma-secretase complex (reviewed in Selkoe and Kopan, 2003). The NICD is translocated to the nucleus (Struhl and Adachi, 1998), where it associates with a co-factor Suppressor-of-Hairless and activates transcription of the *Enhancer-of-Split*

(*E(spl)-C*; Bailey and Posakony, 1995) gene complex encoding a family of bHLH trancription factors. (Thus, Notch can be defined as a membrane-bound transcription factor.) E(SPL)-C proteins, in conjunction with the Groucho co-repressor, in turn repress the expression of proneural genes *achaete* and *scute* (Paroush et al., 1994), which are themselves positive regulators of *Delta* expression (Kunisch et al., 1994). Consequently, Notch signalling results in down-regulation of both proneural genes and *Delta* in the cells receiving the inhibitory Delta signal (reviewed in Lewis, 1996). Thus, the proneural genes are acting both upstream and downstream of the neurogenic genes (Lewis, 1996) - proneural gene activity in prospective neurons induces expression of *Delta*. This, in turn, leads to Notch-mediated down-regulation of proneural gene expression in the neighbouring (prospective) non-neural cell. Thus, there is a regulatory loop between Notch and Delta which is under the transcriptional control of *achaete/scute* and *E(spl)-C* genes (Heitzler et al., 1996). It is thought that this positive regulatory feedback loop enables amplification of the initially small, random differences in the strength of lateral inhibitory signal between the cells of a proneural cluster. Thus, eventually, a cell that initially expresses *Delta* at the highest levels will become a neuroblast/sensory organ precursor whereas its neighbouring cells will adopt epidermal fate (reviewed in Simpson, 1997). This type of lateral inhibition is known as unbiased because any epithelial cell within a proneural cluster can adopt this fate. In contrast, in the event of biased lateral inhibition, the choice of cell fate is non-random so that a cell at a particular position will invariably assume a specific fate (Simpson, 1997).

1.2.4. Evolution of vertebrate *Notch* genes

We extended the cDNA library synthesis method described in Paper I to develop a novel technique for isolating unknown flanking DNA (described in Paper III). As this technique was used to obtain part of the sequence of the amphioxus *Notch* gene that subsequently served as a basis for a phylogenetic comparison of *AmphiNotch* to other vertebrate *Notch* genes (described in Paper IV), I will briefly describe the evolution of these genes in this section.

In addition to their well-established role in mediating lateral inhibition, *Notch* genes are central components of numerous other evolutionarily conserved intercellular signalling pathways across the animal kingdom, both during developmental and physiological processes (reviewed by Artavanis-Tsakonas et al., 1999). Extracellularly, the Notch receptors consist of 10-36 EGF (epidermal growth factor)-like repeats and three LIN (Lin/Notch/Repeat) repeats. Intracellularly, they possess a RAM domain, 6-7 ankyrin/cdc10 repeats flanked by NLS (nuclear localisation signal) sequences, and PEST and OPA (glutamate-rich) regions (reviewed in Nam et al., 2002; in vertebrates, the OPA-region is present only among the members of the *Notch1* subfamily; Nam et al., 2002; Lardelli et al., 1994). In vertebrate *Notch* genes, individual repeats within these subdomains show highest similarity to their counterparts in *Drosophila Notch* (Lardelli et al., 1994), implying that, during evolution, there has been selective pressure for conservation of the overall structure of the protein (Lardelli et al., 1994). Thus, all vertebrate *Notch* genes and the protovertebrate *AmphiNotch* contain the full 'ancestral' complement of 36 EGF repeats (excepting *Notch3* that lacks two EGF repeats; Lardelli et al., 1994) that is also characteristic of *Drosophila Notch* (Wharton et al., 1985). This contrasts sharply with homology relationships between several other families of developmental regulatory proteins where particular domains are strongly conserved whereas the rest of the molecule bears no significant sequence similarity to the corresponding region of its homologues. (For example, the only conserved part of the T-box protein is the T-box domain itself; Wilson and Conlon, 2002; see also section 2 - 'T-box genes in animal development' below).

Notch, like many other developmental genes, is present as a single copy in invertebrates and multiple copies in vertebrates (reviewed in Lardelli et al., 1995). The two copies of *C. elegans*, *lin-12* and *glp-1*, are thought to have arisen as a result of an independent, lineage-specific duplication event (Maine et al., 1995). A single *Notch* homologue also exists in amphioxus (Paper IV). This implies that an increase in the number of *Notch* genes might have contributed to the evolution of vertebrate-specific structures. However, as vertebrate Notch proteins are structurally very similar, the nature of the mechanism(s) by which the additional *Notch* genes exert their novel, vertebrate-specific developmental effects remains unclear (Kortschak et al., 2001). On the one hand, it is possible that the additional *Notch* copies encode

proteins with equivalent biochemical activities in which case their divergent developmental roles may rely on differential expression patterns (Williams et al., 1995). This notion is supported by the observation that vertebrate *Notch* genes are expressed in both complementary and combinatorial patterns (Williams et al., 1995; Westin and Lardelli, 1997). An informative example concerns the *Notch1a* and *Notch1b* genes in zebrafish, apparent duplicate orthologues of the mouse *Notch1* gene: the combined expression domains of *Notch1a* and *Notch1b* genes correspond to the expression pattern of the mouse *Notch1* (Bierkamp and Campos-Ortega, 1993; Westin and Lardelli, 1997). Such 'shared labour' is consistent with the duplication-degeneration-complementation model of gene duplication, whereby newly-duplicated genes become fixed in evolution as a result of complementary mutations in the regulatory regions (Force et al, 1999). Furthermore, partial redundancy exists between the mouse *Notch1* and *Notch2* genes (Conlon et al., 1995). A similar situation occurs in the invertebrate *C. elegans*, where the two *Notch* homologues, *glp-1* and *lin-12*, while possessing distinct developmental roles, appear to be biochemically interchangeable (Lambie and Kimble, 1991; Fitzgerald et al., 1993). Also, most Notch proteins that have been analysed bind to the DNA-binding Su(H)/RBP-Jk protein (Fortini and Artavanis-Tsakonas, 1994; Kato et al., 1996) and appear to activate common downstream targets, the HES proteins (reviewed by Baron, 2003). On the other hand, there is also evidence supporting the notion that different Notch proteins possess different biochemical activities. Namely, while the mouse Notch1 protein participates in the activation of transcription of *HES* genes (Jarriault et al., 1995), Notch3 acts as a repressor of these genes (Beatus et al., 1999). Moreover, Notch1 and Notch2 require responses to different cytokines to mediate their inhibition of myeloid differentiation (Bigas et al., 1998).

Collectively, it appears that acquisitions of both novel expression patterns as well as biochemical activities have played a role in the evolution of vertebrate *Notch* genes. A fuller understanding of the evolution of divergent expression domains of vertebrate *Notch* genes awaits structural and functional characterisation of both the *cis*-acting genomic regulatory regions of the different *Notch* genes as well as the upstream modulators controlling them.

2. T-box genes in animal development

Members of the T-box gene family encode transcription factors with a conserved DNA-binding domain of approximately 160-180 amino acids (Smith, 1997) and function as important developmental regulators of various developmental processes (such as gastrulation, differentiation of the notochord, heart formation, control of limb identity; reviewed in Wilson and Conlon, 2002). Mutations in two human T-box genes cause abnormalities in embryo development. Namely, haploinsufficiencies of *TBX3* and *TBX5* genes result is Holt-Oram (Basson et al., 1997) and ulnar-mammary (Bamshad et al., 1997) syndromes, respectively, that are characterised by abnormalities in heart, forelimb and genital tract development.

The defining characteristic of the T-box gene family, the T-box encoded DNA binding domain, was first characterised in the *Brachyury* gene (Tada and Smith, 2001). The *Brachyury* (*Bra*, Greek for 'short tail' - also known as *T* (tail)) locus was named after the shortened tail phenotype caused by heterozygous mutations at this locus in mice by Dobrovolskaïa-Zavadskaïa in 1927 (cited in Papaioannou and Silver, 1998). Subsequent studies by Gluecksohn-Schoenheimer (1938, cited in Smith, 1997) showed that mouse embryos with homozygous loss of *Bra* are much more drastically affected than heterozygotes: the primitive streak is greatly congealed, the notochord is missing and mesoderm posterior to somite 7 is absent.

More recent studies in mice as well as zebrafish and *Xenopus* have demonstrated that, between vertebrates, the role of *Brachyury* is evolutionarily conserved. In mice lacking *T*, mesodermal progenitor cells fail to undergo proper morphogenetic movements of gastrulation, which eventually will manifest as absence of axial and posterior mesoderm (Wilson, et al., 1995). In zebrafish, the *no tail* (*ntl*) embryos, resulting from heterozygous loss-of-function mutations in the zebrafish *Bra* homologue *ntl*, have a mutant phenotype highly reminiscent of the mouse *Bra* phenotype (Schulte-Merker et al., 1994). In *Xenopus* embryos, (Cunliffe et al., 1992) misexpression of its *Bra* homologue (*Xbra*) mRNA in prospective ectoderm was

sufficient to convert that tissue's fate from (prospective) epidermal into ventral mesoderm.

T gene encodes a protein of 436 amino acids (Herrmann et al., 1990). The T domain of the T protein (or T-box), comprising the most highly conserved N-terminal 180 amino acid portion of the protein, has been shown to act as sequence-specific DNA binding domain (Kispert and Herrmann, 1993) by *in vitro* DNA-binding experiments. These results, combined with the protein's nuclear localisation, suggested that T protein is a transcription factor.

2.1. The T-box gene family in vertebrates and invertebrates

Initially, the T-box motif was thought to be unique and unrelated to any known genes. However, subsequently a homologous sequence shared between *T* and *Drosophila* gene *optomotor-blind* (*omb*) was identified and shown to encode a protein involved in DNA-binding (Pflugfelder et al., 1992). As sequence homology is confined to the T-box encoding region, it was suggested that the T-box might define a novel gene family, the T-box family. Indeed, the *Caenorhabditis elegans* genome possesses 15 T-box related orthologues whereas there are more than 20 orthologues present in the vertebrate genomes (Tada and Smith, 2001). Many T-box orthologues have also undergone recent (on evolutionary timescale) duplications resulting in the expansion of this gene family (Papaioannou and Silver, 1998). Sequence identity between orthologues, while usually not extending further than the T domain, ranges from 43 to 93% (Tada and Smith, 2001).

2.2. Developmental roles of the T-box genes

The initial study of the expression patterns of five different members of the T-box gene family revealed that these genes are expressed in multiple tissues encompassing the derivatives of all three germ layers, suggesting that this gene family has diverse roles in development (Chapman et al., 1996). However, it is not quite clear how different T-box genes exert their specific developmental effects (Smith, 1999). Namely, all the T-box proteins tested so far are able to bind to the same target sequence as the Brachyury protein. Thus, their specificity apparently cannot be

accounted for by differential DNA binding (Tada et al., 1998; Hsueh et al., 2000). An alternative explanation for the specificity of different T-box proteins is that co-factors unique to a given cell type confer specificity to different T-box proteins. However, relatively little is known about the protein partners of T-box proteins - to date, only one interacting protein has been characterised. Namely, T-brain-1 (Tbr-1), a T-box protein required for several early events in the development of the mammalian CNS cortex (Hevner et al., 2001), was identified as an interacting partner of CASK/LIN-2 (Hsueh et al., 2000), a membrane-associated guanylate kinase essential for EGF receptor localisation and signalling in *C. elegans* (Kaech et al., 1998). The complex of CASK and Tbr-1 has, compared to Tbr-1 alone, a 10-fold increased affinity to the 'T-element' (the DNA target sequence for Brachyury).

2.3. T-box genes in the control of mesoderm development

A number of T-box genes, including *Brachyury* and its orthologues in *Xenopus* and zebrafish, have been implicated in the control of the morphogenetic movements of the mesodermal precursors during gastrulation and tail formation as well as in specification of certain mesodermal cell fates (Smith, 1999). In *Xenopus*, two other known T-box genes are expressed in early embryos: *Eomesodermin* and *VegT*. *Eomesodermin*, like *Xbra*, is initially activated pan-mesodermally, but unlike *Xbra*, is excluded from the notochord at later stages (Ryan et al., 1996). *VegT* (or *Xombi*, *Antipodean*, *Brat*) is the only known maternally expressed T-box gene. Its maternal expression is restricted to the vegetal hemisphere of the egg (which will contribute to the endoderm) whereas the zygotic expression domain is pan-mesodermal (Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996; Horb and Thomsen, 1997). Mutational analysis of the possible functions of *VegT* in vertebrate mesoderm formation has been performed in zebrafish, where the mutant phenotype resulting from a null allele in *spadetail*, the zebrafish *VegT* orthologue, has been characterised (Griffin et al., 1998; Smith, 1999). In $spt^{-/-}$ embryos the trunk somites (i.e. paraxial mesoderm) are missing, resulting in the accumulation of misplaced presumptive trunk paraxial cells in the tail (Kimmel et al., 1989). Although *spadetail* is expressed in the progenitors of both the trunk and tail mesoderm, tail somites still form in $spt^{-/-}$

embryos. *no tail* (*ntl*), the zebrafish orthologue of *Brachyury*, is also expressed in the progenitors of trunk and tail paraxial mesoderm (and in the notochord), but is only required in the tail - embryos null mutant for *no tail* have defects only in the tail paraxial mesoderm (and notochord), whereas trunk somites are unaffected (Schulte-Merker et al., 1994). This observation suggests that *spadetail* and *no tail* are redundant in the tail and trunk, respectively. *spadetail* has also been implicated in regulating cell fate - in embryos lacking *floating head* (*flh*), a notochord-specific homeobox gene, *spt* is ectopically expressed in the prospective notochord which develops as muscle rather than notochord (Halpern et al., 1995; Melby et al., 1996; Yamamoto et al., 1998). As this *trans*-fating event does not occur in *flh;spt* double mutant embryos, *floating head* is thought to inhibit (directly or indirectly) the expression of *spadetail* in axial mesoderm. Both *spt* and *ntl* are positively regulated by FGF signalling, which has been implicated as an important player in the mesodermal development of zebrafish, mice and *Xenopus* (Kimelman and Griffin, 2000; see also section 2.4 - 'Downstream targets and upstream regulators of T-box genes' below).

How do *spadetail* and *no tail* exert their control of morphogenetic movements and mesoderm differentiation? Griffin et al. (1998) proposed that a third T-box gene, zebrafish *tbx6*, which from mid-gastrulation onwards is expressed similarly to *spadetail* (Hug et al., 1997), might be an important player downstream of *ntl* and/or *spt*. A null allele in mouse *Tbx6* - which is apparently not an orthologue of the zebrafish *tbx6* but still has a remarkably similar expression pattern - has a drastic effect on mesodermal development resembling the $spt^{-/-}$ phenotype. In these mice, the prospective paraxial mesoderm posterior to the forelimb bud adopts a neural fate rather than mesodermal fate forming two ectopic neural-tube like structures flanking the original neural tube (Chapman and Papaiannou, 1998). These mice also have an abnormal accumulation of mesenchymal cells in the tail bud (Chapman and Papaiannou, 1998). Thus, similarly to *T*, *spadetail* and *Eomesodermin*, *Tbx6* functions in both mesodermal cell fate specification and control of cell movements.

Paraxial protocadherin (papc), a cell adhesion molecule, has been implicated as a downstream effector of *spadetail* function (Yamamoto et al., 1998). Its expression domain resembles that of *spadetail* and embryos injected with mRNAs encoding the

dominant negative version of papc exhibit defects in convergence-extension movements resembling the *spadetail* mutants. It is possible that the role of papc is not conserved in all vertebrates - mice with a targeted disruption to the gene encoding papc (*mpapc*) have no defects in mesodermal derivatives (skeleton) and are viable and fertile (Yamamoto et al., 2000).

In addition to its role in controlling adhesion and motility of mesodermal precursors, *spt* is thought to exert its effect on somite formation by promoting the differentiation of presomitic mesoderm from tailbud progenitor cells (Griffin and Kimelman, 2002).

2.4. Upstream regulators and downstream targets of T-box genes

To date, several upstream and downstream genes interacting with T-box genes have been identified. It now appears that the molecular pathways where different T-box genes function are often similar (reviewed in Wilson and Conlon, 2002). From an evolutionary viewpoint, this is consistent with the observation that complete gene networks are more likely to be conserved completely (or at least partially) as regulatory units than are the separate genes comprising these networks (Raff, 1996).

So far, only a handful of upstream regulators of T-box genes have been identified. Most of the work has concentrated on *Brachyury*'s regulators (reviewed in Smith, 1999). Nevertheless, some evidence has accumulated suggesting that the expression of many different T-box genes can be regulated by similar upstream regulatory factors, such as activin, FGF and Wnt signalling pathways (Wilson and Conlon, 2002).

Various approaches have been used for uncovering the targets of T-box genes, including analysis of genes with expression patterns similar to certain T-box genes (i.e. candidate gene approach) and subtractive hybridisation screens for genes activated by T-box genes (reviewed in Tada and Smith, 2001). *tbx6* and *papc* in zebrafish as well as *Xnr1* (*Xenopus* nodal related) and *eFGF* in *Xenopus* are expressed in a similar pattern to certain T-box genes (reviewed in Smith, 1999). Although *papc*

is genetically downstream of *spadetail* (i.e. zebrafish *VegT*; see above), its expression may not be directly activated by *spadetail* as forced expression of *VegT* does not induce *papc* expression in *Xenopus* animal cap explants (Tada and Smith, 2001). Other studies have shown that *tbx6*, *papc*, *Xnr1* and *eFGF* function downstream of *VegT* in *Xenopus* (reviewed in Tada and Smith, 2001).

Two subtractive hybridisation screens have been conducted in *Xenopus* to uncover target genes of *Xbra* (Tada and Smith, 2001). Among other genes, these screens identified four novel homeobox-containing genes, termed *Bix*1-4 (*B*ra-*i*nduced homeobo*x*). *Bix1* is normally expressed in the gastrula mesoderm and can be induced ectopically by *Xbra* expression in the ectoderm in the absence of protein synthesis, demonstrating that *Bix1* is directly activated by Xbra (Tada et al., 1998). Yet another T-box gene, *Eomesodermin* (that is required for mesoderm formation - Russ et al., 2000), is implicated in the activation of a mouse homologue of the *Bix* gene family, *Mml*. Namely, *Mml* expression is lost in mice embryos with a targeted disruption of *Eomesodermin* (Russ et al., 2000).

The screen for Xbra targets also identified *Xwnt11*, a previously characterised member of the Wnt family of signalling molecules (Ku and Melton, 1993). During morphogenetic movements of gastrulation, *Xwnt11* is expressed similarly to *Xbra* (Ku and Melton, 1993). The fact that loss of *Xbra* function causes more extensive defects compared to loss of *Xwnt11* function (Smith et al., 2000), affecting both convergent extension as well as mesodermal differentiation, indicates that *Xwnt11* mediates only one function of *Xbra*: regulation of cell movements (Smith et al., 2000). Analysis of zebrafish *silberblick* (*slb*) mutants, lacking functional *Wnt11*, supports this notion - in *slb* embryos, convergent extension movements, but not mesodermal specification, are affected (Heisenberg et al., 2000). The fact that *Xwnt11* is involved in control of cell movements but not mesodermal differentiation and that the opposite holds true for *Bix* genes suggest that different downstream genes mediate different functions of *Xbra*.

Taken together, T-box genes have been shown to be crucial for numerous developmental processes in both vertebrate and invertebrate embryos. There is mounting evidence from studies of different vertebrate *T-box* genes suggesting that these genes often function in similar pathways. The conservation of expression

patterns and functions of different vertebrate T-box orthologues implies the existence of conserved regulatory mechanisms controlling such expression patterns.

3. Genetic screens for discovering novel developmental control genes

Systematic large-scale genetic analysis of embryo development consists of two main steps: identification of novel developmental control genes, and a detailed investigation of the regulatory interactions between the newly identified developmental control genes. Several methods exist for uncovering genes regulating embryo development (or any biological process with a major genetic component). Systematic strategies for identifying all the relevant genes controlling a given biological process are known as genetic screens.

3.1. Recessive mutation screens

Mutation screens are based on the notion that loss-of-function mutations (which usually behave recessively) in a gene important for development of a given structure may result in an identifiably mutant phenotype (e.g. developmental abnormality) of that structure. The screens consist of randomly mutagenising the genomes of parental germ cells (in one parent only) and subsequently scoring the mutation-carrying progeny for phenotypes of interest (changes in morphology or cell type formation etc). Mutation screens are advantageous for uncovering genes with unique, non-redundant functions, and have been used very successfully is various model organisms, both invertebrates and vertebrates. Nüsslein-Volhard and Wieschaus (1980) performed the first mutation screen in *Drosophila*; the vertebrate homologues of the genes identified in this pioneering screen served as valuable guidance for studying vertebrate developmental genetics and unravelling signal transduction (Gilbert, 2000). Also, this screen subsequently served as a model for a mutation screen for novel developmental genes in zebrafish (Driever et al., 1996; Haffter et al., 1996).

In mutation screens, mutations are induced either chemically or by inserting exogenous DNA. The main disadvantage of chemical mutation screens is that the

identification of the mutant allele requires positional cloning, which is complicated and laborious. The latter approach, known as insertional mutagenesis, is based on the fact that insertion of DNA - retroviral, transgenic or transposon-mediated - can disrupt the function of a nearby gene (Cooley et al., 1988; Gaiano et al., 1996). In this case, the mutated gene is readily available for standard cloning by isolating the genomic sequences adjacent to sites of exogenous DNA insertion.

Another disadvantage of such screens stems from the fact that most animal genomes are diploid, and thus any recessive mutant allele subsequently has to be made homozygous in order to reveal the mutant phenotype. As this requires extensive crossing mutation screens are relatively arduous, time-consuming and limited to model organisms with low maintenance costs and short generation times. Nevertheless, in spite of the expense, some researchers have embarked on a recessive mutation screen in mice (Miosge et al., 2002; Jun et al., 2003).

Because mutation screens are based on generating visible mutant phenotypes, redundant genes with more subtle phenotypes cannot be readily identified with this screening strategy. Thus, to identify genes with redundant or partially redundant functions, approaches other than mutation screens must be used (see below). Mutation screens have two other fundamental disadvantages. Firstly, when the induced mutations result in a lethal phenotype and the corresponding gene itself is multifunctional, a mutation screen is capable of detecting only the earliest essential function of that gene. Nevertheless, in the modern 'post-genomic era', this drawback can be partially bypassed in organisms for which both a complete genome sequence and methods for inducible gene disruption are available. Thus, to identify novel genes affecting cell division, Gonczy et al. (2000) embarked on a genomic-scale loss-of-function screen of all the putative genes predicted from the sequence of the *C. elegans* genome's third chromosome. This was accomplished by treating the worm embryos with RNAi molecules (i.e. interference RNA, a double stranded RNA which eliminates its cognate transcript; reviewed in Hunter, 1999) against each predicted gene. RNAi based screens are also very convenient as they allow the detection of the mutant phenotype in the single generation. Another fundamental disadvantage of mutation screens is their bias against loci which, when their function is disrupted,

cause reduced viability. However, it is possible to avoid this disadvantage in model animals (e.g. *Drosophila*), where generation of genetic mosaics is feasible.

3.2. Modifier screens and screens with sensitised genetic backgrounds

These screens are mainly used to identify very specific genetic interactions: they aim to identify other genes interacting with a gene of interest. The premise is based on the notion that mutations in the loci interacting with the gene of interest can be identified by their virtue of modifying the mutant phenotype. Modifier screens can identify additional important players in a biological process; however, they cannot reveal whether the identified genetic interaction corresponds to a direct or indirect molecular interaction.

Ideally, in order to be able to identify both positive and negative interactors, it is useful to be able to generate a phenotype that is either quantifiable or of 'intermediate severity'. This can be achieved by using a hypomorphic allele of the gene of interest, by ectopic expression of a wild-type gene or, by using a heterozygous phenotype (if the loss-of-function allele shows a partial phenotype in heterozygous condition). Modifier screens have been mainly used in flies and worms for identifying novel components of numerous genetic pathways.

Screens with sensitised genetic background presume that many redundant (or partially redundant) genes, which have no discernible loss-of-function mutant phenotype, will nevertheless show a phenotype when the dosage of their interacting gene is altered (Rubin et al., 1997). The dosage can be changed by removal or addition of gene copies. In this altered dosage background, an induced mutation in a redundant interacting gene often results in a mutant phenotype.

3.3. Forced expression screens

Expression screen is a variation of modifier screens; it has mainly been used in *Xenopus*, as classical mutation screen in this model organism is not feasible (Gilbert,

2000). This screen is based on forced expression of random cDNAs to produce a detectable 'phenotype', for instance change in cell fate or rescue of a defective phenotype. This strategy was used to identify noggin, a novel factor with mesoderm dorsalising activity and neural induction activities (Smith and Harland, 1992). In this study, random cDNAs were injected into ventralised *Xenopus* embryos (which lack body axis) and assessed for their ability to rescue normal development – i.e. to dorsalise the mesoderm and thus restore the axis.

3.4. Screens for genes with restricted expression patterns (*in situ* transcript hybridisation screens)

As mentioned above, many known developmental regulatory genes are expressed only in a specific region of an embryo. It follows that genes with unknown function(s) exhibiting restricted expression patterns may be important for the development of the regions where these genes are expressed. During an *in situ* hybridisation screen, randomly chosen cDNAs are used to generate antisense cRNAs, which are then used as probes to reveal the expression patterns of the genes corresponding to the random cDNAs. Genes with expression patterns suggestive of a role in the process of interest are then functionally analysed. The main advantage of this approach is the easy access to all genes identified by the screen; moreover, genes with multiple or redundant functions can be detected. However, these screens do not give any immediate information about the function of the identified gene.

In situ hybridisation screens have been used to identify novel developmental control genes in *Xenopus* (Gawantka et al., 1998), mouse (Neidhardt et al., 2000) and zebrafish (Donovan et al., 2002; and Paper I). These screens uncovered known developmental control genes as well as several putative novel developmental regulatory genes. One of the genes identified in our zebrafish *in situ* screen exhibited an expression pattern suggestive of a role in gastrulation/somitogenesis and subsequent experimental disruptions of this gene's function indicated a role in the cell movements of gastrulation (S. Wells, Honours thesis, Adelaide University 2000; and M. Lardelli, personal communication).

3.5. Screens for genes with differential expression patterns

Potential developmental regulatory genes can be identified by comparing gene expression profiles between different tissues, developmental or physiological stages and experimental conditions. This can be achieved by subtractive cDNA cloning and the use of gene-specific cDNA microarrays. Both methods rely on nucleic acid reassociation techniques. In subtractive cloning, cDNAs common to tissues derived from two different sources (e.g. embryonic and adult tissues) can be eliminated since they hybridise to each other whereas cDNAs unique to the tissue of interest are retained (reviewed in Sagerström et al., 1997).

cDNA microarrays are matrices with oligonucleotides/cDNAs of known sequence from a defined set of genes (e.g. all known cDNAs of an organism) at defined positions. Hybridisation of cDNAs present in the tissue or under experimental conditions of interest is compared to that of the 'control' tissue/default conditions. Application of specialised computer algorithms is then used to uncover genes that are under- or overexpressed in the tissue of interest. The great advantages of this method include automation, high throughput and quantifiability. However, the results reported by different groups using comparable tissues are only partially consistent.

4. Basic steps of cDNA library construction

In Paper I, we describe a novel PCR-based method for constructing randomly-primed, directionally cloned cDNA libraries from small amounts of mRNA. Hence, an outline of the basic strategy of cDNA library construction is presented.

Complementary DNA (cDNA) libraries are constructed to isolate the sequences of the genes transcribed specifically during developmental stages and/or in cell-types/tissues of interest (Sambrook and Russell, 2001).

cDNA library construction begins with isolation of cells/tissues expressing the gene(s) of interest. Total RNA or mRNA is then extracted from this tissue material and used as a template to synthesise first-strand cDNA by catalysis with reverse transcriptase, an RNA-dependant DNA polymerase (Sambrook and Russell, 2001). Reverse transcription is primed using either poly(d)T oligonucleotides or random oligonucleotides (usually hexamers). Poly(d)T oligonucleotides are used because they anneal to the polyA tails that are present at the 3' ends of most eukaryotic mRNA molecules. Thus, the use of poly(d)T priming potentially allows synthesis of full-length cDNAs and reduces the likelihood of conversion of non-poly(A)+ RNA into cDNA (McCarrey and Williams, 1994). However, poly(d)T priming is problematic in organisms possessing transcripts with long 3' untranslated regions (3' UTRs) such as zebrafish because the corresponding cDNA clones may not extend into open reading frames (ORFs). Even if the length of 3' UTRs is not prohibitive, the suboptimal processivity of the reverse transcriptase means that 3' regions of mRNAs are frequently over-represented in poly(d)T-primed cDNA libraries (McCarrey and Williams, 1994). To ameliorate this, reverse transcription can be primed using random oligonucleotides (usually hexamers) - that can direct cDNA synthesis from any site in the mRNA template. However, the use of random priming is potentially disadvantageous as it can lead to under-representation of the 3' ends of transcripts in a cDNA library (McCarrey and Williams, 1994). Also, cDNA clones generated by random priming are likely to be shorter than clones produced with poly(d)T priming (Sambrook and Russell, 2001). To enable directional cloning (i.e. whereby the

orientation of every cDNA clone in the cloning vector is the same), a restriction endonuclease recognition sequence can be introduced into the first strand primer.

Following reverse transcription, the product of this reaction, an mRNA-cDNA hybrid, is used as a template for synthesising the second strand of cDNA. Initially, self-priming was the method of choice for performing this step. However, due to this method's relative inefficiency it has been mostly replaced by nick-translation (also known as replacement synthesis) with DNA-dependent DNA polymerase (e.g. T4 DNA polymerase, *E. coli* DNA polymerase I or Klenow fragment thereof) and RNase H acting as catalysts (Sambrook and Russell, 2001). Alternatively, second strand synthesis can be primed using oligonucleotides whose binding sites have been generated by addition of a homopolymer tail (which usually consists of dC residues) to the 3' end of first-strand cDNA (Sambrook and Russell, 2001).

The final step of cDNA library construction involves insertion of double-stranded cDNA into appropriate propagation vectors, commonly based on plasmids or bacteriophage λ. Since it is convenient to work with an oriented cDNA library (i.e. in which all cDNA inserts have the same known orientation), restriction endonuclease recognition sites are usually incorporated into the ends of cDNA molecules (either within linkers or oligonucleotides used for priming cDNA synthesis). Thus, double-stranded cDNA can be digested with appropriate enzymes and attached to the vector that has been previously linearised with the same enzymes. To obtain cDNA inserts of a desired size range, size fractionation is carried out prior to cloning.

A high-quality cDNA library should possess clones corresponding to all expressed mRNA species of the tissue or developmental stage from which the RNA used for constructing the library was originally derived. Furthermore, these clones should be present in frequencies similar to those of their cognate mRNA species in the tissue of origin. The importance of such representative cDNA libraries is underscored by the observation that many transcripts (e.g. mRNAs for different *Notch* genes; Westin and Lardelli, 1997; M. Lardelli, personal communication) encoding developmental control genes occur in very low copy numbers and may thus not be retained throughout the various steps of library preparation (McCarrey and Williams, 1994). Although use of

large quantities of total RNA/mRNA (i.e. in the order of several micrograms) may seem like a valid approach for overcoming this limitation, the quantities of available embryonic tissues, and hence mRNA of interest, are often minuscule. One way to overcome this problem relies on normalisation of cDNA libraries (e.g. a method based on single-stranded reassociation kinetics; Kohchi et al., 1995) that, ideally, results in equal, non-proportional representation of low-abundance and high-abundance transcripts.

Alternatively, to improve retention of all transcripts (including low-abundance mRNAs), cDNA synthesis can be performed on a solid carrier such as oligo(d)T coupled to paramagnetic beads (Lambert and Williamson, 1993). Also, PCR-based methods can be used to amplify cDNAs prior to cloning. However, this generates additional problems associated with relatively low fidelity of PCR-amplified cDNA clones (Ennis et al., 1990) and skewed frequencies of cDNA clones when compared to those of their mRNAs in the source material (Das et al., 2001).

In summary, while a number of methods exist for generating cDNA libraries, many of these have distinct drawbacks. Thus, the choice of the optimal method is largely dictated by the ultimate purpose for which the library will be used.

5. Isolation of unknown flanking DNA sequences

Paper III describes development of a non-specific, nested suppression PCR-based method for isolating unknown DNA sequences adjacent to known DNA regions. Therefore, a brief outline of existing methods used for cloning such DNA is presented below.

Before the invention of PCR, cloning of novel DNA sequences flanking a known DNA sequence required screening of cDNA or genomic DNA libraries for inserts containing additional DNA using radio-labelled known DNA as a probe. However, the library screening approach in 'non-model' organisms, for which genomic and cDNA libraries are not available, requires the construction of these libraries. Furthermore, in some cases, a cDNA library may not contain clones possessing the desired unknown flanking DNA region, e.g. if they do not contain full-length inserts that include sequences corresponding to the 5' ends of mRNAs in poly-(d)T-primed cDNA libraries. Since the advent of PCR, numerous techniques (including various methods for rapid amplification of cDNA ends - RACE) have been developed for cloning unknown flanking DNA (reviewed in Schaefer, 1995; Hui et al., 1998; Das et al., 2001). These techniques are considerably less laborious and, frequently, as reliable as library screening.

To conduct PCR under stringent reaction conditions (whereby the PCR primers only anneal to entirely complementary template sequences) requires knowledge of the sequences of the binding sites of both primers. PCR primers can only be designed if the DNA sequence is known. Thus, one primer is designed so that it will bind in the known region and direct DNA synthesis 'towards' unknown DNA. To create the second ('upstream') primer binding site, an adaptor of known sequence is ligated to the end of unknown DNA. (The procedure is termed 'vectorette' PCR; Arnold and Hodgson, 1991).

Two other types of PCR based approaches for 'walking' in unknown DNA region do not require adaptor ligation. One of these methods, inverse PCR (Ochman et al, 1988;

Triglia et al, 1988) is based on digestion of DNA with a restriction endonuclease that does not cut within the known sequence. The fragments containing sequences of interest (which are identified by Southern analysis) are re-circularised by intramolecular ligation and the resultant template amplified with primers designed to bind in known DNA sequences flanking the unknown DNA. However, this method requires the presence of restriction endonuclease sites within unknown DNA sequence not present within known sequence.

Alternatively, the phenomenon of non-specific priming (also known as random priming) can be utilised. Under certain PCR conditions (e.g. reduced annealing temperature and/or elevated concentration of Mg^{2+} ions), a primer can anneal to partially complementary target sequences (in addition to annealing to its complementary binding site) and direct synthesis of DNA. Thus, a single primer - which binds specifically in the known DNA region as well as non-specifically in the unknown flanking region - can be used to amplify the unknown flanking DNA between the two primer binding sites (Parker et al, 1991; Parks et al, 1991). As the non-specific primer binding can occur in multiple sites along the unknown DNA region, multiple PCR products (of various sizes) are generated (Dominiguez et al, 1994). Theoretically, all these PCR products should possess identical DNA sequences at one end (i.e. corresponding to known DNA where specific priming has occurred) while the sequences of the other end of these PCR products should be non-identical (as non-specific priming has occurred at various positions along the unknown DNA sequence). However, because non-specific binding can occur on a single-stranded molecule which itself has been generated as a result of non-specific PCR, products containing no desired sequences can also be amplified along with PCR products that include the DNA region of interest. Consequently, to be able to obtain a sample enriched for the desired sequences, the desired product(s) have to be identified among the products of the initial non-specific PCR by Southern hybridisation (to confirm their presence) and then specifically re-amplified.

6. Zebrafish as a model system for vertebrate developmental biology

Zebrafish was the model system used for conduction of the *in situ* transcript hybridisation screen (Paper I). As it has become an important model system for vertebrate developmental genetics relatively recently, an overview of the basic embryological and genetic characteristics of this species is presented.

Zebrafish (*Danio rerio*) was introduced to developmental genetics by George Streisinger in early 1980s (reviewed in Grunwald and Eisen, 2002). Because vertebrates share a common basic body plan (Raff, 1996), zebrafish is especially useful for studying early stages of vertebrate development, which are more likely to share common genetic bases than later stages. Zebrafish have a small adult body size (approximately 3 cm) and a large number of progeny (which develop reasonably synchronously) – an average of 200 or more per female per week (compared to an average progeny of 10 per female mice per every three weeks; Driever, 1998). These characteristics, coupled with the fact that zebrafish can tolerate high population densities mean that zebrafish maintenance is relatively inexpensive. However, another important factor for the practicality of genetic analysis, the generation time, is longer in zebrafish than in mice (three months versus six weeks, respectively; Driever, 1998).

6.1. Embryological characteristics of zebrafish

Zebrafish embryos develop rapidly and the basic body plan forms within 24 hours post fertilisation. Since zebrafish embryos develop externally, they can be easily observed (and manipulated) at all stages of embryogenesis (Kimmel et al., 1995). A unique feature of early zebrafish embryos (when compared to embryos of other model organisms) is their optical transparency facilitating both embryological experiments and the screening for phenotypes resulting from mutations perturbing embryonic development. For example, optical transparency is extremely useful for cell ablation,

and for fate mapping by injection of fluorescent and by transplantation of cells/tissue either between two embryos or two different embryonic regions (reviewed in Grunwald and Eisen, 2002). Optical clarity also enables uncomplicated whole-mount histochemical and *in situ* hybridisation procedures for characterising the localisation of proteins and gene transcripts (Grunwald and Eisen, 2002). The only embryological disadvantage of zebrafish is that certain structures are formed by developmental processes not used in other vertebrate embryos. For example, the primordium of the nervous system, the neural keel, forms by a process distinct from invagination of the ectoderm and thus initially becomes a rod-like structure (rather than a tubular one; Kimmel et al., 1994) that cavitates secondarily (Raible et al., 1992; Schmitz et al., 1993). Nevertheless, the relationship between the initial medio-lateral co-ordinates of the neural plate and the dorsoventral coordinates of the neural keel is the same as in the neural tube (Papan and Campos-Ortega, 1994).

6.2. Genetic characteristics of zebrafish

The combined favourable genetic and embryological characteristics have made the zebrafish the most amenable vertebrate model system for conducting genetic screens (reviewed in Grunwald and Eisen, 2002). Two independent large-scale screens for novel mutations affecting the developmental of the zebrafish embryo resulted in the identification of approximately 2000 mutations (Driever et al.1996; Haffter et al., 1996). The conceptual design of these screens was based on the mutation screen in *Drosophila* (Nüsslein-Volhard and Wieschaus, 1980). While these two screens used chemical mutagens, a retroviral-mediated pilot-scale insertion screen has been performed more recently (Gaiano et al. 1996; Amsterdam et al., 1999). More specialised mutation screens aiming to detect more subtle phenotypic changes, usually based on identifying alterations in the expression patterns of marker genes, are underway (reviewed in Patton and Zon, 2001).

To enable cloning and functional analysis of the genes identified in mutation screens, several genetic and genomic tools have been developed. To date, numerous high density genetic maps have been constructed, based on various markers, including

simple-sequence repeat polymorphism and radiation hybrid maps (Shimoda et al., 1999; Geisler et al., 1999). Large-insert libraries (based on yeast, bacterial or P1 artificial chromosomes) have been constructed recently and are commercially available (reviewed in Talbot and Hopkins, 2000). Methods for producing genetic mosaics have been devised to analyse the consequences of transplanting lineage-labelled donor cells from embryos of one genotype (e.g. mutant for the gene of interest) to a host embryo of another genotype (e.g. wild-type) (Grunwald and Eisen, 2002).

Transgenic technologies in zebrafish are currently less advanced than in mice; however, development of new methods or adaptation of existing methods common to other model systems is very rapid (reviewed in Udvadia and Linney, 2003). Genetic manipulation of zebrafish is usually achieved by forced gene expression by injection of cDNA/mRNA. It is relatively easy to obtain embryos expressing an injected reporter gene (under the control of foreign promoters in a mosaic pattern (Westerfield et al., 1992), moreover, it is possible to recapitulate a specific expression pattern by using constructs containing a reporter gene coupled to endogenous regulatory regions (Higashijima et al., 1997). Although targeted gene manipulation via homologous recombination is as yet unavailable, translation of a transcript of interest can be blocked by administering morpholino oligonucleotides complementary to that transcript (Nasevicius and Ekker, 2000). However, as both generation of germ-line chimeras from embryo cell cultures (Ma et al., 2001) and cloning of fertile zebrafish from genetically modified cultured cells is now possible (Lee et al., 2002), it can be envisaged that homologous recombination-mediated gene manipulation technique will become available in zebrafish in the near future (Udvadia and Linney, 2003). For the analysis of spatial and temporal aspects of gene function, inducible transgenics (using heat shock promoters; Halloran et al., 2000) and the GAL4-UAS system of tissue- and stage-specific misexpression have been successfully adopted (Scheer and Campos-Ortega, 1999; Scheer et al., 2001). Remarkably, the optical clarity of zebrafish embryos can be utilised at the level of single cells to induce temporally controlled gene expression that have been placed under the control of a heat shock promoter by focusing a laser-beam (heat source) onto the cell(s) of interest (Halloran et al., 2000).

Ultimately, genetic analysis of zebrafish development provides novel guidance and complementary insights to phenomena observed in other vertebrate model organisms. For example, the function of mammalian homologues of zebrafish genes identified in mutation screens can be further analysed by targeted gene disruption in mice.

6.3. Neuronal classes of the zebrafish developing spinal cord

The work presented in Paper II describes the identity and rostrocaudal distribution of the *spt*-expressing cells of the developing zebrafish spinal cord. Hence, a brief overview of the neuronal classes present in the developing spinal cord is included below.

As in other anamniote embryos, the zebrafish spinal cord possesses both primary and secondary neurons (reviewed in Roberts, 2000; Lewis and Eisen, 2003). Primary neurons, born during gastrulation, are present in fewer numbers, have large somata and complete axogenesis by 24 hpf (Myers et al., 1986). Secondary neurons, born after gastrulation and at later stages, have smaller somata, are present in larger numbers (Myers et al., 1986; Kimmel et al., 1994; Appel et al., 2001), and frequently have thinner axons (Lewis and Eisen, 2003). Various neuronal cell types can be identified among primary and secondary neurons by their unique morphological and molecular characteristics. These include the size and dorsoventral/antero-posterior position of the cell body, neurotransmitter profile, the routes taken by the axons as well as by the protein products and/or transcripts derived from marker genes expressed in these cells.

The zebrafish embryonic spinal cord has a simple structure (Bernhardt et al., 1990; Kuwada et al., 1990), both in terms of its cellular composition and the sterotyped axonal trajectories. This allows many developing neurons to be identified individually (Bernhardt et al., 1990; Kuwada et al., 1990; Figure 2). In zebrafish, as in all vertebrate embryos, the three main neuronal cell types of the developing spinal cord (i.e. motor neurons, interneurons and sensory neurons) are located at different dorso-

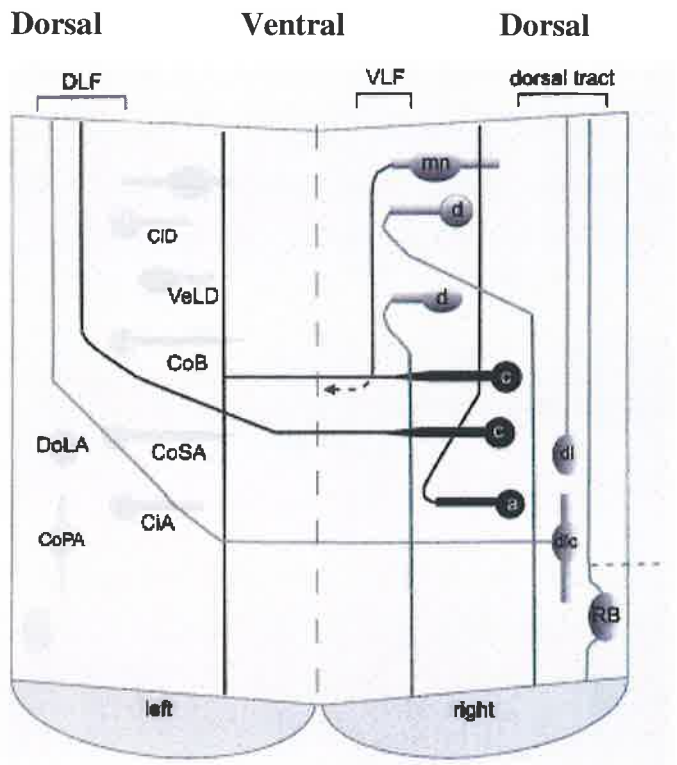

Figure 2. Schematic representation of the neuronal classes of the zebrafish developing spinal cord. One spinal segment is shown as though it had been cut sagittally along the midline and then 'flat-mounted'. Thus, the dashed vertical line indicates the ventral midline. Rostral is up. Defined neuronal types are shown on the left side, and the general neuronal classes are shown on the right side. Thick and thin processes represent dendrites and axons, respectively. Putative excitatory and inhibitory neurons are shaded grey and dark grey, respectively. **Abbreviations**: **a** - ascending neurons; **c** - commissural neurons; **CiA** - circumferential ascending neuron; **CiD** - circumferential ascending neuron; **CoB** - commissural bifurcating neuron; **CoPA** - commissural primary ascending neuron; **CoSA** - commissural secondary ascending neuron; **d** - descending neurons; **dlc** - dorsolateral commissural interneurons; **DLF** - dorsal longitudinal fasciculus; **DoLA** - dorsal longitudinal ascending neuron; **mn** - motor neuron; **RB** - Rohon-Beard neuron; **VeLD** - ventral longitudinal ascending neurons; **VLF** - ventral longitudinal fasciculus. Modified from Roberts (2000) with permission.

ventral levels of the spinal cord (Kuwada et al., 1990). Thus, while motoneurons develop ventrally and sensory neurons dorsally, interneurons arise in the medial part of the spinal cord (Lewis and Eisen, 2003). In the embryonic zebrafish spinal cord, each of the three main neuronal cell types can be further divided into distinct classes according to their characteristic axonal morphology and/or positions along the antero-posterior (rostrocadual) and dorsoventral axes of the embryonic spinal cord (Lewis and Eisen, 2003). Thus, at 18-20 hpf, there are approximately 18 lateral and

apparently postmitotic cell bodies per each spinal hemisegment (Kuwada et al., 1990). Approximately 8-11 of these neurons have projected growth cones by 18 hpf and can be grouped into five classes of neurons (Bernhardt et al., 1990). These include mechanosensory Rohon-Beard (RB) neurons, three classes of interneurons (dorsal longitudinal ascending - DoLA, ascending commissural, and VeLD) and three classes of primary motor neurons (Eisen et al., 1986). Several of these neuronal classes disappear by larval stages. For example, RB neurons die and are subsequently replaced by dorsal ganglion root neurons whereas the later fate of DoLA neurons is unclear (Lewis and Eisen, 2003).

There are several molecular markers that specifically label developing spinal neurons (Lewis and Eisen, 2003). For instance, several members of the *islet* family (*isl1*, *isl2*, *isl3*) are expressed in the primary sensory (i.e. RB) neurons and subsets of the primary motor neurons (Korzh et al., 1993; Inoue et al., 1994; Appel et al., 1995; Tokumoto et al., 1995) while *lim3* is expressed in primary and secondary motoneurons and VeLD interneurons (Appel et al., 1995). CoSA interneurons express *pax2a* and possibly also *evx1* (Mikkola et al., 1992; Thaeron et al., 2000). DoLA interneurons apparently express *spadetail* and *isl1*, *isl2*, and *isl3* (Paper II). VeLD, KA, DoLA and a subset of CoSA interneurons are also recognisable by labelling with anti-GABA antibodies (Bernhardt et al., 1992).

The spacing of motor, sensory and interneurons along the rostrocaudal axis appears to be less ordered when compared to the dorsoventral distribution of these neuronal classes. While the three neuronal classes comprising the primary motoneurons are bilateral, segmentally repeated and present in consistent numbers in each hemisegment (with the exception of VaP neurons, Kuwada et al., 1990; Eisen et al., 1990), the other six classes (various interneurons and sensory neurons) appear to be organised non-segmentally (Kimmel et al., 1991). Their numbers and positions along the longitudinal axis between either different hemisegments of the same embryo or identical segments of different embryos are variable (Kuwada et al., 1990). However, all nine classes of early neurons project stereotyped axons and occupy consistent positions along the dorsoventral axis (Kuwada et al., 1990; Figure 2). For example, Rohon-Beard sensory neurons are located most dorsally and DoLA interneurons are situated immediately ventral to Rohon-Beard cells.

7. Amphioxus as a model organism in evolutionary developmental biology

We used a comparative approach to begin to understand the evolution of *Notch* genes (Paper IV) by characterising the sequence and embryonic expression pattern of the *Notch* gene in amphioxus (*Branchiostoma floridae*). This primitive chordate is the closest living invertebrate relative of the vertebrates (Wada and Satoh, 1994), and has thus been extensively used for studying the relationships between genome complexity and body plan evolution in vertebrates. Like other chordates (i.e. tunicates and vertebrates), amphioxus has a mesodermally-derived notochord located ventrally to a hollow neural tube and bilateral segmented muscle blocks (Shimeld and Holland, 2000). However, morphological structures characteristic to the jawed vertebrates (*Gnathostoma*) such as an endoskeleton, a morphologically segmented neural tube and the neural crest, are lacking. Interestingly, this simple body plan correlates well with a relative lack of genome complexity - in the amphioxus genome, members of many gene families are often present in fewer copy numbers than in vertebrates (Holland et al., 1994; Panopoulou et al., 2003). The vertebrates' duplicate genes/gene families encode proteins with diverse functions, such as transcription factors (Hox, En, Otx, Msx, Pax, bHLH), signaling molecules (BMP, Hh, IGF), as well as 'housekeeping' proteins (cholinesterase, actin, keratin; Shimeld and Holland, 2000). According to the initial comparative studies performed in the early- and mid-1990s, many single-copy amphioxus (and *Drosophila*) genes/gene clusters appear to be represented by four orthologues in vertebrates. For example, while only one *Hox* cluster exists in amphioxus, four clusters are present in vertebrates (Holland et al., 1994). (In *Drosophila*, a single *Hox* cluster has been split into two after the divergence of this lineage form the future chordates; reviewed in Gilbert, 2000). These studies implied that novel, vertebrate-specific morphological characters might have evolved as a result of the expansion of gene families and subsequent co-option of duplicated genes into novel gene control networks (Garcia-Fernandez and Holland, 1996). This notion is consistent with Ohno's (1970; cited in Sidow, 1996) theory of the role of gene duplication in the evolution of morphological complexity that states that a newly-duplicated (and thus initially redundant) gene can acquire additional

role(s) as it is freed from the genetic constraints imposed by natural selection. Acquisition of a novel function also ensures retention of the duplicate gene (Sidow, 1996). This hypothesis is further supported by the observation that vertebrate gene duplicates are often expressed in vertebrate-specific structures (Mazet and Shimeld, 2002).

Since a 1:4 ratio of amphioxus/*Drosophila*-vertebrate orthologues has been observed to occur frequently, it was also proposed that the multiple gene copies in vertebrates originated as a result of two rounds of whole-genome duplication that occurred soon after the divergence of the vertebrate lineage (Holland et al., 1994; Sidow, 1996; Spring, 1997). Subsequent studies revealed that the number of gene copies may have increased either due to one round of whole-genome duplication followed by selective duplication of certain genes/chromosome segments or two rounds of whole-genome duplication followed by an extensive loss of particular duplicates. This conclusion was based on detailed calculation of duplication rates for vertebrate duplicates by comparing the numbers of orthologous groups of genes that are represented by a single copy in *C. elegans*, *D. melanogaster* and *S. cerevisae* to those of the corresponding homologues from amphioxus, a tunicate (*C. intestinalis*), mouse and humans genomes that exist in one or more copies (Panopoulou et al. 2003). While these calculations showed that there had been a twofold increase in the average number of duplicates since the emergence of amphioxus, it is currently unclear whether one or two complete genome duplications have occurred within the vertebrate lineage (Panopoulou et al. 2003; also reviewed in Durand, 2003).

SUMMARY OF PAPERS I-IV AND CONTEXTUAL LINKAGES BETWEEN THEM

Paper I: Simple, directional cDNA cloning for *in situ* transcript hybridisation screens

One of the research aims of my postgraduate research studies was identification of novel candidate genes important for early CNS and somite development in vertebrates. We decided to achieve this by conducting an *in situ* hybridisation screen with riboprobes prepared from randomly chosen cDNA templates derived from gastrulation/neurulation stage vertebrate embryos. A cost-effective *in situ* screen requires abundant supplies of embryos that develop freely and rapidly. Also, to facilitate further studies, the model system should be genetically tractable. Since zebrafish (*Danio rerio*) is the only vertebrate model organism for developmental biology that fulfils these requirements we chose it as a model vertebrate for the screen. Moreover, zebrafish embryos are especially suitable for conducting *in situ* screens due to their optical transparency – this feature greatly facilitates the detection of gene expression patterns of interest.

Another main prerequisite for conducting an *in situ* screen is possession of a cDNA library derived from embryos of appropriate developmental stage (i.e. from 6-9 hours post fertilisation (hpf) zebrafish embryos that are undergoing gastrulation and beginning neurulation; Kimmel et al, 1995). Ideally, the cDNA library used for *in situ* screens should meet a set of criteria. First, the cDNAs should be directionally-cloned, in which case all cDNA inserts have the same known orientation and thus only riboprobes synthesised from one strand have to be tested by *in situ* hybridisation, halving the amount of resources required for conducting a screen. Second, it is important that cDNA clones extend into open reading frames instead of containing sequences corresponding solely to 3' untranslated regions (3' UTRs). This enables the use of sequence analysis to determine whether the cDNA clones that reveal genes with expression patterns of interest encode novel or known protein products. However, at the onset of this project, all available directionally cloned zebrafish embryonic cDNA libraries had been produced by using poly(dT) priming of a reverse

transcription reaction. In such libraries, many cDNA inserts may contain 3' UTRs instead of ORFs due to the fact that the reverse transcriptase used for first strand synthesis frequently may not have elongated the first strand of the cDNA clone to the extent that it encompassed part of the open reading frame (ORF) of the corresponding transcript. Hence, it frequently cannot be determined whether the cDNA clones revealing genes with expression patterns of interest encode novel or known protein products. This is problematic since sequence analysis is the first step in the analysis of such cDNA clones. This obstacle could be alleviated by using directionally-cloned cDNA libraries produced by random priming of the first cDNA strand synthesis. As there were no such libraries available at the time (1997), we set out to construct one. However, existing methods of random-priming frequently require large amounts (i.e. microgram quantities) of mRNA, whereas the quantities of available embryonic tissues, and hence mRNA of interest, are often minuscule. We decided to develop a simpler method that would allow the use of small quantities of mRNA of interest. A brief outline of the procedure follows below.

First, to increase the probability that cDNA clones would extend into ORFs, the reverse transcription reaction was performed using random priming. In addition to an 8 bp stretch of random nucleotides, the first strand primer consisted of two other parts: a restriction endonuclease recognition site to allow directional cloning and an area identical to a region in the second strand primer that would allow the amplification of cDNAs of appropriate size by suppression-PCR. Second, to compensate for the small amounts of mRNA, synthesis of the first and second cDNA strands was conducted on a solid carrier, i.e. streptavidin-coated magnetic beads. (This was achieved by the use of a 5'-biotinylated first strand primer). In addition to the use of solid carrier, the cDNAs were also amplified by PCR prior to cloning. Third, the second cDNA strand synthesis was primed non-specifically at low annealing temperature with the same primer that was then used to amplify the cDNA by suppression-PCR at stringent annealing temperature. This 'shortcut' abolished the need for ligation of a binding site for the second strand primer to the 3' end of the first cDNA strand. Following cloning of cDNAs, redundancy of the library was determined by sequence analysis. This revealed that, out of 62 unique sequences, 59 (11%) were present in one copy whereas 7 clones (11%) were represented by more

than one replicate. 28 (45.2%) of the 62 unique clones exhibited at least 96% identity to known ESTs and 21 (38%) sequences encoded putative open reading frames.

63 cDNA clones were then used for preparing antisense cRNA to reveal the expression patterns of their cognate transcripts. The proportions of clones corresponding to genes whose expression patterns was either ubiquitous (68%), restricted (17%) or undetectable (i.e. when no expression signal was observed even after 4 days of staining) were similar to those described for an *in situ* screen conducted by Gawantka et al. (1998) with 1765 cDNAs derived from neurula-stage *Xenopus* embryos. This observation indicates that the non-specific priming based cDNA synthesis can generate cDNA libraries with the proportion of developmentally expressed genes (as loosely determined by the proportion of restricted expression patterns) comparable to other embryonic-stage cDNA libraries. A further RT-PCR based analysis of three cDNA clones not revealing any expression suggested that the cDNA library contains clones representing mRNAs undetectable by *in situ* hybridisation, suggesting that our cDNA synthesis method is effective in the detection of transcripts present at very low abundance.

Paper II: The identity and distribution of the neural cells expressing the mesodermal determinant *spadetail*

Several cDNA clones from the 6-9 hpf zebrafish cDNA library (see above) revealing corresponding genes with restricted expression patterns were subjected to further analysis (S. Wells, Honours Thesis, Adelaide University 2000; M. Lardelli, personal communication, and Paper II). One of these cDNA clones identified a gene whose expression is confined to the presomitic mesoderm and isolated, apparently irregularly distributed cells of the developing spinal cord at 22 hpf. As our aim was to identify novel candidate developmental control genes, we sequenced this clone to determine whether it represented a known or a previously uncharacterised ORF. Sequence analysis showed that it encoded a fragment of *spadetail/Tbx16* (*spt*), a member of the T-box gene family of transcription factors (Griffin et al, 1998; Ruvinsky et al., 1998). However, although *spt* was known to have a role in mesoderm development (Kimmel et al., 1989), its function in neural development had not been investigated. Also, the identity of the spinal cord cells expressing *spt* had not been conclusively determined:

they had been putatively identified as Rohon-Beard neurons (Ruvinsky et al., 1998; Griffin et al., 1998), a type of sensory neurons located in the most dorsal part of the developing spinal cord.

The work described in Paper II was performed to conclusively identify the *spt*-expressing cells as well as to characterise their distribution along the rostrocaudal axis and to obtain some insight into the mechanisms that might be responsible for the control of this distribution. The *spt*-expressing neural cells were identified as dorsal longitudinal ascending (DoLA) neurons based on their co-expression of neural marker genes *huC*, (a general early neuronal marker) and *isl1*, *isl2*, *isl3* (putative markers of Rohon-Beard neurons) and their dorsoventral position (i.e. immediately ventral to the most dorsally located Rohon-Beard neurons). These cells also fail to express *valentino*, another putative marker of Rohon-Beard neurons. We also showed that *spt* mRNA is transported into rostral processes emanating from *spt*-expressing cells. Statistical analysis of these cells' rostrocaudal distribution revealed that, at 24 hpf, rostral spinal segments caudal of the 5th-formed somite contain higher numbers of *spt*-expressing neurons. Extended staining of 24 hpf embryos for *spt* expression showed that the dorsocaudal regions of somites express *spt* at the same dorsoventral level as the *spt*-expressing neurons of the spinal cord. The observation that the somitic cells expressing low levels of *spt* are frequently juxtaposed to *spt*-expressing neurons implies that both neural and somitic *spt* expression is either dependent on a common positional signal or that *spt*-expressing somitic cells pattern the flanking *spt*-expressing neurons in an irregular fashion.

Paper III: Nonspecific, nested suppression PCR method for isolation of unknown flanking DNA

A number of research projects in our lab included isolation of unknown DNA sequences neighbouring known sequences. As these projects involved charaterisation of the *Notch* homologue of amphioxus (*Branchiostoma floridae*) - whose cDNA was had not yet been cloned into bacteriophage libraries of randomly-primed cDNA, we had to resort to PCR-based methods for amplifying these sequences. However, most existing methods proved to be either insufficiently sensitive or technically

complicated. Thus, we decided to adapt the cDNA library construction technique we had developed (described in Paper I) - which is based on non-specific priming of cDNA 2nd strand synthesis/suppression-PCR - to devise an improved method for obtaining unknown flanking DNA. This method, termed nonspecific, nested suppression-PCR (NSPS-PCR) consists of two rounds of PCR. In the initial reaction, a gene-specific single primer was used at a low annealing temperature so that it would anneal both specifically within known DNA sequence and non-specifically further upstream (please see Figure 1, Paper III). Consequently, PCR products of different lengths (including those containing undesired sequences) were generated. (In the first round of PCR, the use of a single primer results in the formation of inverted repeats at the termini of PCR products; this, in turn, allows size selection of PCR products by suppression PCR). Subsequently, to achieve specific reamplification of the desired DNA sequences, the initial reaction was re-amplified using a primer that was otherwise identical to the first reaction primer apart from having been extended by 6 nucleotides from the 3' end of the original primer. This technique allowed us to us to obtain novel flanking DNA sequences from known DNA derived from two different templates, namely amphioxus cDNA (*AmphiNotch*) and zebrafish genomic DNA (tyrosinase gene exonic sequence) suggesting that this technique is applicable to a range of substrates with various degrees of complexity.

Paper IV: Characterisation and developmental expression of the amphioxus homolog of *Notch* (*AmphiNotch*): evolutionary conservation of multiple expression domains in amphixous

One of the main interests of our laboratory concerns the evolutionary relationships between *Notch* genes of invertebrates and vertebrates. We have investigated this both by bioinformatical approaches (based on sequence data generated by ourselves and other researchers) as well as comparing the domains of embryonic expression of *Notch* genes in various species.

The genomes of *Drosophila* and lower deuterostomes (i.e. ascidian tunicates and sea urchins) possess a single *Notch* gene (Artavanis-Tsakonas et al., 1999; Hori et al., 1997; Sherwood and McClay, 1997). Whereas the fly *Notch* is expressed in multiple

embryonic and larval tissues, its ascidian and tunicate counterparts have very limited expression domains (Hori et al., 1997; Sherwood and McClay, 1997, 1999). In contrast, multiple *Notch* genes exist in vertebrates (four in the mouse and the zebrafish), all of which are expressed in many tissues in a partially overlapping manner. This leads us the question as to when on the timescale of vertebrate evolution might the different copies of the *Notch* and their complex expression domains have originated.

To examine this problem, we isolated the single *Notch* gene from amphioxus (*Branchiostoma floridae*), an invertebrate chordate, and characterised its intron/exon structure, coding sequence and embryonic expression pattern. Amphixous, despite having been separated for the vertebrate evolutionary branch for ~500 million years, is the closest extant relative of vertebrates, and is often used as a 'substitute' for the most recent common ancestor of the cephalocordate and vertebrate lineages (Wada and Satoh, 1994; Garcia-Fernàndez and Holland, 1996). Various standard cloning methods had enabled us to clone all but the most 5' region of *AmphiNotch* cDNA. As there were no randomly-primed cDNA libraries available from this organism (which are more likely to possess cDNA inserts corresponding to 5' ends of transcripts), we had attempted to use various RACE techniques, including ones commercially available as kits without success. We then decided to use the NSPS-PCR method that we had developed (described in Paper III). This allowed us to clone a 0.5 kb region of cDNA predicted to lie ~500 bp downstream from the translation start site. (The sequence of the remaining 0.5 kb region was deduced from alignment of vertebrate *Notch* gene sequence against sequence information from genomic cosmids containing *AmphiNotch* sequences). Southern analysis revealed that, like *Drosophila* and lower deuterostomes, amphioxus only has one copy of the *Notch* gene. This is consistent with the notion that the genome of early vertebrates underwent two rounds of (at least partial) duplication. Sequence alignments with *Drosophila* and mouse *Notch* genes revealed that the extracellular domain of AmphiNotch has the complete set of 36 EGF repeats. This, and additional phylogenetic analyses suggest that *AmphiNotch* is similar to the ancestral *Notch* gene from which all vertebrates genes have been derived. This notion is also supported by the observation that the expression pattern of *AmphiNotch* (including mesendoderm, nerve cord and the amphioxus equivalent of kidney) corresponds to the expression domains of all vertebrate *Notch* homologues combined.

PAPER I

Tamme, R., Mills, K., Rainbird, B., Nornes, S. and Lardel, M. (2001). Simple, Directional cDNA Cloning for In Situ Transcript Hybridization Screen. *BioTechniques*, v. 31(4), pp. 938-946.

PAPER II

BMC Developmental Biology

BioMed Central

Research article

The identity and distribution of neural cells expressing the mesodermal determinant *spadetail*

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Published: 18 July 2002

BMC Developmental Biology 2002, **2**:9

Received: 26 March 2002
Accepted: 18 July 2002

This article is available from: http://www.biomedcentral.com/1471-213X/2/9

Abstract

Background: The *spadetail* (*spt*) gene of zebrafish is expressed in presomitic mesoderm and in neural cells previously suggested to be Rohon-Beard neurons. The mechanism(s) generating the apparently irregular rostrocaudal distribution of *spt*-expressing cells in the developing CNS is unknown.

Results: *spt*-expressing neural cells co-express *huC*, a marker of neurons. These cells also co-express the genes *islet-1*, *-2* and *-3* but not *valentino*. The *islet-1* gene expression, irregular distribution and dorsolateral position of *spt*-expressing cells in the developing CNS are characteristic of dorsal longitudinal ascending (DoLA) interneurons. Shortly after their birth, these neurons extend processes rostrally into which *spt* mRNA is transported. At 24 hours post fertilisation(hpf), *spt*-expressing neurons occur most frequently at rostral levels caudal of the 5th-formed somite pair. There is no apparent bias in the number of *spt*-expressing cells on the left or right sides of embryos. Extended staining for *spt*-transcription reveals expression in the dorsocaudal cells of somites at the same dorsoventral level as the *spt*-expressing neurons. There is frequent juxtaposition of *spt*-expression in newly formed somites and in neurons. This suggests that both types of *spt*-expressing cell respond to a common positional cue or that neurons expressing *spt* are patterned irregularly by flanking somitic mesoderm.

Conclusions: *spt*-expressing cells in the developing CNS appear to be DoLA interneurons. The irregular distribution of these cells along the rostrocaudal axis of the spinal cord may be due to "inefficient" patterning of neural *spt* expression by a signal(s) from flanking, regularly distributed somites also expressing *spt*.

Background

The spinal cord of vertebrates shows no apparent morphological metamerism. However, the pattern of motor and sensory axonal projection from the spinal cord shows a metameric distribution that is patterned by the flanking somites [1,2].

In developing zebrafish, both metameric and non-metameric patterns of neuron distribution can be observed.

When primary motoneurons first arise in the developing ventral spinal cord, three such cells are present per hemisegment [3,4]. Mutation of the gene *spadetail* (*spt*) causes changes in somite formation that affect this pattern of motoneuron formation. This shows that motoneuron patterning is controlled by signals from the somites [5–7]. In contrast, the Rohon-Beard sensory neurons in the dorsal central nervous system (CNS) show no segmental distribution and are not affected by mutations affecting somite formation [5]. However, mutations such as *bmp2b/swirl*, *bmp7/snailhouse* affecting signalling by members of the bone morphogenic protein (BMP) family, [8]) and changes in Notch signalling [9][10][11] can affect the number/differentiation of these cells.

Rohon-Beard neurons, when they arise, are sufficiently numerous to be found adjacent to every somite (i.e. in each "hemisegment"). However, a third type of neural cell distribution exists with less than one cell per hemisegment. For example, dorsal longitudinal ascending (DoLA) interneurons are found at a frequency of 0.06 per hemisegment for the 5th- to 8th-formed flanking somite pairs in embryos at 18 hpf [12]. The mechanisms that control these irregular distributions are unknown.

The *spt* mutation was originally described by Kimmel et al. in 1989 [13] as a γ ray-induced mutation affecting trunk development including somite formation. Closer analysis of the effect of this mutation on development has shown that *spt* controls convergence movements and the differentiation fate of mesodermal precursors of the trunk [13–17].

The locus for *spt* mutations was identified by Griffin et al. in 1998 [18]. They showed that the *spt* gene encodes a T-box protein similar to those encoded by the *Xenopus* gene *Xombi* (also known as *Antipodean*, *BraT* or *VegT*) and the chick gene *Tbx6L*. *spt* is transcribed in caudal paraxial mesoderm before its differentiation to somitic mesoderm. *spt* is also expressed in irregularly distributed neural cells that have been suggested, on the basis of their position and distribution, to be Rohon-Beard neurons [19].

In the work described in this paper, we show that the neural cells expressing *spt* have the characteristics of DoLA interneurons. We then examine the distribution of *spt*-expressing neurons on the rostrocaudal axis and on the left and right sides of embryos. Intriguingly, we have discovered low-level expression of *spt* in the dorsocaudal extremities of newly formed somites that corresponds in dorsoventral level and, frequently, rostrocaudal position, to newly formed neurons expressing *spt*. This distribution of *spt* expression suggests the possible existence of an "inefficient" mechanism producing an irregular pattern of neuron distribution based on a regularly patterned flanking structure (somitic mesoderm).

Results

Neural spt-expressing cells have the characteristics of DoLA neurons

Cells expressing *spt* in the developing central nervous system have previously been suggested to be Rohon-Beard neurons [18,19]. To confirm their neuronal nature, we double-stained embryos for *spt* expression and the neuronal marker gene *huC*[20]. We observed coexpression of *spt* with *huC* confirming that these cells are neurons (Figure 1D).

To test the idea that *spt*-expressing neurons are Rohon-Beard neurons we double-stained embryos for expression of *spt* and the *islet* (*isl*)*-1*, *-2* or *-3* genes [6,7,21] or *valentino* (*val*, [22]) that have been stated to be expressed in these cells. Interestingly, the *spt*-expressing neurons also express all three known *isl* genes but not *val* (Figures 1E,1G,1H,1I). In embryos at 22 hours post fertilisation (hpf, at 28.5°C), *spt*-expressing cells express *isl-1* from the moment of their first detection at the caudal end of the developing CNS. *isl-2* and *isl-3* coexpression with *spt* is more easily visible at more rostral levels. In every case, the cells co-expressing the *spt* and *isl* genes are located just ventral to dorsally located cells expressing *isl* genes alone, i.e. Rohon-Beard neurons. The *isl-1* expression, rostrocaudal distribution and dorsolateral position of these cells are characteristic of DoLA interneurons [6,21]. We cannot state with certainty that all DoLA neurons express *spt*, only that all DoLA neurons expressing *isl-1* also appear to express this gene. Contrary to an earlier report [7], we observed expression of *isl-2* and *isl-3* in these interneurons. This might be explained by difficulty in distinguishing DoLA neurons from Rohon-Beard neurons at the rostral levels where *isl-2* and *-3* expression is more easily observed.

spt mRNA is transported into neurite-like structures

Soon after their differentiation in the central nervous system, a rostrally-projecting process of the *spt*-expressing neurons can be observed to contain *spt* mRNA (Figure 1B). This process may, in fact, become the future ascending axon of the DoLA neurons. The transport of *spt* mRNA into this process presumably is an active rather than passive process since other mRNAs, such as those of *huC* and the *islet* genes, are not similarly localised (data not shown).

We attempted to observe the pattern of axonal projection from *spt*-expressing neurons at later times after their differentiation. We stained embryos at 22 hpf to reveal both *spt*-transcription and the presence of acetylated tubulin (that labels axons). Confocal imaging of *spt*-expressing neurons

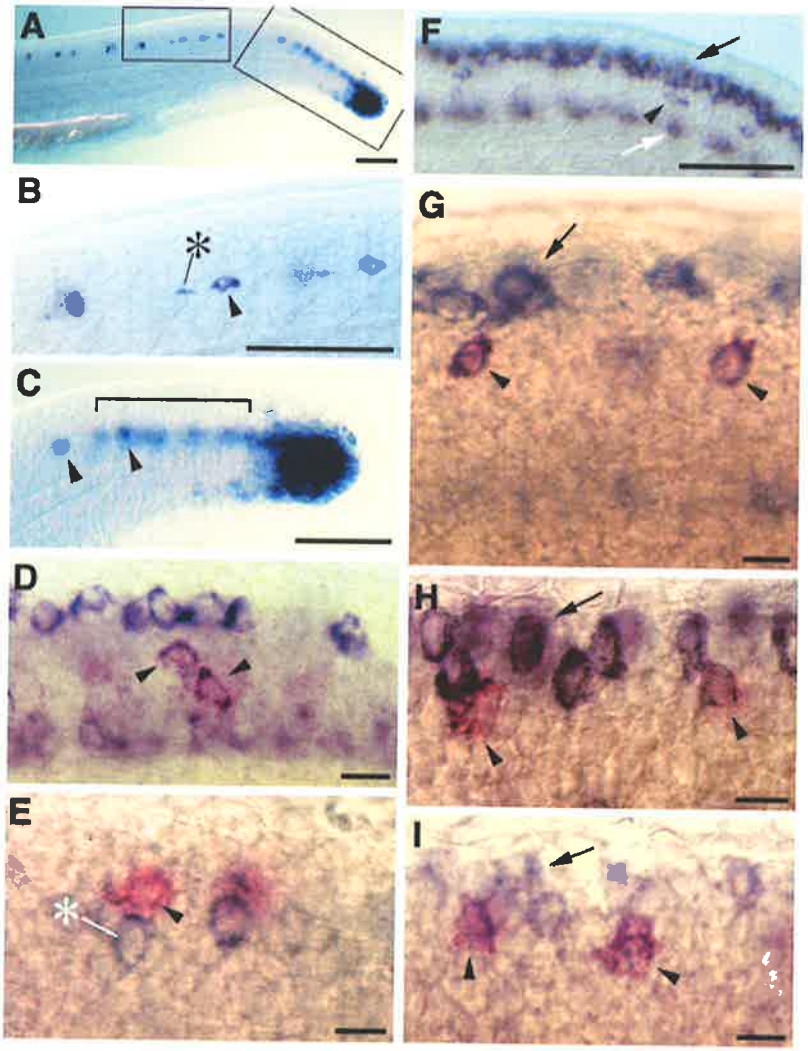

Figure 1
Whole mount *in situ* transcript hybridisation analysis of the expression of *spt* and other genes in the tail and trunk of zebrafish embryos at approximately 22 hpf. In all images, dorsal is up and rostral is to the left. An apparently irregular rostrocaudal distribution of *spt*-expressing cells is seen in the developing CNS rostral to the domain of expression in the presomitic mesoderm of the extending tail (A). Boxed areas in A indicate parts of the image magnified in B and C. Shortly after their birth, these cells extend a process rostrally (indicated by a black asterisk in B) into which *spt* transcript is transported. *spt* is expressed in newly formed somites in a restricted region, the "somitic trail" (bracketed in C), at the same dorsoventral level as *spt*-expressing cells in the developing CNS (black arrowheads in any panel). The *spt*-expressing cells in the developing CNS (red stain) co-express *huC*, a marker of neurons (blue stain in D). A probe that identifies cells transcribing *val* (blue stain) shows that the *spt*-expressing neurons (red stain) are not identical with these (E). Transcription of the *isl-1* gene (see F) is seen dorsally in Rohon-Beard neurons (black arrows in any panel), and ventrally in motoneurons (white arrow). Intermediate between these two levels are DoLA neurons that also express *isl-1* (black arrowhead). Double staining with *isl-1* (blue) and *spt* (red) shows that these intermediate-level neurons express *spt* (G). Costaining of *spt* (red) with *isl-2* (blue in H) and *isl-3* (blue in I) shows that the DoLA neurons also apparently express these genes, although the onset of expression occurs more rostrally than for *isl-1*. Scale bars equal 100 μm in A, B, C and F and 20 μm in D, E, G, H, I.

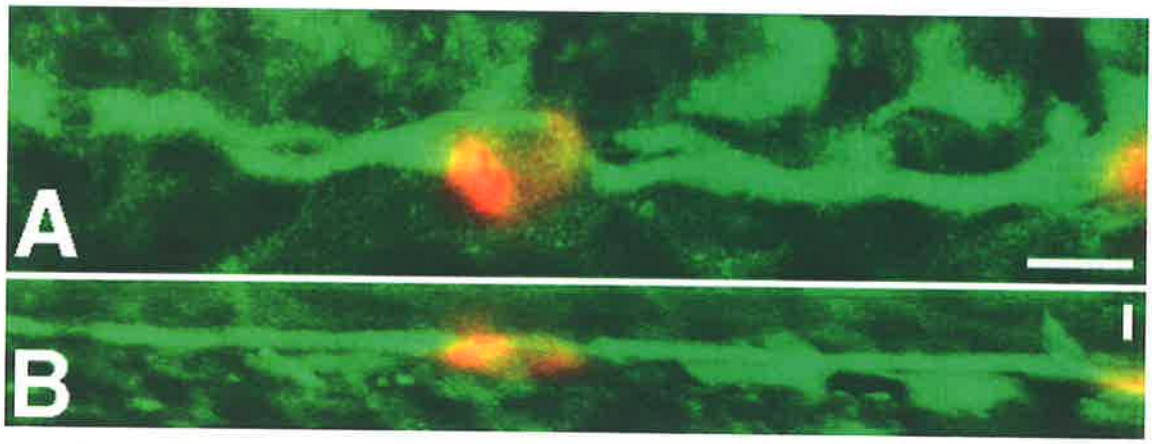

Figure 2
Close association of neurons expressing *spt* with the dorsal longitudinal fasciculus. Images shown are projections of serial 0.5 μm optical sections through a 22 hpf embryo stained to reveal *spt* transcripts (red) and acetylated tubulin (green) that marks axons. The cell shown lies in that part of the developing spinal cord midway along the yolk extension. Rostral is to the left in both images. A shows a lateral projection with dorsal to the top. B shows a dorsal projection with medial to the bottom and lateral to the top. The size bar in A indicates 10 μm. B has an identical rostrocaudal dimension but the mediolateral dimension is compressed. The size bar in B indicates 10 μm in the mediolateral dimension.

in the region of the spinal cord dorsal to the yolk extension showed that these cells lie alongside the dorsal longitudinal fasciculus (DLF, Figure 2). Their proximity to the DLF obscured the pattern of axonal projection from these cells. We did not observe the presence of *spt* transcript in axons near these cells.

Dorsoventral and rostrocaudal correspondence of caudal spt expression in the somitic mesoderm and developing CNS

Extended staining for *spt* expression allowed us to observe *spt* mRNA in recently-formed somites at 24 hpf just rostral to the previously observed, high-level expression of *spt* in the presomitic mesoderm. This expression is not present throughout the somites but, rather, only at the same dorsoventral level as *spt*-expressing cells in the developing spinal cord. From a lateral perspective, this gives the impression of a "trail" of *spt*-expressing cells in the somitic mesoderm left behind by the extending tail tip (Figures 1C, 3A,3B,3D).

The somitic expression of *spt* is strongest in the dorsocaudal cells of these structures (Figure 3). Observation of this region from a dorsolateral perspective shows that cells expressing *spt* in the developing spinal cord most commonly form so that they are in direct juxtaposition with these cells across the basal lamina (Figures 3C,3E; at least 76% of observed cases, n = 25). However, they do not form adjacent to every somite. This distribution suggests that: 1) the *spt*-expressing neurons are either generated in response to signals from the dorsocaudal cells of each somite or, 2) that neural and somitic cells express *spt* in response to a common patterning signal(s). In either case, an "inefficient" stimulation of neural cells to transcribe *spt* would result in the observed distribution of *spt*-expressing neurons.

Occasionally, neural cells transcribing lower levels of *spt* can be observed adjacent to the most posterior somites (see asterisk in Figure 3E). We have not observed such cells at more rostral levels so these might represent cells in the process of activating *spt* transcription. Alternatively, neural cells transcribing *spt* at lower levels might be lost or might repress *spt* transcription later in spinal cord development.

The earliest formation of spt-expressing neurons

The somitic expression of *spt* at 24 hpf is only seen in the most recently formed somites. We wished to observe whether newly born *spt*-expressing neurons are always flanked by *spt* expression in somitic mesoderm, and to determine the earliest time at which *spt*-expressing neurons could be observed.

To gain an indication of the time at which *spt*-expressing neurons might first arise, we observed the somitic juxtaposition of the most rostral *spt*-expressing neuron in 11 embryos at approximately 24 hpf. The majority of the embryos (n = 10) possessed at least 6 somite pairs rostral to the most rostral *spt*-expressing neural cell (Figures 4A,4B).

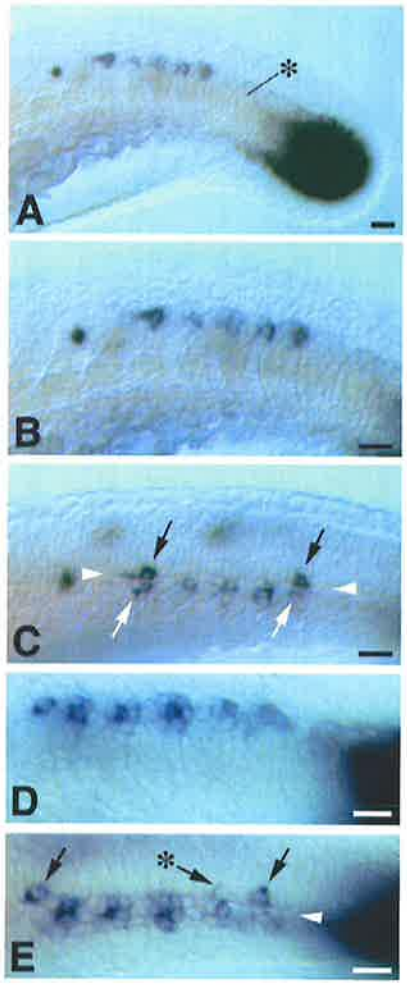

Figure 3
The juxtaposition of *spt* expression in newly formed somites and the developing CNS at approximately 22 hpf. In all images dorsal is uppermost and rostral is to the left. A, B and C are views from one embryo. A and B show the appearance from a lateral view of the tail in the region of the "somitic trail" of *spt* expression. A black asterisk indicates the most recently formed somite. *spt* expression is concentrated to the dorsocaudal extremity of somites. In an optical (DIC) section through the same embryo viewed from a dorsolateral perspective (C), the basal lamina separating the developing CNS and the somitic mesoderm can be seen clearly (arrowheads). Cells expressing *spt* in the developing CNS (black arrows) are juxtaposed to somitic cells expressing *spt* (white arrows). The "somitic trail" region of a second embryo is shown in D (lateral view) and E (dorsolateral view). The black asterisk in E indicates a neural cell expressing a lower level of *spt*. Scale bars equal 20 μm.

Only one embryo showed a lower number (at least 5 rostral somite pairs). Thus, *spt*-expression in the spinal cord is flanked by the region of somitic mesoderm that shows slower somite formation (occurring after the initial rapid formation of the first six somite pairs, [23]). Since the 5th somite pair forms at approximately 12 hpf (at 28.5°C), we examined embryos between 12 hpf and 16 hpf for *spt* staining in the CNS. The CNS primordium is relatively flattened at this time and the basal laminae separating CNS, mesoderm and individual somites are difficult to observe in fixed embryos. Nevertheless, the earliest time at which we could observe *spt* expression confidently in the developing CNS was 15.5 hpf (13 somite pairs). At 16 hpf (14 somite pairs), five of six embryos examined for which *spt*-expressing neurons could be seen had at least 9 somite pairs rostral to the most rostral *spt*-expressing neuron (see Figure 5). This observation implies that the *spt*-expressing neurons at more rostral positions (i.e. adjacent to the 6th to 9th somite pairs) differentiate at later times or that *spt*-expressing cells migrate rostralwards after their birth (see later). At 16 hpf, the *spt*-expressing neural cells are also flanked by low level *spt* expression in somites (white arrowheads in Figure 5). Thus, low level somitic expression of *spt* occurs during most of somitogenesis. Low level *spt* expression is observable at 14.5 hpf in laterocaudal cells. However, we could not determine whether these cells were neural or mesodermal (data not shown).

Analysis of left-right bias in spt-expressing neuron number
The irregular distribution of *spt*-expressing neurons may conceal a left or right bias in the number of these neurons. To investigate this we examined the numbers of neurons on the left and right sides of 48 embryos at 24 hpf. The mean number of cells on the left sides of embryos was found to be 10.5 with a standard deviation of 2.1. The mean number of cells on the right sides of embryos was found to be 10.7 with a standard deviation of 1.9. The differences in the mean number of *spt*-expressing neurons on the left and right sides of the embryos is considerably smaller than the standard deviations of left and right. This argues against any left-right bias.

The analysis above might not reveal a left or right bias when the variability in the number of *spt*-expressing neurons in each embryo is high. Thus, we also examined the difference in the numbers of *spt*-expressing neurons between the left and right sides of individual embryos. For each of the 48 embryos, the number of *spt*-expressing cells on the left of the embryo was subtracted from the number on the right. The mean difference was +0.2 with a standard deviation of 2.0. Since the standard deviation is far larger that the mean difference, this also argues against any left or right bias in *spt*-expressing neuron number. Finally, we tested whether there is simply a tendency for an absolute difference in the numbers of *spt*-expressing cells

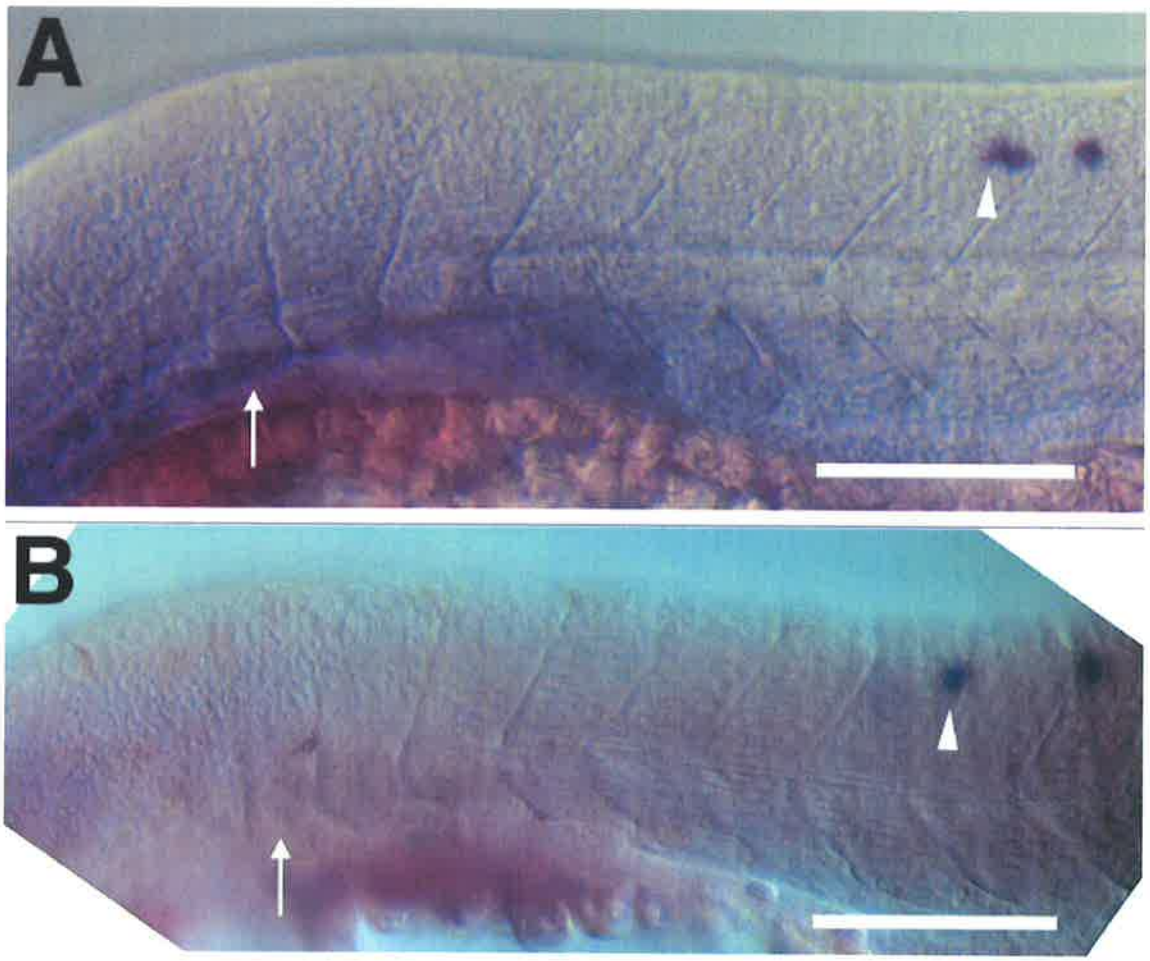

Figure 4
Lateral views of two embryos (A and B) at approximately 24 hpf stained to reveal *spt* transcription. Dorsal is up and rostral is to the left. DIC microscopy was used to reveal somite boundaries. Consequently, *spt*-expressing cells in the developing CNS are not seen clearly because they lie in a different focal plane. However, the most rostral cell in each embryo is indicated by a white arrrowhead. The most rostral visible discernible somite is indicated by a white arrow. In both cases there are 6 somites rostral to the most rostral *spt*-expressing neuron. Scale bars equal 100 μm.

to exist between the two sides of the embryo, regardless of any left-right bias. The mean absolute bilateral difference for the 48 embryos was 1.5 cells. The standard deviation for this value was 1.4. Thus, there is no significant difference in the numbers of *spt*-expressing cells between the two sides of embryos.

Preferred positions of spt-expressing neurons on the rostrocaudal axis

While the distribution of spt-expressing neurons along the rostrocaudal axis of the spinal cord appears to be irregular, preferred positions may, nevertheless, exist. To analyse this, 20 embryos were fixed at 24 hpf and stained to reveal expression of spt. The left and right sides of the trunk and tail of the embryos were then photographed under differential interference contrast (DIC) optics to show simultaneously the spt-expressing neurons and the boundaries between the flanking somitic tissue. We then counted the neurons occurring adjacent to each particular somite on the left and right sides of the embryo. Since we have shown that there is no left-right bias in the number of spt-expressing neurons, we combined the data from the two

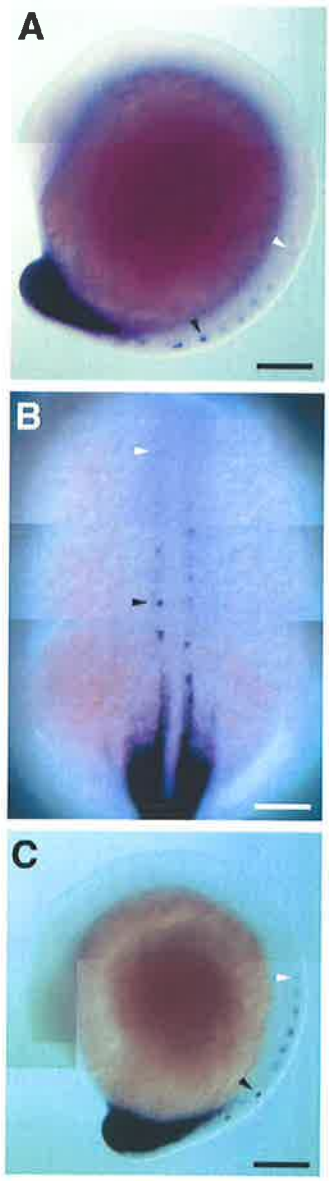

Figure 5
Early *spt* expression in the developing CNS and somites. A and C show lateral views of two embryos at 16 hpf. Rostral is up and dorsal is to the right. B shows a dorsal view of the embryo in A. Rostral is up. White arrowheads indicate the most rostral somitic domain of *spt* transcription visible. Black arrowheads indicate the most rostral neural cell expressing *spt*. For B, the light source was concentrated behind the yolk to give greater visibility of staining. All images are composites of smaller images. Scale bars equal 100 μm.

sides. The number of somite pairs present in embryos at 24 hpf can vary [23], as can the visibility in fixed embryos of the most anterior somite boundaries and the most recently formed somite boundaries. Therefore, to make the results from each embryo comparable, we identified the somite pair directly dorsal to the most caudal extent of the yolk extension as somite level 0. We then numbered the other somite pairs according to this reference point (Figure 6). Somite pairs rostral to somite level 0 were given a "+" designation while caudal somite pairs were given a "-" designation. The mean number of cells present at each somite level was then calculated (Table 1).

A tendency to higher numbers of cells at rostral somite levels is evident. The highest mean number observed was at somite level +11 (1.9 cells per embryo for left and right sides combined). At 24 hpf, somite level +11 commonly corresponds to the 7th somite pair formed. Lower numbers of *spt*-expressing neurons are observed at somite levels caudal to somite level 0 (commonly the 18th somite pair formed). However, there is great variability between embryos in the number of cells at any somite level (as indicated by the large standard deviation values in Table 1). The increase in cell number at rostral levels is not explained by the increase in the rostrocaudal dimension of somites as they mature since the segmental pattern of neuron distribution in the spinal cord expands correspondingly [2]. The higher number of *spt*-expressing neurons found rostral to somite level 0 could be due to: 1) continuing birth of these neurons at rostral positions as the CNS develops, 2) programmed cell death of neurons at caudal positions, or 3) rostralwards migration of neurons after their birth. Two observations support the last possibility. First, the mean number of *spt*-expressing neurons along the entire rostrocaudal axis per embryo was determined for 76 embryos at 24 hpf (21.4 neurons, standard deviation 3.4) and 45 embryos at 30 hpf (22.7 neurons, standard deviation 2.9). Somitogenesis ends at approximately 24 hpf but differentiation along the rostrocaudal axis continues in a rostral to caudal manner. Thus, any later, rostral generation of *spt*-expressing neurons or programmed cell death of caudal neurons as spinal cord development continues after 24 hpf might be expected to alter the average number of neurons by a greater number than that observed. Second, ipsilateral juxtaposition of *spt*-expressing neurons (which we defined as instances in which the cell bodies of the neurons appear to contact each other) occurred for 5.5% of cells in the region of somite levels -11 to +4, but for 11.3% of these cells in the region of somite levels +5 to +12. These data, together with the observation of greater neuron numbers at rostral levels, suggest that these neurons accumulate at rostral levels due to rostralwards migration after their birth.

Table 1: Numbers of *spt*-expressing neurons per somite level (pair of hemisegments) at 24 hpf

Somite level	Somite number	Number of embryos	Mean cell number	Standard deviation
-14	32	3	0	0
-13	31	7	0	0
-12	30	12	0	0
-11	29	15	0.07	0.26
-10	28	19	0.16	0.50
-9	27	19	0.47	0.61
-8	26	19	0.47	0.61
-7	25	19	0.63	0.76
-6	24	20	0.55	0.69
-5	23	20	0.50	0.61
-4	22	20	0.70	0.92
-3	21	20	0.55	0.69
-2	20	20	0.55	0.60
-1	19	20	0.95	0.83
0	18	20	0.95	1.05
+1	17	20	0.70	0.66
+2	16	20	0.70	0.73
+3	15	20	0.95	1.00
+4	14	20	0.75	0.79
+5	13	20	1.00	0.92
+6	12	20	0.75	0.72
+7	11	20	1.40	0.68
+8	10	20	1.45	1.19
+9	9	19	1.11	0.88
+10	8	17	1.47	1.01
+11	7	13	1.92	1.38
+12	6	7	1.14	0.69
+13	5	4	0.75	0.50
+14	4	2	0	0

Somite level 0 represents the somite pair immediately dorsal to the most posterior extremity of the yolk extension. Negative values are more caudal to somite level 0 and positive values are more rostral. The common identity of each somite pair (i.e. disregarding variability between embryos) in terms of its order of formation is given as the somite number

No *spt*-expressing cells were observed rostral of somite level +13, commonly corresponding to the 5th somite pair formed. This could be an artefact of the low number of embryos for which these somite levels could be distinguished during observation. However, this result is consistent with our earlier failure to observe *spt*-expressing neurons more rostral than the 5th most rostral somite pair (see Figure 4 and above).

At first glance, the numbers of *spt*-expressing neurons we observe at each somite level (i.e. per two hemisegments) at 24 hpf does not appear to be comparable to the previous observations of Bernhardt et al. in 1990 [12] of 0.06 DoLA interneurons per hemisegment (0.12 per somite level) flanked by the 5th- to 8th-formed somite pairs in embryos at 18 hpf. However, the fact that we rarely observe *spt*-expressing neurons anterior to the 6th-formed somite pair at 24 hpf combined with the possibility that these neurons migrate rostrally after birth (see above) suggests that fewer DoLA neurons may be found in the region flanked by the 5th- to 8th-formed somite pairs at 18 hpf compared to 24 hpf. Also, these authors identified DoLA neurons by their pattern of arborisation whereas we have identified these cells by *spt* expression. At 18 hpf many *spt*-expressing cells may not yet have developed characteristic DoLA arborisation patterns. In contrast, in a study of GABAergic DoLA neurons in embryos at 27 hpf by Bernhardt et al. in 1992 [24], a mean of 3.89 cells (standard deviation 1.17) were observed in the region of hemisegments 6 to 10. At 24 hpf, we observed a mean of 3.64 cells (standard deviation 1.08) in the same region. The close correspondence of these figures supports that *spt*-expressing neural cells are DoLA neurons.

Discussion

The identity of spt-expressing neural cells

The *spt*-expressing cells in the developing spinal cord show coexpression of a number of neural markers such as

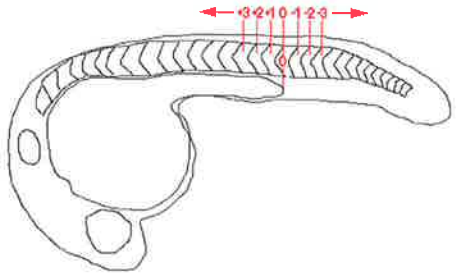

Figure 6
Diagram of somite level designations relative to the caudal tip of the yolk extension in a 24 hpf embryo.

huC and the *islet* genes. This, together with the position of these cells just ventral to the Rohon-Beard neurons and their rostrocaudal distribution establishes that these cells are likely to be the DoLA neurons originally described by Bernhardt et al. [12]. Indeed, we are able to observe a rostrally projecting process of these cells similar to the ascending axon of DoLA neurons due to the active transport of *spt* mRNA along this process.

That DoLA neurons express *spt* conflicts with observations of the expression in *Xenopus* embryos of the *spt* orthologous gene, *Xombi*. *Xombi* is transcribed in a very similar pattern to *spt* in the developing spinal cord. In 1996, Stennard et al. [25] and Zhang and King [26] suggested that this gene (they named it *Antipodean* and *VegT* respectively) might be expressed in Rohon-Beard neurons based on the dorsal/dorsolateral position of expressing cells in the spinal cord. However, in a simultaneous publication, Lustig et al. [27] suggested that *Xombi* expression was in the dorsolateral area of interneuron formation. We expect that closer examination will show that *Xombi* is expressed in *Xenopus* DoLA-equivalent cells, probably dorsolateral interneurons (see review by Roberts [28]).

The observation of *spt* mRNA in an anterior growth process/axon suggests a number of possibilities. First, the mRNA may not be translated but may perform some other (or no) role in the process. Second, Spt protein may be required in this process for a function other than gene regulation. Third, *spt* mRNA may be required in the process for production of protein that is used to signal back to the nucleus. There is some precedence for the expression of transcription factors in neurites since these are known to be found in dendrites where it is thought that they may be involved in activities such as long term potentiation [29]. Finally, and most intriguing, is the possibility that *spt* mRNA might be involved in signalling to cells with which the process makes contact. It has been demonstrated that the transcription factor Engrailed and the homeodomains of other proteins can be transported between cells [30–33]. Testing of these possibilities will require observation of the distribution of Spt protein.

spt-expressing DoLA neurons possibly migrate rostrally

Higher numbers of *spt*-expressing neurons are observed rostrally compared to caudally in the spinal cord. It may be that *spt*-expressing neurons continue to be born as the developing CNS matures in a rostral to caudal progression or that caudal neurons undergo programmed cell death. However, the marginal change in the number of these neurons between 24 and 30 hpf argues against this. Also, ipsilateral juxtaposition of these neurons is more common at rostral compared to caudal sites. The increased juxtaposition rostrally could be caused by rostral migration of *spt*-expressing neurons when an anterior limit exists for the migration. *spt*-expressing neurons are rarely seen anterior of the 6th-formed somite pair suggesting that this position on the rostrocaudal axis may represent such a limit.

spt is expressed in somitic mesoderm

Extended staining for *spt* mRNA revealed that this gene is transcribed at low levels in the dorsocaudal cells of recently formed somites. It has previously been assumed that *spt* expression marks only presomitic mesoderm. The function of *spt* expression in these somitic cells is unknown. Discovery of other genes expressed in a similar pattern in newly formed somites may reveal more of the function or fate of these cells.

The irregular pattern of spt-expressing neurons may be based on an underlying regularity

The question of how irregular patterns of cell distribution or gene expression are controlled is not commonly addressed in studies of developmental biology. Nevertheless, these patterns are common in the central nervous systems of most animals and occur in many other tissues. In the spinal cord of the developing embryo, Rohon-Beard neurons occur at a frequency of more than one per hemisegment [12]. Their positions are not highly ordered and do not depend upon signals from mesoderm [5]. Instead, short-range intercellular interactions controlled by Notch signalling appear to play a role in their differentiation from a field of progenitor cells [10,11].

The ascending commissural neurons that are located just ventral to Rohon-Beard neurons are also found at a frequency of more than one per hemisegment. However, subclasses of these neurons exist with lower frequency. For example, anti-CON1 antibody labels a subclass of ascending commissural neurons in the embryo that probably become commissural primary ascending (CoPA)

interneurons in the larva. These are present in an irregular pattern on the rostrocaudal axis at a frequency of 0.87 per hemisegment flanking the 6^{th}- to 11^{th}-formed somite pairs at 28 hpf [12]. Ascending commissural neurons are located at a similar dorsoventral level to the DoLA neurons. We have shown that *val* expression in the spinal cord occurs just ventral to *spt*-expressing neurons. Thus, it is possible that *val* labels a subclass of ascending commissural neurons.

The neuromasts of the posterior lateral line – while part of the peripheral nervous system – are, nevertheless, an example of a neural cell type distributed at a frequency of less than one per hemisegment. These neurons are deposited by the migrating lateral line primordia along the myoseptum at the boundary between somites at four or five positions along each side of the embryo. While their rostrocaudal distribution is not completely irregular, there is considerable variation in the actual position of any one neuromast. The position at which a neuromast is deposited appears to depend more strongly on the distance from the previously deposited neuromast rather than the precise position on the rostrocaudal axis [34]. Interestingly, the recessive, homozygous viable mutation *hypersensitive* (*hps*) results in neuromast deposition at nearly every somite boundary [35]. The fact that this (presumably) loss-of-function mutation can increase the regularity of a pattern indicates that the distribution of neuromasts probably results from the combined effect of at least two patterning mechanisms – one controlling inter-neuromast distance and one controlling neuromast localisation to intersomitic boundaries. This raises the question as to whether mutations might exist that increase the frequency of generation of *spt*-expressing neurons, for example, by increasing the strength of a patterning signal from the mesoderm to the developing CNS.

The dorsoventral and rostrocaudal correspondence of *spt* expression in newly formed somites and the CNS suggests a functional connection between the *spt* expression in these two tissues. The somitic and neural cells may be responding to a common patterning signal. Alternatively, the somitic *spt* expression may mark the source of a signal from the somite to neural tissue. A precedent for the latter alternative exists in the influence of flanking mesoderm on primary motoneuron formation [5–7]. However, the formation of most primary motoneurons occurs with complete regularity (one neuron per hemisegment). An interesting exception to this is the Variable Primary (VaP) motoneuron that occurs at a frequency of less than 0.5 per hemisegment. VaPs arise adjacent to Caudal Primary (CaP) motoneurons midway between hemisegment boundaries [36]. VaPs normally extend an axon to the horizontal myoseptum in the myotomes after which the VaP dies. In contrast, the CaP axon continues from the myoseptum into ventral muscle. These two neurons actually represent an equivalence pair since ablation of a CaP causes the neighbouring VaP to develop a CaP-like arborisation pattern [37]. Thus, rather than VaP formation occurring with less than complete regularity, we can regard this situation as CaP formation at greater than one cell per hemisegment followed by regulation to one cell per hemisegment.

We suggest that the *spt*-expressing DoLA interneurons might be "inefficiently" patterned by flanking somitic mesoderm. Thus, the initial distribution of these neurons would represent an incomplete pattern based on a regular template. Migration and tissue growth might then scramble this pattern. We are currently testing this hypothesis by examining the role of *spt* expression and mesodermal signals in DoLA neuron differentiation and distribution.

Conclusions

spt-expressing cells in the developing central nervous system appear to be DoLA interneurons. The irregular distribution of these cells along the rostrocaudal axis of the spinal cord may be due to "inefficient" patterning of neural *spt* expression by flanking, regularly distributed somites also expressing *spt*. Rostral migration of *spt*-expressing neurons might then scramble any residual regularity in their distribution. The idea that irregular patterns of neuron distribution may arise in partial correspondence to regular templates is a parsimonious explanation for the evolution of such patterns.

Materials and Methods

Double whole mount in situ transcript hybridisation

(Cloning of probe sources)

A cDNA clone, (26 M), corresponding to transcription from *spt* was isolated in a whole mount *in situ* transcript hybridisation screen of zebrafish embryos [38]. cDNAs corresponding to parts of transcripts from the genes *huC*, *isl-2* and *valentino* were amplified by RT-PCR from embryos at 24 hpf using the oligonucleotide primers described in Table 2. All cDNA fragments were cloned into the pGEMT vector (Promega Corporation, Madison, WI, USA). The inserts of these clones were amplified by PCR using M13 primers and then transcribed with T3 or SP6 RNA polymerase to produce digoxigenin- or fluorescein-labelled antisense RNA probes (see [38]). The clones for production of probes against *isl-1* and *isl-3* transcripts were obtained from Hitoshi Okamoto [6,7].

Double whole mount *in situ* transcript hybridisation was performed essentially as described in [39] but the first staining reaction was with BCIP/NBT, inactivation of the first alkaline phosphatase staining reaction was by heating

Table 2: Oligonucleotides used for cDNA fragment isolation for probe synthesis

Gene transcripts detected	oligo name	PCR oligonucleotide sequence
huC	#277	5' CAG ATG ACA GCA AAA CTA ACC 3'
	#278	5' AGA GCA ATA GTG ACT AGG CC 3'
isl2	#351	5' GAC GGC AAG ACT TAT TGC 3'
	#352	5' CAT CTT CGG AGA TCA TGC 3'
val	#322	5' GGT CCC CCT GTC GCC TC 3'
	#323	5' CCA CGA GCG ACA ACC CG 3'

to 65°C for 45 min in PBS and the second staining reaction used the Alkaline Phosphatase Substrate Kit I (Vector Laboratories Inc., Burlingame, CA, USA).

Combined whole mount in situ transcript hybridisation and immunohistochemistry

Staining for the presence of *spt* transcript and acetylated tubulin was performed essentially as described above for double whole mount *in situ* transcript hybridisation except that *spt* staining using the Alkaline Phosphatase Substrate Kit I ("Vector Red", Vector Laboratories Inc.) was performed first followed by washing for 10 min in 100 mM Tris HCl pH 8.5 then 10 min in PBS + 0.1% Tween 20 (Sigma, St. Louis, MO, USA) (PBT) before fixation in 4% formaldehyde in PBT. Embryos were then washed 4 × 5 min in PBT, then 3 h in PBT + 0.3% IPEGAL (Sigma) (PBTI) + 2% BSA (Fraction V, Sigma), then 1 h in PBTI + 2% BSA at 4°C before incubation overnight at 4°C in a 1:2500 dilution of anti-Acetylated Tubulin antibody (Sigma Cat. No. T6793) in PBTI + 0.2% BSA. Embryos were then washed 6 × 1 h in PBTI then 2 × 30 min in PBTI + 2% BSA before incubation overnight at 4°C in a 1:200 dilution of anti-mouse IgG labelled with Alexa Fluor 488 (Molecular Probes Inc., Eugene, OR, USA) in PBTI + 0.2% BSA. Finally, embryos were washed 7 × 1 h in PBTI before equilibration with 80% glycerol in PBT before imaging. Note that all wash series were preceded by three rinses in the wash solution and were at room temperature unless otherwise indicated.

Observation and statistical analysis of cell distribution

Embryos were dechorionated at 15–18 hpf, 22 hpf, 24 hpf or 30 hpf and fixed in 4% formaldehyde in PBS at 4°C before *in situ* transcript hybridisation with a probe for *spt*. To ensure observation of all cells expressing *spt* including any expressing *spt* at low levels, the staining reaction was allowed to proceed overnight at 4°C before the embryos were fixed in 4% formaldehyde in PBS and then equilibrated with 80% glycerol.

Light field observation of the embryos was conducted under a Zeiss Axiophot™ microscope (Carl Zeiss Jena GmbH, Jena, Germany) at 200× magnification using DIC optics. For examination of cell positions, the trunk-tail region of an embryo was removed from the rest of the body and then laid flat on a slide. Photographs were taken such that the intersomitic boundaries and the *spt*-expressing neural cells were simultaneously visible. Confocal imaging of embryos was conducted on a Bio-Rad MRC-1000 UV Confocal Laser Scanning Microscope System (Bio-Rad Laboratories Inc., Hercules, CA, USA) using a Nikon Diaphot 300 inverted microscope (Nikon Instech Co., Ltd., Kawasaki, Kanagawa, Japan). Fluorescence was observed using a krypton/argon laser with excitation at 488/10 nm and emission at 522/35 nm excitation for Alexa 488 and with excitation at 568/10 nm and emission at 605/32 nm for Vector Red. Images were processed with Adobe Photoshop version 5.0 (Adobe Systems Inc. San Jose, California, USA) and Confocal Assistant version 4.02 (Todd Clark Brelje).

List of abbreviations used

BCIP, 5-Bromo-4-chloro-3-indolyl-phosphate.p-toluidine-salt

BSA, bovine serum albumin

CaP, Caudal Primary

CNS, central nervous system

CoPA, Commissural Primary Ascending

DIC, differential interference contrast

DLF, dorsal longitudinal fasciculus

DoLA, dorsal longitudinal ascending

isl, *islet*

hpf, hours post fertilisation

NBT, Nitroblue tetrazolium chloride

PBS, phosphate buffered saline

PBT, PBS + 0.1% Tween 20

PBTI, PBT + 0.3% IPEGAL

RT-PCR, reverse transcription polymerase chain reaction

spt, spadetail

val, valentino

VaP, Variable Primary

Authors' contributions

RT carried out the majority of the *in situ* transcript hybridisation, cell counting, and statistical analyses and some photography.

SW performed the *in situ* transcript hybridisation analyses on 12 – 16 hpf embryos and staining for acetylated tubulin.

JGC contributed to the statistical analysis

ML directed the research, performed observation of staining patterns, some cell counting and statistical analysis, some photography and drafted the manuscript.

Acknowledgements

The authors wish to thank Dan Kortschak and Judith Eisen for valuable discussion and Simon Koblar for critical reading of the manuscript. However, the conclusions drawn by the authors are theirs alone. We thank Meredith Wallwork for assistance with confocal microscopy. Clones for production of probes against the *isl-1* and *isl-3* genes were the kind gift of Hitoshi Okamoto. This work was supported by an Australian Research Council small grant and by funds from the Special Research Centre for the Molecular Genetics of Development. RT was supported by an International Postgraduate Research Scholarship from The University of Adelaide.

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PAPER IV

Developmental Biology 232, 493–507 (2001)
doi:10.1006/dbio.2001.0160, available online at http://www.idealibrary.com on IDEAL®

Characterization and Developmental Expression of the Amphioxus Homolog of *Notch* (*AmphiNotch*): Evolutionary Conservation of Multiple Expression Domains in Amphioxus and Vertebrates

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Notch **encodes a transmembrane protein that functions in intercellular signaling. Although there is one *Notch* gene in *Drosophila*, vertebrates have three or more with overlapping patterns of embryonic expression. We cloned the entire 7575-bp coding region of an amphioxus *Notch* gene (*AmphiNotch*), encoding 2524 amino acids, and obtained the exon/intron organization from a genomic cosmid clone. Southern blot and PCR data indicate that *AmphiNotch* is the only *Notch* gene in amphioxus. *AmphiNotch*, like *Drosophila Notch* and vertebrate *Notch1* and *Notch2*, has 36 EGF repeats, 3 Notch/lin-12 repeats, a transmembrane region, and 6 ankyrin repeats. Phylogenetic analysis places it at the base of all the vertebrate genes, suggesting it is similar to the ancestral gene from which the vertebrate *Notch* family genes evolved. *AmphiNotch* is expressed in all three embryonic germ layers in spatiotemporal patterns strikingly similar to those of all the vertebrate homologs combined. In the developing nerve cord, *AmphiNotch* is first expressed in the posteriormost part of the neural plate, then it becomes more broadly expressed and later is localized dorsally in the anteriormost part of the nerve cord corresponding to the diencephalon. In late embryos and larvae, *AmphiNotch* is also expressed in parts of the pharyngeal endoderm, in the anterior gut diverticulum, and, like *AmphiPax2/5/8*, in the rudiment of Hatschek's kidney. A comparison with *Notch1* and *Pax5* and *Pax8* expression in the embryonic mouse kidney helps support homology of the amphioxus and vertebrate kidneys. *AmphiNotch* is also an early marker for presumptive mesoderm, transcripts first being detectable at the gastrula stage in a ring of mesendoderm just inside the blastopore and subsequently in the posterior mesoderm, notochord, and somites. As in sea urchins and vertebrates, these domains of *AmphiNotch* expression overlap with those of several *Wnt* genes and *brachyury*. These relationships suggest that amphioxus shares with other deuterostomes a common mechanism for patterning along the anterior/posterior axis involving a posterior signaling center in which the *Notch* and *Wnt* pathways and *brachyury* interact.**

Key Words: Brachyury; pattern formation; Notch; amphioxus; lancelet.

INTRODUCTION

Notch genes encode single-pass transmembrane receptors which mediate intercellular communication. The extracellular domain includes up to 36 EGF repeats and 3 Lin/Notch repeats, while the intracellular domain includes 6 ankyrin repeats. Notch undergoes proteolytic cleavage at three sites during maturation and signaling (reviewed in Annaert and De Strooper, 1999; Weinmaster, 2000). Notch proteins function during embryogenesis in cell fate deci-

0012-1606/01 $35.00

sions in the neuroectoderm and other tissues as well as in formation of borders as in the *Drosophila* wing and eye, vertebrate limbs, and somitic mesoderm (Beatus and Lendahl, 1998; Jiang *et al.*, 1998; Lewis, 1998; Irvine, 1999). The core Notch signaling pathway (reviewed in Bray, 1998; Jiang *et al.*, 1998; Kimble *et al.*, 1998; Fleming, 1998; Weinmaster, 2000) is highly conserved between the ecdysozoans (*Drosophila* and *Caenorhabditis elegans*) and higher deuterostomes (vertebrates), although there is evidence that Notch can act in an alternate pathway(s) (Rusconi and Corbin, 1998). The Notch signaling pathway is modulated at multiple levels by interaction with proteins such as Wingless, Dishevelled, Big Brain, Numb, and Hairless (reviewed in Panin and Irvine, 1998; Wu and Rao, 1999), Fringe (Munro and Freeman, 2000), and Scute (Cooper *et al.*, 2000). Several downstream targets of the Notch signaling pathway have been identified, including *Brachyury* in the notochord of ascidian tunicates (Corbo *et al.*, 1997, 1998), *sticks-and-stones*, involved in myoblast fusion in *Drosophila* (Bour *et al.*, 2000), *vestigial* and *wingless* in the *Drosophila* wing (Rulifson and Blair, 1995; Axelrod *et al.*, 1996; Artavanis-Tsakonas *et al.*, 1999), and *HES1* and *her1* in presomitic mesoderm in vertebrates (Takke and Campos-Ortega, 1999; Jouve *et al.*, 2000).

In *Drosophila* and lower deuterostomes (ascidian tunicates and sea urchins) there is a single Notch gene, while in vertebrates there are multiple *Notch* genes (four in the mouse). An independent gene duplication has resulted in two Notch genes in *Caenorhabditis* (Yochem and Greenwald, 1989). In vertebrates, *Notch1, 2,* and *3* are expressed in numerous tissues, and their expression domains partially overlap. These domains include the central nervous system, otic vesicle, presomitic mesoderm, pancreas, hemopoietic cells, limb bud, hair, tooth, and kidney (Coffman *et al.*, 1990; Bierkamp and Campos-Ortega, 1993; Lardelli and Lendahl, 1993; Conlon *et al.*, 1995; Williams *et al.*, 1995; Beatus and Lendahl, 1998; Lammert *et al.*, 2000; Singh *et al.*, 2000). The divergent *Notch4* has more restricted expression in maturing macrophages, the pancreas, and endothelial cells (Lewis *et al.*, 1998; Lammert *et al.*, 2000; Singh *et al.*, 2000). In contrast, the single *Notch* genes in sea urchins and ascidian tunicates have very limited expression domains. Ascidian *Notch* is expressed chiefly in the neural plate and anterior adhesive organ (Hori *et al.*, 1997), while zygotic expression of sea urchin *Notch* is limited in the late blastula to cells at the boundary of the future secondary mesoderm and endoderm and later to the secondary mesenchyme cells (Sherwood and McClay, 1997, 1999). The restricted expression in these lower deuterostomes raises the question of when the many domains of *Notch* expression arose in vertebrate evolution and how the evolution of these domains correlates with duplications of the *Notch* gene.

To address this question, we cloned the single *Notch* gene from the invertebrate chordate, amphioxus, and determined its intron/exon organization and embryonic expression. Amphioxus is the closest living invertebrate relative of the vertebrates (Wada and Satoh, 1994; Holland and Garcia-Fernàndez, 1996). Although the cephalochordate and vertebrate lineages separated about 500 million years ago, amphioxus is proving to be a relatively good proxy for their most recent common ancestor. Amphioxus development up to the gastrula stage is sea urchin-like: cleavage produces a hollow blastula, which then invaginates from the vegetal pole to form a gastrula. However, subsequent development is vertebrate-like. After gastrulation, the embryo develops a notochord, segmentally arranged somites, a dorsal hollow nerve cord, and a pharynx with gill slits. The larva develops homologs of the vertebrate thyroid gland, kidney, and pancreatic islet cells; however, a complete vascular endothelium, an otic vesicle, and paired eyes are lacking.

Our results indicate that amphioxus has a single *Notch* gene with a full complement of 36 EGF repeats. The gene has 30 exons (Table 2). The positions of several introns are conserved between amphioxus *Notch*, *Drosophila Notch*, and vertebrate *Notch4*. Thus, amphioxus *Notch* is probably representative of the ancestral deuterostome *Notch* gene. Fewer EGF repeats in *Notch* genes of lower deuterostomes and *Notch3* and *4* of vertebrates appear to be due to independent losses. Expression of amphioxus *Notch* throughout development closely parallels that of the multiple *Notch* genes of vertebrates put together, except that the vertebrate genes are also expressed in structures that are unique to vertebrates, supporting the idea that gene duplications in the vertebrate lineage may have facilitated the evolution of new structures. Comparisons of early developmental expression in amphioxus, vertebrates, and lower deuterostomes suggest that patterning along the anterior/posterior axis in the ancestral deuterostome embryo involved a posterior signaling center including the *Notch* and *Wnt* pathways.

MATERIALS AND METHODS

Cloning of AmphiNotch cDNAs

Adult amphioxus (*Branchiostoma floridae*) were obtained by shovel and sieve from Old Tampa Bay, Florida. Spawning was induced by electric shock and embryos were raised at 23°C as previously described (Holland and Holland, 1993). Total RNA was isolated from 2- to 4-day larvae by the method of Chomczynski and Sacchi (1987) and used for cDNA synthesis by random priming. cDNAs encompassing EGF and lin-12/Notch (LN) repeat sequences of amphioxus *Notch* were amplified by PCR with the degenerate primers Mila3 [5′ TG(T/C)CA(A/G)AA(T/C)GIGGIACITG 3′] and Mila4 [5′ (A/G)CA(T/C)TCIGC(A/G)TT(A/G)CAICC 3′] as previously described (Westin and Lardelli, 1997). cDNA from a 200-μl PCR was purified on a Wizard PCR preps column (Promega, Inc., Madison, WI), precipitated with ethanol, and redissolved in 50 μl of 25 mM Tris–Cl (pH 8.0), 20 mM NaCl, 1 mM dithiothreitol, 0.1 mg/ml BSA, and 0.1 *S*-adenosyl methionine. Possible *Srf*I sites were methylated to prevent cleavage by *Srf*I during subsequent ligation by incubation with 45 units of *Hpa*II methylase (Fermentas AB, Vilnius, Lithuania) at 37°C for 1 h. To "polish" the cDNA ends, after methylation the NaCl concentration was increased to 50 mM, dNTPs to 70 μM each, and 2 units

TABLE 1
PCR Primers and Conditions

Oligo name	Oligo sequence (5′–3′)	Binding site	Paired with	T (°C) Annealing	Extn. time (s)
Mila190	TTG ACG ATG TCA GAG TGC	ANK7	Mila191	63	180
Mila191	AAC TGT GAC CAG CAG TGC	LNR2	Mila190	63	180
Mila207	ATC ATC GCC AGT GGA CC	A-T	Mila190	63	180
Mila227	TAG CTC GCA GTT GTC TCC ACC	EGF3,4	Mila281	50	270
Mila269	GAC GAT GTC AGA GTG CAT GC	ANK4	Mila270	60	60
Mila270	CTC AAC TCG CAC GCT GAT GC	ANK7	Mila269	60	60
Mila281	TAT GCA GTC CCC AAA CAT CTG C	EGF8	Mila227	62	270
Mila339	TTC CGC AGT TCA AGC AGA TGT TTG GG	EGF8	(Self)[a]	[a]	[a]
Mila379	GAT TCG CAC GTC CCT CCG TGT T	EGF6	Mila381	64	120
Mila381	TGT GCG GAG GAA AGG CGT CGC	5′UTR	Mila379	64	120

Note. PCR cycling was 35 cycles of 94°C/30 s, annealing temperature for 30 s, then ramp of +0.5°C/s to 72°C, then 72°C/(extension (Extn.) time). Binding sites are EGF, EGF repeat; LNR, LN repeat; ANK, ankyrin repeat; T-A, between transmembrane domain and ankyrin repeat 1.

[a] See Tamme *et al.* (2000).

of T4 DNA polymerase (Boehringer Inc., Mannheim, Germany) was added, and the mixture was incubated at 25°C for 30 min. The reaction was heated to 70°C for 10 min to inactivate the enzyme, cooled, and ligated to pCR-Script SK(+) (Stratagene Inc., La Jolla, CA). Thirty-five clones containing inserts of 0.6 kb or greater were sequenced (Westin and Lardelli, 1997).

To isolate DNA corresponding to the ankyrin repeats of the amphioxus *Notch* gene, a genomic library constructed in the Lawrist 7 vector (Lawrence Livermore Laboratory, Livermore, CA) was screened with the insert of one of the cDNA clones (Amph26) obtained by PCR. High-density colony filters on Hybond N^+ membrane (Amersham Life Sciences, Inc., Arlington Heights, IL) were hybridized with 10^6 cpm/ml of the probe labeled with ^{32}P by random priming in 6.95% SDS, 1 mM EDTA, 0.1 mg/ml tRNA, 0.5 M sodium phosphate buffer (pH 7.2) at 65°C. Washes were 3× 20 min in 1× SSC, 0.1% SDS at 60°C.

One of the cosmid clones obtained (E1080) was restricted with *Hin*cII and the resultant fragments were subcloned into the *Sma*I site of the pBluescript SK(+) vector (Stratagene). To detect clones containing *Notch* exons, DNA from these subclones was screened on Southern blots with cDNAs spanning the entire open reading frame of mouse *Notch3* using low-stringency hybridization and washes in 6× SSC at 65°C. Two subclones approximately 1 kb long were identified and sequenced at their termini. Alignment of these sequences to vertebrate *Notch* genes revealed exons encoding regions corresponding to *Notch1* EGF repeats 7 to 10 and *Notch1* ankyrin repeats 3 to 7. Oligonucleotides (see Table 1) corresponding to these sequences and to the initially isolated cDNA region were designed and used in reverse transcriptase PCR on mRNA from 2-day-old larvae to amplify additional regions of amphioxus *Notch* cDNA.

Additional PCR with primers and conditions listed in Table 1 yielded much of the remainder of the Notch cDNA sequence. To obtain the ankyrin repeat and 3′ region, cDNA from an oligo(dT)-primed cDNA library of amphioxus gastrula–neurula stages in Lambda ZAP II (Stratagene) was amplified with oligonucleotide primers Mila269 and Mila270 corresponding to ankyrin repeat sequences. One clone was obtained containing Notch cDNA, which was then used to screen the same library. A single clone containing the remaining 3′ extent of the open reading frame of amphioxus *Notch* was obtained. To clone cDNA sequences corresponding to EGF repeats 4 to 8, we used primer Mila339 in the nonspecifically primed RT-PCR technique of Tamme *et al.* (2000). Sequence of EGF repeats 8 through 34 was obtained by PCR with primer pairs Mila227 and Mila281 from first-strand cDNA synthesized from total RNA of 36-h larvae with primer Mila280 (5′-TGA GGA TGT GGA TGA ATG TAT GC-3′). Finally, the complete sequencing of cosmid E1080 permitted the identification of a putative translational start codon.

Genomic Sequencing

Cosmid E1080 was subsequently sequenced in its entirety by the shotgun strategy (Deininger, 1983; Wilson, 1993; Rowen *et al.*, 1996). Five micrograms of cosmid DNA was sheared by sonication, repaired with the large fragment of DNA polymerase I (Klenow fragment) to generate blunt ends, and size fractionated on Chroma Spin-1000 columns (Clontech, Inc., Palo Alto, CA). Fragments larger than 1.0 kb were ligated into the *Sma*I site of pUC19 and transformed into *Escherichia coli* strain DH5α. Approximately 400 recombinant pUC19 clones were sequenced. Individual sequences were minimally edited to remove vector sequences, transferred to a SPRAC station (Sun Microsystems, Palo Alto, CA) on the TCP/IP protocol, and assembled into contiguous sequences with the GENETYX-S/SQ software (SDC: Software Development Co., Tokyo). Remaining gaps or areas of ambiguity were analyzed either by sequencing PCR amplification products or by sequencing the clones in pUC19 with custom primers.

Sequence Comparisons

Sequence alignments were done with the ClustalW program [written by Des Higgins (e-mail: Des.Higgins@ebi.ac.uk)] and manually adjusted. The parameters for the comparison were pairwise similarity parameters—*K*-tuple length, 1; gap penalty, 3; number of diagonals, 5; diagonal window size, 5; scoring method—percentage; multiple alignment parameters—gap penalty (fixed), 10.00; gap penalty (varying), 0.05; gap separation penalty range, 8; percentage identity for delay, 40%; list of hydrophilic residues, GPSNDQEKR; protein weight matrix—blosum.

The Notch cDNA sequence has been deposited in the EMBL and GenBank databases under Accession No. Y12539.

Phylogenetic Analysis

Phylogenetic analysis by the neighbor-joining method was based on the 114-amino-acid sequence of the Lin/Notch domain. Sequences were aligned with the ClustalX program and only conserved portions were used for the phylogenetic analysis. The distance measure was estimated with the Protdist program (categories model George Hunt/Barker categorization of amino acids) of the Phylip program (v3.5c). The reliability of clustering was tested by bootstrapping (100 samples). Only values greater than 49 are shown. The tree was either unrooted or rooted with the *Drosophila* and blowfly Notch sequences with the assumption that the duplications giving rise to the vertebrate *Notch* genes occurred after the deuterostome/protostome split. Sequences used and their accession numbers are *Drosophila Notch* (K03508), blowfly (*Lucilia cuprina*) *SCL* (U58977), sea urchin (*Lytechinus variegatus*) *Notch* (AF000634), zebrafish *Notch1* (Y10352), zebrafish *Notch5* (Y10353), goldfish (*Carassius auratus*) *Notch3* (U09191), *Xenopus Notch1* (M33874), chicken *Notch1* (AF159231), mouse *Notch1* (Z11886), mouse *Notch2* (D32210), mouse *Notch3* (X74760), mouse *Notch4* (U43691), rat *Notch2* (M93661), human *Notch1* (M73980), human *Notch3* (NM_000435), and human *Notch4* (D63395).

Southern Blot Analysis

DNA was extracted from adults in guanidinium isothiocyanate and purified according to methods in Holland *et al*, (1996). Fifteen 10-μg aliquots of genomic DNA were each digested with a different restriction enzyme, subjected to electrophoresis on an 0.7% agarose gel in 1× TAE buffer, and transferred to Hybond N^+ (Amersham Life Sciences, Cleveland, OH) according to L. Z. Holland *et al.* (1995). Probes were labeled to a specific activity of 1×10^8 cpm/μg by random priming and used at a concentration of 1×10^6 cpm/ml. For low-stringency hybridization to determine the number of *Notch*-related genes in amphioxus, the probe was an 850-bp Mila207–Mila190 clone of the ankyrin repeat region of amphioxus *Notch* (Table 1). Hybridization was in 10× Denhardt's, 0.1 mg/ml tRNA, 0.2% SDS, 6× SSC, 1 mM EDTA at 50°C. Washes were at 55°C in 2× SSC, 0.1% SDS. For a high-stringency blot to determine the specificity of the riboprobe, the Southern blot was stripped and rehybridized with the 850-bp insert of a clone in pCR-Script (Stratagene) containing EFG and LN repeats. Hybridization was as above with the temperature raised to 65°C; washes were 3 × 20 min in 1× SSC, 0.1% SDS at 65°C (L. Z. Holland *et al.*, 1995). Since the probe hybridized with a single band of amphioxus genomic DNA cleaved with 7 of 10 enzymes (data not shown), we concluded that the riboprobe is specific for *Notch* mRNA.

In Situ Hybridization

Expression of amphioxus *Notch* was determined by *in situ* hybridizations on developmental stages of *B. floridae* fixed at intervals during the first 2 days of development. Fertilization envelopes were removed from prehatching stages to facilitate penetration of reagents. The same 850-bp clone used to probe the Southern blot was used as a template for a reverse-sense riboprobe. Methods of fixation, probe synthesis, and hybridization were according to Holland *et al.* (1996). After photography of hybridized embryos as whole mounts, they were counterstained with 1% Ponceau S in 1% acetic acid, dehydrated in ethanol, embedded in Spurr's resin, and sectioned at 3 μm.

RESULTS

Amphioxus Has a Single Notch Gene

Our PCRs with first-strand cDNA of larval *B. floridae* as a template yielded overlapping clones that constituted the entire 7575-bp reading frame of an amphioxus *Notch* gene, which we call *AmphiNotch* (Fig. 1). This sequence corresponded with minor polymorphisms to the open reading frame deduced from the sequence of genomic cosmid clone E1080 that contained the entire *Notch* gene. Thus, there appears to be only one *Notch* gene in amphioxus, since all 21 *Notch* clones obtained from PCR with degenerate primers to the highly conserved EGF region represented the same gene. In addition, on a low-stringency Southern blot probed with the most conserved domain of *Notch* (the ankyrin repeat region) 6 of 10 enzymes resulted in one major hybridizing band (Fig. 2). Since probing a similar blot under somewhat more stringent conditions with the 3′UTR of one of the two muscle actin genes in amphioxus gave multiple bands in all lanes (Kusakabe *et al.*, 1997), the very weakly hybridizing bands on the *Notch* blot are likely due to the hybridization of the probe with the ankyrin repeats of distantly related genes such as *ankyrin* and not to the presence of a second *Notch* gene. Indeed, when probed under higher stringency with a longer probe (1000 bp) to the EGF and Notch/Lin-12 repeat region, 7 of 10 enzymes revealed a single band, and no weakly hybridizing bands were detected (data not shown).

Structure of the AmphiNotch Protein

The AmphiNotch protein (Fig. 1) is 2524 amino acids long and includes 36 EGF repeats, 3 Notch/lin-12 repeats, a transmembrane region, a RAM23 domain, 6 ankyrin repeats (Fig. 1), and an additional highly conserved domain just C-terminal of the ankyrin repeats. There are also S1 (furin), S2 (TACE), and S3 sites for proteolytic cleavage. All of these domains are conserved among AmphiNotch, *Drosophila* Notch, and mouse Notch1 proteins. In addition, most of the EGF repeats have sites for residues of O-linked fucose residues or glucose residues or both (Fig. 3A). These sites are highly conserved among *Drosophila*, amphioxus, and mouse *Notch* proteins. Conserved sites for Ca^{2+} binding and for Asx hydroxylation also occur on most of the EGF repeats (Fig. 3B). With one or two exceptions, these sites cooccur on the same EGF repeats, which is not surprising because some of the Asx residues in the hydroxylation sites are also part of the Ca^{2+}-binding sites.

Exon/Intron Structure of AmphiNotch

Analysis of the genomic clone shows that the entire translated sequence of *AmphiNotch* is contained in 30

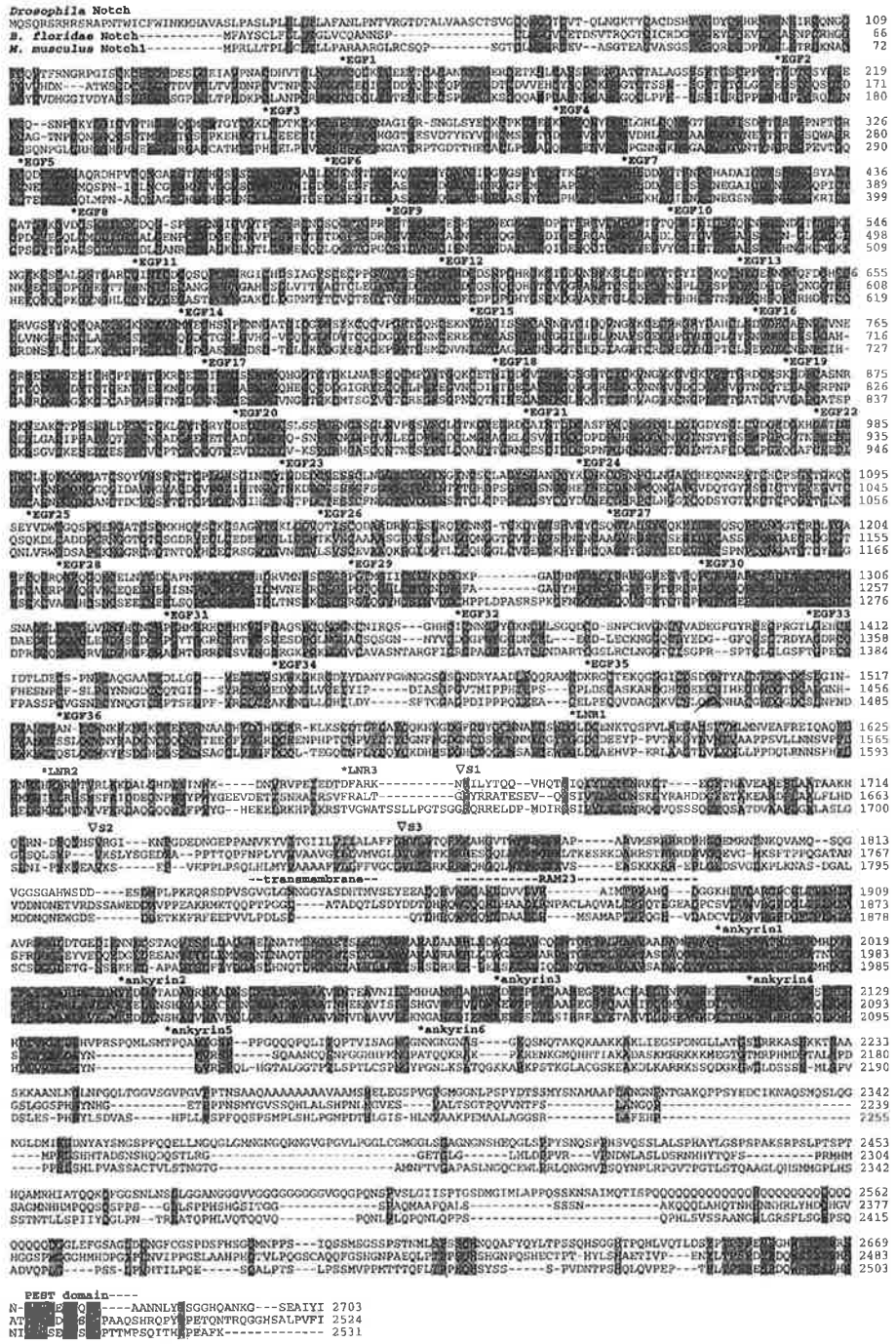

FIG. 1. Alignment of the AmphiNotch, *Drosophila* Notch, and mouse Notch1 proteins. Asterisks indicate the starting residues for EGF, Notch/Lin-12 (LNR), and ankyrin repeats. Shading indicates identities or conserved substitutions among all three (dark gray) or two (light gray) of the homolog proteins. There are three proteolytic cleavage sites, S1 (furin), S2 (TACE), and S3 (dependent on PS/γ-secretase activity); a transmembrane region; the RAM23 domain [which binds effectors of Notch signaling such as Su(H), CBF1, and lag-1]; and a PEST domain.

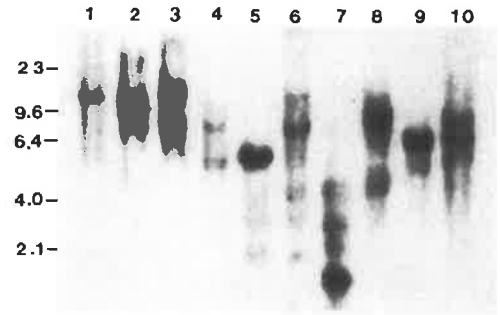

FIG. 2. Low-stringency Southern blot of genomic DNA from *B. floridae* probed with the ankyrin repeat region of *AmphiNotch*. Enzymes are (1) *Bam*HI, (2) *Bgl*II, (3) *Bst*EI, (4) *Eco*0109, (5) *Eco*RI, (6) *Hin*dIII, (7) *Kpn*I, (8) *Nco*I, (9) *Pst*I, (10) *Sal*I. Molecular size markers in kb are indicated at left.

exons (Table 2, Fig. 4). A comparison of intron positions with those of *Drosophila Notch* and human *Notch4* (a vertebrate *Notch* gene for which all intron positions have been published) (Li *et al.*, 1998) shows that three of the three most 5′ introns in amphioxus are conserved among all three organisms (Fig. 4). Furthermore, even through human *Notch4* has only 29 EGF repeats compared to 36 for both the *Drosophila* and the amphioxus *Notch* genes, there are an additional six conserved intron positions between the amphioxus and the vertebrate gene.

Phylogenetic Analysis

To determine the phylogenetic relationship between AmphiNotch and the four vertebrate Notch proteins, we constructed both unrooted (Fig. 5) and rooted (data not shown) trees by the neighbor-joining method. In both trees, AmphiNotch lies at the base of the vertebrate Notch genes, suggesting that duplications of the vertebrate *Notch* genes occurred after the split between amphioxus and the vertebrates.

Expression of AmphiNotch

In situ hybridization reveals no detectable expression of *AmphiNotch* in cleavage stages or the blastula. Transcripts of *AmphiNotch* are first detectable in the midgastrula in a ring of presumptive mesendoderm just inside the widely open blastopore and dorsally in the presumptive notochord and somites (Figs. 6A and 6B). These cells are columnar, and the transcripts of *AmphiNotch* are most abundant in the perinuclear cytoplasm near the cell apices (Fig. 6B). As gastrulation proceeds and the neural plate begins to flatten dorsally, transcripts of *AmphiNotch* (Figs. 6C and 6D) spread throughout the cytoplasm of the mesodermal cells. Dorsally there is a gradient of *AmphiNotch* expression in the presumptive somites and notochord with a high level of expression posteriorly, tapering off anteriorly. Expression progressively decreases ventrally and is undetectable in ventral endoderm (Figs. 6E and 6F). At this stage there is no ventral mesoderm. Ventral mesoderm forms at the midneurula stage as ventral extensions from the somites. In histological sections of the late gastrula/early neurula weak expression is also visible in the posterior neural plate (Fig. 6F, arrow).

During the first phase of amphioxus neurulation, the ectoderm bordering the neural plate on either side moves medially across the open neural plate and fuses in the midline, except at the extreme anterior end where the neuropore remains open to the exterior. Only after the ectoderm has covered the neural plate does the neural plate gradually roll up to form the neural tube (Fig. 6H). At the start of neural tube formation, the first four somites evaginate from the wall of the archenteron (Figs. 6G and 6H). The strongest *AmphiNotch* expression is in the posterior mesoderm and in the anteriormost three somites, especially in the dorsal portion of each (Figs. 6G and 6H). In

TABLE 2
Exon Positions of *AmphiNotch*

	Genomic DNA		cDNA		
Exon	Start	End	Start	End	aa position
1	1592	1713	1	122	41
2	15379	15632	123	376	126
3	16024	16359	377	712	238
4	17943	18065	713	835	280
5	20613	20846	836	1068	357
6	21172	21972	1069	1870	624
7	22187	22297	1871	1981	661
8	22571	22749	1982	2160	720
9	23137	23296	2161	2320	774
10	23726	23958	2321	2554	852
11	24311	24463	2555	2707	903
12	25907	26135	2708	2936	979
13	26843	27044	2937	3138	1046
14	27631	27784	3139	3292	1098
15	28144	28461	3393	3610	1204
16	28926	29263	3621	3948	1316
17	29708	30011	3949	4252	1418
18	30256	30311	4253	4308	1436
19	30575	30768	4309	4502	1501
20	31326	31528	4503	4705	1569
21	31921	32092	4706	4877	1626
22	32404	32555	4878	5029	1677
23	33090	33327	5030	5267	1756
24	34029	34146	5268	5385	1795
25	34665	34872	5386	5593	1865
26	35270	35419	5594	5743	1915
27	35750	35901	5744	5895	1965
28	36205	36352	5896	6043	2015
29	36672	36769	6044	6141	2047
30	36895	38328	6142	7575	2524

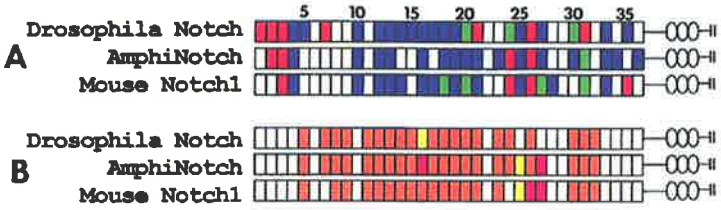

FIG. 3. Comparison of EGF repeat region of *Drosophila* Notch, AmphiNotch, and mouse Notch1 showing sites for (A) glycosylation and (B) Ca^{2+} binding. Red, sites for O-linked fucose. Blue, sites for O-linked glucose. Green, sites for both O-linked fucose and glucose. Yellow, aspartic acid/asparagine hydroxylation sites. Pink, Ca^{2+} binding sites. Orange, sites for both aspartic acid/asparagine hydroxylation and Ca^{2+} binding.

the most recently-formed somite (i.e., the most posterior), there is no detectable *AmphiNotch* expression. As successive somites are added, *AmphiNotch* transcription begins in the second-youngest somite. At this stage *AmphiNotch* is also expressed weakly throughout the neural plate and forming notochord (Fig. 6H).

The pattern of *AmphiNotch* expression within the somites changes with time. By hatching at 11 h, expression in the somites is still predominantly dorsal (Fig. 6I), but by 13 h it is also strong in the posterior half (Figs. 6J and 6L). Transcripts in the posterior mesoderm remain conspicuous in elongating embryos (Figs. 6I–6K, 6M, 6O, and 6S). Moderate expression continues in the notochord while that in the neural plate intensifies as it begins to roll up (Fig. 6L). In the late neurula the pattern of *AmphiNotch* transcripts in somites and posterior mesoderm remains unchanged (Figs. 6M and 6N), but there is a new zone of expression in the anterior pharyngeal endoderm (arrow, Fig. 6M). At this stage, the neural tube has rolled up and most of its cells still contain a low level of *AmphiNotch* transcripts (Fig. 6N). By 22 h (Figs. 6O and 6P) expression of *AmphiNotch* is downregulated in the somites and notochord but is upregulated in cells in the dorsal half of the cerebral vesicle. Transcripts remain conspicuous in the posterior mesoderm, the anterior pharyngeal endoderm, and the wall of the anterior left gut diverticulum (Hatscheck's left diverticulum) (Figs. 6O, 6P, and 6Q). In the late embryo (28 h) in which the mouth and first gill slit are forming, *AmphiNotch* is still expressed in the posterior mesoderm and in cells of the cerebral vesicle, but is downregulated in all but a few cells of the posterior nerve cord (Figs. 6S and 6T). In larvae older than about 30 h (the time the mouth opens) expression decreases in the posterior mesoderm (data not shown). Expression in the anterior pharyngeal endoderm remains strong and is also detectable in mesothelial cells that are apparently part of Hatschek's nephridium (Figs. 6S and 6T).

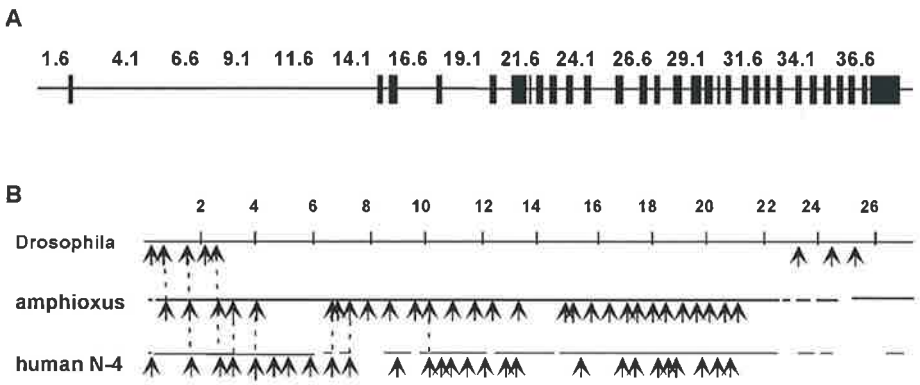

FIG. 4. Intron/exon organization of *Notch* genes. (A) Intron and exon positions in *AmphiNotch*. Exons are indicated by black bars. Numbers indicate positions within the *AmphiNotch* locus in kb. (B) Comparison of intron positions (arrows) among the *Drosophila* Notch, AmphiNotch, and human Notch4 cDNAs. Numbers are amino acid position $\times 10^{-2}$. Dotted lines indicate identical intron positions. Gaps in sequences of AmphiNotch and human Notch4 are introduced for alignment with the longer *Drosophila* cDNA.

DISCUSSION

Molecular Evolution of Notch Genes

In our phylogenetic tree, the single *AmphiNotch* gene branches at the base of the four vertebrate *Notch* genes. Together with the presence of single *Notch* genes in sea urchins and ascidian tunicates, this result suggests that *Notch* duplicated within the vertebrate lineage. However, *Notch4*, which is known only in mammals (Li *et al.*, 1998), branches before the divergences of the other *Notch* genes. Given the low bootstrap value (50) and long branch length, the position of vertebrate *Notch4* may simply reflect the extreme divergence of this gene. Indeed, homologs of three of the vertebrate *Notch* genes (*Notch1, 2, 3*) have been found both in mammals and in birds, while those of *Notch1* and *3* occur in fish and amphibians as well (Maine *et al.*, 1995; Williams *et al.*, 1995; Larsson *et al.*, 1994; Westin and Lardelli, 1997), suggesting that they must be basal to the mammalian *Notch4*. Notch genes have not yet been described in agnathans. However, if the current paradigm of one round of whole genome duplication at the base of the vertebrates and a second round after the split of gnathostomes and agnathans (Holland *et al.*, 1994) holds true, two *Notch* genes, a *Notch1* and a *Notch2/3* gene, would be expected in lampreys and hagfish.

Comparisons of Notch Genes and Proteins

Variability among EGF repeats may be responsible for the tissue-specific expression of different *Notch* homologs. EGF repeats can differ in their affinity for Ca^{2+} and in the presence or absence of O-linked fucose and/or glucose. In addition, the number of EGF repeats varies among *Notch* genes both within an organism and among different organisms. Calcium-binding sites on EGF repeats have been recognized as including a consensus sequence, Cys_3-x-ASP/Asn-x-x-x-x-Tyr/Phe-x-Cys_4, necessary for β-hydroxylation of Asp/Asn residues, plus the sequence As/Asn/Glu-Ile/Val-Asp/Asn/Glu-Glu/Asp/Glyn-Cys_1 preceding the first Cys (Rand *et al.*, 1997). As Fig. 3B shows, these sites are highly conserved between *Drosophila* Notch, AmphiNotch, and mouse Notch1 and are absent from the 4 most N-terminal and 4 most C-terminal EGF repeats. EGF repeats 11 and 12 are necessary for Ca^{2+}-dependent ligand-mediated cell aggregation and bind Ca^{2+} directly (Rand *et al.*, 1997). The arrangement of Ca^{2+}-binding and non-Ca^{2+}-binding EGF repeats in *Notch* proteins together with differences in Ca^{2+} affinity may modulate ligand binding. In addition, differences in relative position of Ca^{2+}-binding EGF repeats among the four mammalian Notch homologs may contribute to the differences in their ligand specificity (Rand *et al.*, 1997).

In contrast, O-linked glycosylation of EGF repeats does not appear to affect the affinity of ligand binding. However, it can modulate the functions of EGF-containing proteins induced by ligand binding (Rabbani *et al.*, 1992; Moloney *et al.*, 2000). As Fig. 3A shows, these sites are moderately conserved between *Drosophila* Notch, AmphiNotch, and mouse Notch1; however, they are not as conserved as the Ca^{2+}-binding sites. Interestingly, two EGF repeats in all three Notch homologs, Nos. 22 and 31, lack sites both for Ca^{2+} binding and for glycosylation.

All *Notch* genes described to date have three Notch/lin-12 repeats. In contrast, the number of EGF repeats is variable, with a maximum number of 36 in insect *Notch* (Wharton *et al.*, 1985), vertebrate *Notch1* and *2* (Coffman *et al.*, 1990; Weinmaster *et al.*, 1991, 1992), and *AmphiNotch*. However, there are fewer EGF repeats in *Notch* genes of *Caenorhabditis* (Yochem and Greenwald, 1989), lower deuterostomes (sea urchin and ascidian) (Sherwood and McClay, 1997; Hori *et al.*, 1997), and vertebrate *Notch3* and *Notch4* (Lardelli *et al.*, 1994; Uyttendaele *et al.*, 1996). Amino acid alignments (data not shown) show that the positions of the missing EGF modules vary from organism to organism. For example, sea urchin EGF repeat 15 and ascidian EGF repeats 2 and 5 are missing. Mouse Notch3 lacks repeat 21 and parts of 2 and 3, while vertebrate Notch4 is missing Nos. 15, 17, 19, 21–23, 25, and 31. The *Caenorhabditis Notch* homologs, glp-1 and lin-12, are missing a total of 26 and 23 EGF repeats, respectively (Yochem and Greenwald, 1989). Since neither the number nor the position of the absent EGF repeats (with the exception of repeat 15 in sea urchin Notch and vertebrate Notch4) correlates with the phylogenetic position of the organisms, it seems likely that both the ancestral bilaterian and the ancestral deuterostome *Notch* genes had 36 EGF repeats and that losses of EGF repeats have occurred independently.

Different ligand specificities have been ascribed to different EGF modules. For example, modules 11 and 12 are involved in binding of Delta, and modules 19–36 bind Wingless (Wesley, 1999). Thus, the absence of specific EGF repeats could affect tissue-specific expression. Notch genes with 36 repeats (e.g., amphioxus Notch, *Drosophila* Notch, and vertebrate Notch1 and 2) are typically expressed widely in early embryos. In contrast, *Notch* genes of lower deuterostomes, *Caenorhabditis*, and *Notch3* and *4* of vertebrates, which all have fewer EGF repeats, tend to be expressed in fewer tissues. For example, zygotic expression of the ascidian *Notch* is largely restricted to ectodermal lineages during gastrulation and later to the neuroectoderm, particularly in the dorsal anterior portion (Hori *et al.*, 1997). Whether the restricted expression of ascidian *Notch* is related to the absence of specific EGF repeats remains to be determined. Expression of sea urchin *Notch* is initially restricted to the animal half of the early blastula and then becomes localized to a ring of cells around the vegetal plate which corresponds to the boundary between the presumptive secondary mesoderm and the endoderm (Sherwood and McClay, 1997, 1999; Sweet *et al.*, 1999); neural expression has not been described. Mammalian Notch4, which has only EGF modules, is apparently expressed only in developing macrophages, endothelial cells, mammary gland tissue, and the pancreas (Uyttendaele *et al.*, 1996, 1998; Lammert *et al.*, 2000; Singh *et al.*, 2000). Furthermore, mutations in human

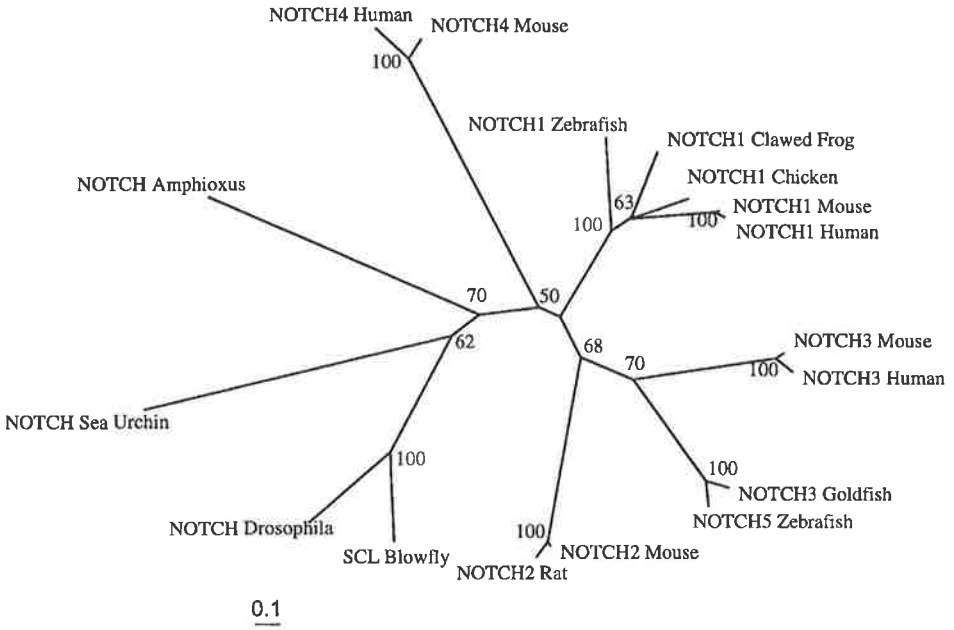

FIG. 5. Phylogeny of Notch proteins based on the Lin/Notch repeat regions, neighbor-joining method. Bootstrap values >50 are given. Scale line for branch lengths is the number of changes between character-states.

Notch3, which cause a defect in vascular smooth musculature that affects the function of several organs, tend to be clustered in the 5 most N-terminal EGF repeats (Joutel and Tournier-Lasserve, 1998). In addition, the EGF repeat that is missing in sea urchin *Notch* is the same repeat mutated in the *split* mutation of *Drosophila Notch*, which has an eye-specific phenotype (Hartley *et al.*, 1987).

Evolutionary Conservation of the Notch Pathway in Patterning the Mesendoderm

Notch is expressed in the mesendoderm in a wide variety of protostome and deuterostome embryos. In *Drosophila*, *Notch* functions in both mesoderm and endoderm, in patterning of the heart and somatic musculature, and in the midgut (Corbin *et al.*, 1991; Hartenstein *et al.*, 1992; Schnabel, 1994; Park *et al.*, 1998; Rusconi and Corbin, 1998), while in *Caenorhabditis*, a role in morphogenesis of the intestine has been described (Hermann *et al.*, 2000). Vertebrate *Notch* genes are expressed like *AmphiNotch* in the posterior mesoderm and forming somites as well as in the gut. They are also expressed in several gut derivatives such as the pancreas and lung (Weinmaster *et al.*, 1992; Conlon *et al.*, 1995; Lammert *et al.*, 2000). Thus, the lack of mesendodermal expression in ascidian tunicates (Hori *et al.*, 1997) may represent a loss, which might have evolved in connection with early determination of cell fate and reduction of the embryonic gut to an endodermal strand.

In most deuterostomes, *Notch* genes are expressed very early in the posterior mesendoderm. They are coexpressed with genes of the *Wnt* signaling pathway and transcription factors such as *brachyury*. This coincidence suggests the interaction of these genes in patterning along the anterior/posterior axis. Indeed, there is considerable experimental evidence for interaction of the *wingless* and *Notch* pathways and *brachyury* at several levels in a number of tissues in embryos of several species (reviewed in Panin and Irvine, 1998; Dierick and Bejosovec, 1999). In both *Drosophila* and vertebrates, *Notch* and *wingless/Wnt* can play opposing roles in developing tissues (Brennan *et al.*, 1999; Uyttendaele *et al.*, 1998). In *Drosophila*, Wingless is a ligand of Notch, binding to the EGF repeats (Wesley, 1999; Wesley and Saez, 2000). Wingless may also affect Notch signaling through interaction of Dishevelled, a downstream component of the *Wnt*-signaling pathway, with the intracellular domain of Notch (Axelrod *et al.*, 1996). Conversely, Notch signaling can regulate *wingless* expression (Rulifson and Blair, 1995; reviewed in Panin and Irvine, 1998).

There is evidence that *brachyury* is a target of both the *Notch* and the *wingless* pathways. In ascidian tunicates, Suppressor of Hairless [Su(H)]/RBP-J_κ binds to the *brachyury* promoter and activates *brachyury* expression in

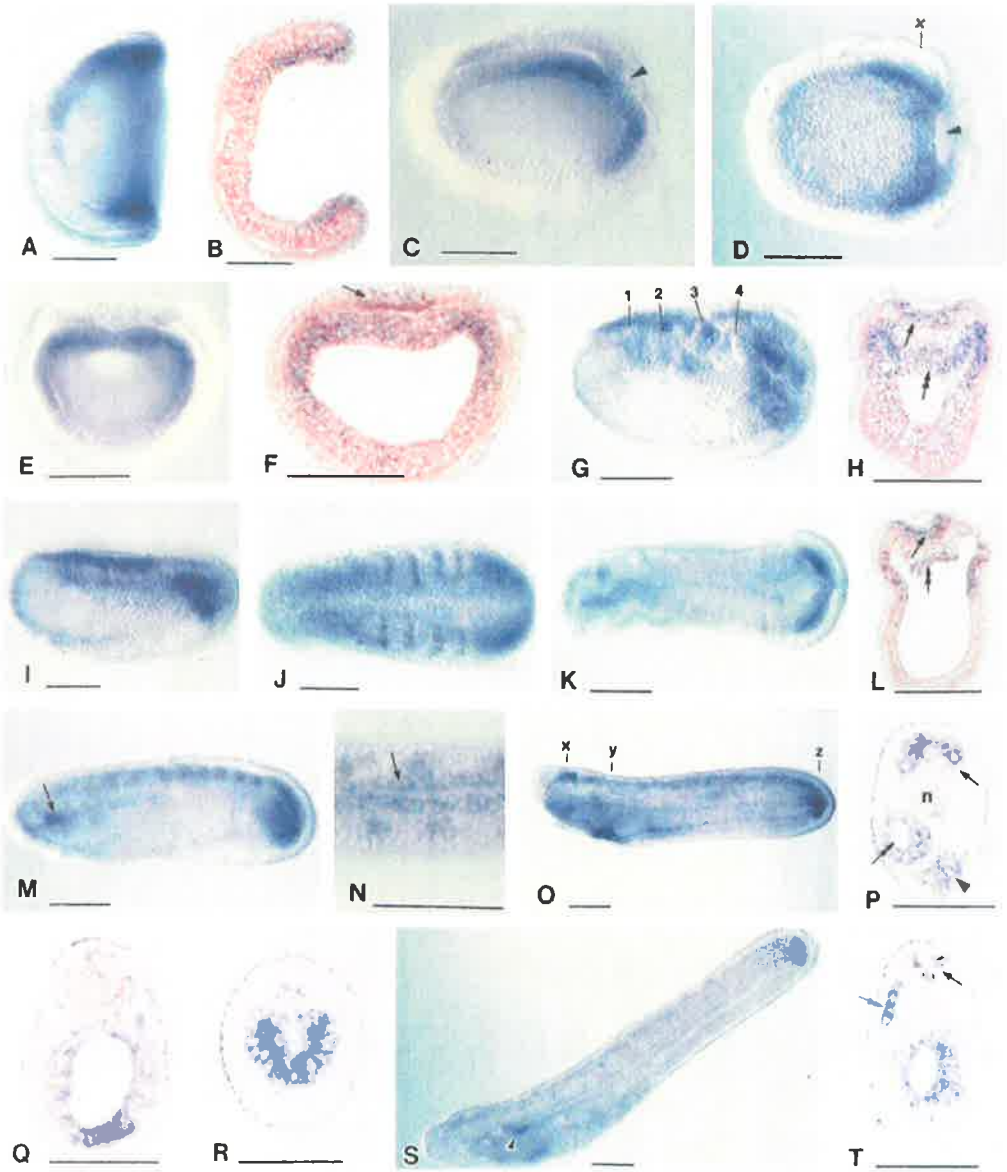

FIG. 6. *In situ* hybridization of *AmphiNotch* showing expression in amphioxus embryos shown as whole mounts (scale, 50 μm) with anterior toward the left (A, C–E, G, I–K, M–O, S) or cross sections (scale, 25 μm) (B, F, H, L, P–R, T). In all side-view whole mounts and sections dorsal is up. (A) Side view of cup-shaped gastrula (6 h) with blastopore opening toward the right. *AmphiNotch* is expressed in the presumptive mesendoderm around the blastopore and dorsally in presumptive notochord and somites. (B) Sagittal section of embryo in A showing expression in mesendoderm. (C) Side view of late gastrula (8 h). Blastopore (arrowhead) is at top right. Expression of *AmphiNotch* is strongest in the dorsolateral mesoderm and just within the blastoporal lip. (D) Dorsal view of the previous embryo showing mesodermal expression just within the blastopore (arrowhead) and extending dorsolaterally. (E) Posterior view of embryo in D in optical section through level X; expression is strong dorsally and dorsolaterally in presomitic and prechordal mesoderm. (F) Cross section through level X in D;

the notochord (Corbo *et al.*, 1997, 1998). Su(H) is a downstream component of the Notch signaling pathway; upon binding to the ankyrin repeat region of Notch, Su(H), either alone or together with the Notch intracellular domain, is translocated to the nucleus where it acts as a transcription factor (reviewed in Wu and Rao, 1999). Not surprisingly, expression of a constitutively activated Notch receptor alters tail morphology (Corbo *et al.*, 1998). In addition, in *Xenopus* the *brachyury* promoter also binds the downstream component of the Wnt signaling pathway, LEF-1/β-catenin (Arnold *et al.*, 2000). Thus, interactions between *Notch* and *wingless* pathways and *brachyury* are evidently complex.

In amphioxus, *Notch, wingless/Wnt,* and *brachyury* are all expressed around the blastopore in the early gastrula. The first of these posterior markers to be expressed is *brachyury* (*AmBra1* and *AmBra2*), which turns on in a ring around the equator of the late blastula/very early gastrula—the future blastoporal lip (P. W. H. Holland *et al.*, 1995; Terazawa and Satoh, 1995; Zhang *et al.*, 1997). Next, *Wnt1* turns on in the blastoporal lip (Holland *et al.*, 2000a), followed by *Wnt8* (Schubert *et al.*, 2000a; M. Schubert pers. commun.), then by *Notch, Wnt4,* and *Wnt7b* (Schubert *et al.*, 2000b), and finally in the late gastrula by *Wnt11* (Schubert *et al.*, 2000c). Expression of other amphioxus *Wnt* genes has not been determined. In the late gastrula and neurula, expression of *Wnt1* remains restricted to the region of the blastopore. However, as expression of *Notch* and *brachyury* expands into the somites, notochord, and neural plate, expression of *Wnts 4, 8,* and *7b* also expands into some of these domains. For example, *Wnts 4, 8,* and *11* and *brachyury* are coexpressed with *Notch* in the presomitic mesoderm with expression continuing into the somites (Schubert *et al.*, 2000a,c; P. W. H. Holland *et al.*, 1995). The spatiotemporal expression of these genes in amphioxus suggests that in amphioxus the *Notch* and *Wnt/wingless* pathways and *brachyury* may cooperate in patterning the mesendoderm.

Although sea urchin embryos form neither somites, a notochord, nor a nerve cord, there is also a posterior/vegetal *Notch* and *Wnt* signaling center in the early embryo. In the late blastula, the vegetal pole flattens to form the vegetal plate, which will give rise to the mesoderm and invaginate to form the embryonic gut. At this stage, Notch protein becomes localized to the apical surfaces of cells at the edges of the vegetal plate. Subsequently, by midgastrula, Notch is localized on the apical surfaces of cells around the blastopore and in the invaginating endoderm, predominantly along the dorsal side (Sherwood and McClay, 1997, 1999; Sweet *et al.*, 1999). This pattern is reminiscent of *Notch* expression in early amphioxus embryos. Similarly, genes of the Wnt-signaling pathway (*Wnt8* and β-catenin) are localized to the vegetal region of the sea urchin embryo (reviewed in Angerer and Angerer, 2000). Experimental evidence shows that *Wnt* signaling is involved in patterning along the anterior/posterior (= animal/vegetal) axis and that the Wnt and Notch pathways interact. Treatment of embryos with LiCl, which upregulates the *wingless* signaling pathway by inhibiting the negative regulator GSK3βI, alters the pattern of *Notch* expression and vegetalizes embryos (Sherwood and McClay, 1997). Moreover, effects of manipulating GSK3β levels are in agreement with the presence of a posterior Wnt-signaling center involved in patterning along the anterior/posterior axis (Emily-Fenouil *et al.*, 1998). In sea urchins, *Brachyury* is also expressed in the vegetal plate and later in the secondary mesenchyme

expression is conspicuous through the cells of the dorsolateral mesoderm and also beginning in the basal cytoplasm of cells of the neural plate (arrow). (G) Side view early neurula (10 h); expression is detectable in the posterior mesoderm and in the dorsal part of somites 1–3, but not in somite 4. (H) Cross section of the embryo in G through the level of somite 2; the strongest expression is in dorsal cells of the forming somites; weaker expression is visible in cells of the forming notochord (tandem arrow) and in cells of the neural plate (single arrow), which is overgrown by epidermis. (I) Side view of whole mount of hatched neurula (13 h) showing expression in the posterior mesoderm, in all but the most posterior (youngest) somite, in the neural plate and notochord. (J) Dorsal view of a whole mount of the 6-somite neurula in I with the dorsal portion of the somites in focus. (K) The same embryo as in J viewed in optical section through the gut showing strong expression in the posterior mesoderm and weak expression in anterior endoderm. (L) Cross section through the embryo in J and K at the level of somite 5. *AmphiNotch* is expressed in the walls of the forming somites, in the neural plate (single arrow), and in the forming notochord (tandem arrow). (M) Side view of a late neurula (18 h) showing strong expression in the posterior mesoderm, somites, and anterior pharyngeal endoderm (arrow). (N) Enlargement of the preceding embryo in dorsal view with the neural canal in focus. Expression is detectable in the somites and in many cells of the dorsal nerve cord (arrow). (O) Side view of 22-h embryo; the most conspicuous expression is in dorsal cells of the cerebral vesicle (level x), in some ventral pharyngeal cells (level y), and in the posterior mesoderm (level z). There is less conspicuous expression in the remainder of the nerve cord and in the somites. (P) Cross section through level x of the embryo in O showing strong expression in dorsal and lateral cells of the cerebral vesicle (single arrow), in the wall of the left anterior gut diverticulum (tandem arrow), and in the anterior extremity of the pharynx (arrowhead). The notochord (n) no longer contains detectable transcripts of *AmphiNotch*. (Q) Cross section through y in the embryo in O showing strong expression in the pharyngeal endoderm especially on the ventral side. (R) Cross section through level z of the embryo O showing conspicuous expression in the posterior mesoderm. (S) Side view of a 28-h embryo with strong expression in the posterior mesoderm, in some cells of the dorsal nerve cord, in the pharynx, and in some mesothelial cells (arrowhead) that may be forming part of Hatschek's nephridium. (T) Cross section through the embryo in S at the level indicated by the arrowhead; expression is in the dorsal nerve cord (single arrow) and in mesothelial cells (tandem arrow) that are apparently part of Hatscheck's nephridium. Expression is undetectable in the notochord.

(Harada *et al.*, 1995), while in starfish it is expressed around the blastopore (Shoguchi *et al.*, 1999).

Both vertebrate and amphioxus *Notch* genes are expressed in the posterior mesendoderm, in the forming somites, and later in the tailbud (Bierkamp and Campos-Ortega, 1993; Westin and Lardelli, 1997; Beck and Slack, 1999). *Brachyury* and several *Wnt* genes are expressed in patterns overlapping with that of *Notch* (Gont *et al.*, 1993; Beck and Slack, 1999; Tada and Smith, 2000). Although in the *Xenopus* blastula the *Wnt*-signaling pathway first establishes dorsoventral polarity, there is a second late phase of *Wnt* signaling, in which β-catenin is translocated to nuclei of cells around the lateral and ventral margins of the blastopore (Schneider *et al.*, 1996). This phase is involved in posteriorization of the neuroectoderm, formation of paraxial mesoderm, and tailbud extension. There is experimental evidence for the interaction of the *Notch* and *Wnt* pathways and *Brachyury* both in elongation of the tailbud and in patterning of the somites. In *Xenopus*, expression of *Notch* together with *Xwnt3a* provokes elongation and formation of neural tubes in animal caps and has been implicated in outgrowth of the tailbud (Beck and Slack, 1999), as has *Brachyury* (Gont *et al.*, 1993). It has been suggested that the mechanism for tail extension involving *Notch* and *Wnt3a* may be common among vertebrates. Wnt3a and Wnt11 are targets of Brachyury during gastrulation and in paraxial mesoderm, respectively (Tada and Smith, 2000; Yamaguchi *et al.*, 1999). Conversely, *Brachyury* can also be a target of the Wnt-signaling pathway, at least in embryonic stem cell cultures (Arnold *et al.*, 2000). Our results suggest that *Notch*, *Wnts*, and *Brachyury* may also cooperate in patterning the amphioxus mesoderm and in elongation of the tailbud. Although the Notch-signaling pathway has not been shown to be a direct target of Brachyury, in amphioxus, expression of *Brachyury* in the future blastoporal lip, before both *Wnt1* and *Notch* are turned on in the same cells, suggests that *Brachyury* may act upstream of *Notch* either directly or via signaling through the *wingless* pathway. These possibilities could be tested experimentally and by *in vitro* analyses of the *Notch* promoter.

In vertebrates, expression of *Notch* in the presomitic mesoderm and in early somites is required for normal segmentation, acting upstream of cyclically expressed genes such as *her1*and *HES1* (Jouve *et al.*, 2000; Aulehla and Johnson, 1999; Takke and Campos-Ortega, 1999). The Wnt signaling pathway is also involved in somitogenesis, although a direct link between Notch and Wnt signaling in somitogenesis has not been shown. In amphioxus, the somites extend the full-length of the body. The anteriormost somites are formed by enterocoely and the more posterior ones by schizocoely, more like the somites of higher vertebrates. Although there are some differences in gene expression in the two types of somites (e.g., *engrailed* is expressed during segmentation in the anteriormost somites only), *Notch* and *Wnt* genes are expressed in both the anterior and the posterior somites, indicating that later development involves common genetic pathways. Although homologs of vertebrate genes with cyclic expression in the somites (e.g., *her1*, *HES1*) have not been cloned from amphioxus, it is likely that they will similarly be expressed in amphioxus as in vertebrates.

Roles of AmphiNotch in Neurogenesis

In both early and late amphioxus development, the expression of *AmphiNotch* in the neural plate and nerve cord closely parallels that in vertebrate embryos. In amphioxus, ectodermal expression begins in the posterior neural plate, extends to the entire neural plate, and later becomes restricted to anterior regions of the neural tube, chiefly in dorsal cells in the cerebral vesicle. Similarly, in the mouse and *Xenopus*, *Notch* homologs are expressed in the neural plate and neural tube, especially in dorsal regions of the hindbrain, brachial spinal cord, and infundibular recess of the diencephalon (Coffman *et al.*, 1990, 1993; Bierkamp and Campos-Ortega, 1993). In the zebrafish, *Notch* homologs are expressed in the neural plate and later on in much of the brain (Westin and Lardelli, 1997). Thus, in both amphioxus an the vertebrates, *Notch* genes are initially broadly expressed in the neural plate and later become restricted to anterior regions of the nerve cord.

Activation of *Notch1* in the zebrafish in turn activates the bHLH gene *her4*, suppresses *neurogenin* expression, and reduces the number of primary neurons (Takke *et al.*, 1999). In amphioxus, *neurogenin* is broadly expressed in the posterior part of the dorsal ectoderm of the early gastrula, but by the late gastrula turns off in the posterior region of the neural plate in a pattern complementary to that of *AmphiNotch*. As the neural tube forms, *AmphiNotch* becomes weakly but broadly expressed in the neural plate, unlike neurogenin, which becomes restricted to two columns of cells on either side of the floor plate. Subsequently, both *Notch* and *neurogenin* (Holland *et al.*, 2000b) become restricted to subsets of cells in the nerve cord, particularly in dorsal regions of the cerebral vesicle, the homolog of the diencephalon. These domains are not entirely congruent, although the possibility cannot be excluded that they may include some of the same cells. These expression patterns suggest that Notch may have similar roles in neurogenesis in amphioxus and in vertebrates.

Notch Expression in the Developing Kidney

The homology of vertebrate kidneys and amphioxus nephridia has long been controversial. The controversy has centered on whether the amphioxus larval kidney is ectodermal (and thus homologous to protostome nephridia) or mesodermal (and thus homologous to the vertebrate kidney). More recent morphological studies indicate a mesodermal origin for the amphioxus larval kidney (Ruppert, 1996; Stach and Eisler, 1998). In amphioxus, Notch is expressed in the primordium of the larval kidney. Similarly, mouse *Notch* homologs are also expressed in the early kidney (Franco del Amo *et al.*, 1992; Williams *et al.*, 1995).

The finding that both express *Notch* homologs as well as homologs of *Pax2/5/8* in early development (Kozmik *et al.*, 1999) supports ideas of the common ancestry of the vertebrate pronephros and amphioxus kidney.

In summary, the presence in amphioxus of a single *Notch* gene with a full complement of 36 EGF repeats expressed in multiple tissues in embryogenesis in patterns similar to those of all the vertebrate *Notch* genes put together underscores the utility of amphioxus as a stand-in for the ancestral vertebrate. From the accumulating evidence, it is becoming increasingly apparent that the amphioxus and vertebrate body plans are established by very similar mechanisms. Given the simple genome of amphioxus and the diagrammatic clarity of embryogenesis, amphioxus embryos promise to be a simplified model for helping to elucidate the evolution of developmental mechanisms.

ACKNOWLEDGMENTS

We thank Ray Wilson for laboratory space in St. Petersburg, Florida, during the amphioxus breeding season. We thank Jonatan Westin and Helena Vretman for excellent technical assistance in cloning and sequencing and Klas Kullander and Finn Hallböök for advice on evolutionary analyses. This research was supported by Grants IBN 96-30938 and 00-78599 from the U.S.A. National Science Foundation (to L.Z.H. and N.D.H.) and by funds from the Department of Molecular Biosciences of The University of Adelaide (to M.L.). We thank Pierre Pontarotti for initiating the sequencing of the cosmid clone.

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Received for publication October 4, 2000
Accepted December 20, 2000
Published online March 13, 2001

CONCLUDING REMARKS

During the research studies reported in this thesis I have attempted to develop simple and efficient cloning strategies to facilitate isolation and characterisation of novel developmental control genes. This has enabled us to fulfill one of the main goals of our laboratory - identification of novel vertebrate genes regulating formation of the nervous system and somites. As many developmental genes have spatially and temporally restricted expression patterns, we accomplished this by screening cDNAs derived from embryos undergoing gastrulation/neurulation and somitogenesis for clones revealing genes with neural- and somite-specific expression patterns. The lack of suitable cDNA libraries and complications associated with existing methods for cDNA library production prompted us to devise a simplified method for producing randomly-primed, directionally cloned cDNA libraries from small amounts of embryonic tissue. To achieve this, we combined several useful techniques. First, most steps of cDNA synthesis occurred on a solid carrier, thus facilitating retention of all cDNA species, including those corresponding to low-abundance transcripts. Second, we used random priming during reverse transcription to increase the likelihood of cloning ORFs. Third, we circumvented the requirement for linker addition and the use of a separate second strand primer for second cDNA strand synthesis by relying on non-specific priming of this reaction. Fourth, as the same primer was used both for second strand synthesis and the subsequent cDNA amplification by suppression PCR, it was possible to combine the two reactions into one tube, thus obviating the need for any steps of purification between these two stages. This simplifies the procedure and should also improve the overall yield of cDNA.

The use of PCR in cDNA amplification may introduce a bias in the frequencies of different sequences when compared to the frequencies of the corresponding mRNAs in the original tissue material (Das et al., 2001). To ascertain whether this also applies to the cDNA library produced by our method, we assessed the quality of this library by subjecting 66 cDNA clones to sequence analyses. These analyses revealed that 11% of the clones were redundant, most likely due to incomplete non-specificity of second strand priming. (However, such moderate levels of redundancy could be

reduced or eliminated by various existing methods of 'normalisation'.) We also found that a significant proportion of the cDNAs encoded putative ORFs and zebrafish ESTs, suggesting that random priming had enabled successful recovery of cDNAs containing coding regions. In addition to the sequence analysis, the observation that, following an *in situ* screen, the proportions of cDNA clones representing genes with ubiquitous, restricted or undetectable expression patterns are similar to those described by Gawantka et al. (1998) indicates that this method is a valid approach for the construction of cDNA libraries for use in *in situ* screens. The main disadvantage of a library produced by this method stems from the use of random-priming. Namely, randomly-primed cDNA libraries are less likely to contain inserts representing full-length transcripts than poly(d)T-primed libraries (McCarrey and Williams, 1994; Sambrook and Russell, 2001). Thus, such libraries are not optimal for cloning missing parts of coding regions of interest. On the other hand, many organisms, including zebrafish, possess long 3' UTRs, in which case use of randomly-primed libraries may be preferable.

In the subsequent stage of the project, the above cDNA library was used in a pilot-scale *in situ* screen. We uncovered several genes with neural and somitic expression patterns, including both novel (subsequently termed *angiotensin receptor-like protein*) and known (*spadetail*, *fibroblast growth factor receptor 1*) genes. Currently, the developmental roles of all three genes are being investigated in the Lardelli Laboratory. The next stage of my postgraduate research focused on *spadetail*'s neural expression pattern. *spadetail* is expressed both in the presomitic mesoderm and apparently irregularly distributed cells of the spinal cord. It was known to have a role in mesoderm development, yet its role in the neural tissue had not been established. Moreover, the identity of the *spt*-expressing cells remained ambiguous. Therefore, in the subsequent stage of this project, we performed a detailed analysis of the expression pattern of *spadetail* in the isolated cells of the developing spinal cord. This analysis was based on investigation of their co-expression profile (these cells express *huC*, *isl1*, *2*, *3*) and dorsoventral location (i.e. just ventral to the Rohon-Beard neurons). We inferred that *spt*-expressing neural cells are DoLA interneurons. This contrasts with the previous conclusion - that was based solely on the location these cells - stating that these are Rohon-Beard neurons. Although it is not absolutely

proven that all DoLA neurons express *spt*, this gene nevertheless constitutes a valuable marker of this neuronal subtype (or a subset thereof).

Interestingly, the rostrocaudal distribution (i.e. spacing) of *spt*-expressing neurons appeared to be irregular or even random. This is potentially important since the genetic mechanisms regulating the formation of irregularly spaced structures are poorly understood whereas the mechanisms responsible for the development of regularly distributed structures have received far greater attention. To begin to understand such mechanisms, we characterised the distribution of *spt*-expressing neurons statistically. This showed that while considerable variation exists in the numbers of these cells in the corresponding spinal segments of different embryos and between different segments of the same embryo, there is a tendency to higher cell numbers in rostral spinal segments. It is possible that the observed tendency results form the fact that either more neurons are born rostrally or die caudally. However, the observation that ipsilateral juxtapositions are twice as common in these segments than in more caudal ones argues that *spt*-expressing neurons may migrate rostrally as the spinal cord matures. Such tendency to higher rostral neuron numbers is not rare among the different spinal neuronal classes. For example, in *Xenopus*, both Rohon-Beard neurons and commissural interneurons are present in higher numbers in rostral segments (Hartenstein et al., 1993). This implies that the observed distribution of cells expressing *spt* and, by extension, the patterning mechanisms responsible for its formation, may be conserved among the neuronal classes found in lower vertebrates. Interestingly, in embryos stained for *spt* expression for extended periods, we saw frequent juxtaposition of *spt*-expressing neurons to somitic cells also expressing *spt* at low levels. This suggests that the distribution of *spt*-expressing neurons may be 'inefficiently' patterned by *spt*-expressing somitic cells or that the expression of *spt* in both tissues is induced by a common positional cue. These descriptive studies set the stage for the future functional studies of the genetic mechanisms regulating non-segmental (i.e. irregular) rostro-caudal distributions of neurons.

The final stage of the project involved extending the non-specific priming of DNA synthesis to develop a simple and efficient technique for cloning unknown DNA sequences flanking known DNA. This enabled us to clone several parts of the genes under investigation in the Lardelli Laboratory (i.e. *amphioxus notch* and 5' regulatory

sequences from the zebrafish *tyrosinase* gene). The initial non-specific PCR amplification was performed with a single primer that binds specifically within known sequence and non-specifically in the unknown DNA region. In the second PCR reaction, the sequences of interest were amplified from a reaction mixture also containing undesired sequences with nested PCR using a primer that had been extended further downstream from the primer used in the initial PCR. This technique can be used both for rapid amplification of cDNAs as well as cloning unknown genomic sequences and is thus potentially widely applicable. It is valuable for isolation of unknown sequences from organisms for which there are no conventional bacteriophage genomic or cDNA libraries available. Thus, in our case, this technique enabled to isolate a 0.5 kb region located 500 bp downstream from the 5' end of *AmphiNotch* cDNA that could not be cloned by other methods. This sequence was used in the final assembly of the full-length coding sequence of *AmphiNotch*. The subsequent sequence/phylogenetic and expression pattern analysis showed that this gene was ancestral to the vertebrate *Notch* genes.

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