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Original Article

Expression of *axial* and *sonic hedgehog* in wildtype and midline defective zebrafish embryos

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ABSTRACT We present a description of the expression of the *HNF-3ß* homolog *axial (axl)* in the developing zebrafish up to larva stages and compare it with that of *sonic hedgehog (shh)*. Both genes are expressed in derivatives of all three germ layers in dynamic patterns that show substantial overlap, consistent with mutual regulatory interactions between the two genes. However, we also describe unique sites of expression of both *axl* and *shh* indicating that some aspects of their regulation are independent of one another. In *no tail (ntl*, zebrafish *Brachyury)* and *floating head (flh*, zebrafish *Xnot1)* mutants, both of which affect notochord development, early expression of *axl* in the organizer is unaffected, excluding a function for these genes in establishment of *axl* expression. At later stages, *ntl* and *flh* mutants show different effects on the expression of both *axl* and *shh* in the neuroectoderm of the trunk and tail reflecting their distinct contributions to the development of the midline mesoderm; in contrast to *flh* and *ntl* mutations whose effects are restricted to the trunk and tail, mutation of *cyclops (cyc)* affects *axl* and *shh* expression along the entire midline of the neuroectoderm. Endodermal expression of *axl* and *shh* is not affected by the mutations showing that development of the endoderm is under distinct control.

KEY WORDS: *axial, sonic hedgehog, HNF3/forkhead, cyclops, no tail, floating head, floor plate, notochord, zebrafish*

Introduction

The complex organization of the vertebrate body is established during embryogenesis by a series of embryonic inductions (for review see Slack, 1991). One of the best characterized examples of such a process is the induction of floor plate differentiation in the ventral neural tube by the underlying notochord, a derivative of the axial mesoderm (see Placzek *et al.*, 1993 and references therein). The expression patterns of several members of the *forkhead/HNF3* family of winged-helix transcription factors identify them as genes potentially involved in notochord and floor plate development (Dirksen and Jamrich, 1992; Knöchel *et al.*, 1992; Ruiz i Altaba and Jessell, 1992; Ang *et al.*, 1993; Monaghan *et al.*, 1993; Ruiz i Altaba *et al.*, 1993b, 1995a; Sasaki and Hogan, 1993; Strähle *et al.*, 1993). In *Xenopus*, ectopic expression of *HNF3β* and the closely related gene *pintallavis* leads to ectopic activation of floor plate specific marker genes suggesting a role for this *forkhead/HNF3* family gene in floor plate specification (Ruiz i Altaba *et al.*, 1993a, 1995b). Misexpression of *HNF3β* at the midbrain/hindbrain boundary of mouse embryos leads to similar results (Sasaki and Hogan, 1994), whereas mouse embryos homozygous for targeted mutations in *HNF3β* fail to form notochords and lack expression of floorplate markers in the ventral neural tube (Ang and Rossant, 1994; Weinstein *et al.*, 1994). Such a floor plate marker is *sonic hedgehog (shh)*, one of several vertebrate homologs of the *Drosophila* segment polarity gene *hedgehog*. Both the *in vitro* inducing capabilities of the Shh protein and its expression in the midline mesoderm make *shh* a likely candidate for the floor plate inducing signal that emanates from the notochord (Echelard *et al.*, 1993; Krauss *et al.*, 1993; Riddle *et al.*, 1993; Roelink *et al.*, 1994). Shh protein has been shown to induce floor plate and motoneurons in neural plate explants in a concentration-dependent manner suggesting that it may act as a morphogen that directs the patterning of the ventral neural tube (Marti *et al.*, 1995; Roelink *et al.*, 1995; Tanabe *et al.*, 1995). One of the likely targets of *shh* activity is *HNF-3β*, which appears to be an immediate response gene to floor plate induction, its expression being activated when *shh* is ectopically expressed in the neuroectoderm (Echelard *et al.*, 1993; Krauss *et al.*, 1993; Ruiz i Altaba *et al.*, 1995a,b; Tanabe *et al.*, 1995). Thus, *HNF-3β* apparently acts both upstream and downstream of *shh* in

Abbreviations used in this paper: HNF3, hepatocyte nuclear factor 3; *shh, sonic hedgehog; axl, axial; cyc, cyclops; ntl, no tail; flh, floating head; Xnot1, Xenopus notochord 1;* FPL, floor plate lateral; zn12, zebrafish neuron 12; HNK1/L2, human natural killer 1/L2 glycolipid; MLF, medial longitudinal fascicles; *Xbra, Xenopus brachyury.*

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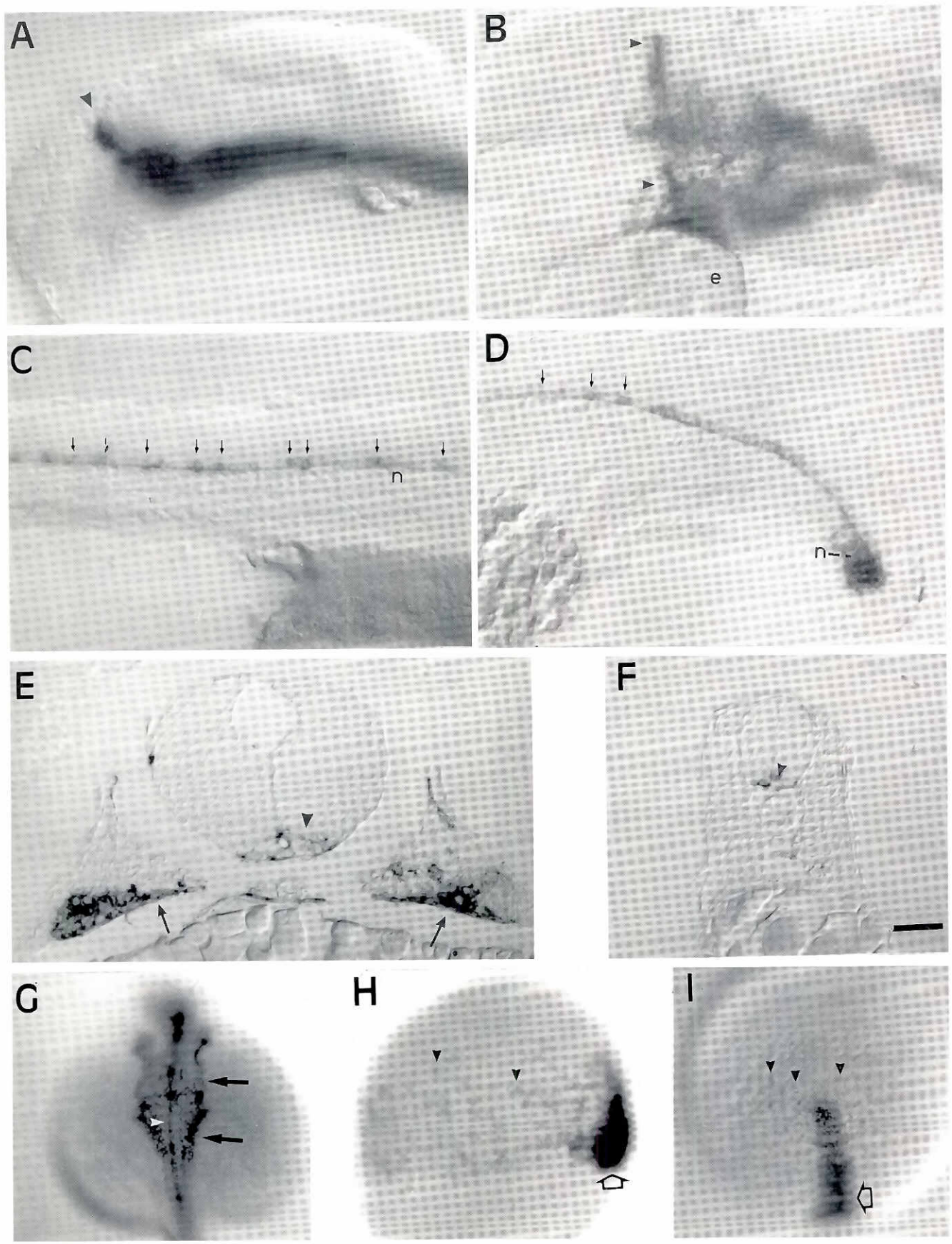
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the pathways controlling the development of the notochord and floor plate.

Previous reports of the expression patterns of *axl* (zebrafish *HNF-3β*, Strähle *et al.*, 1993) and *shh* (Krauss *et al.*, 1993) along the developing midline of gastrula and neurula stage zebrafish embryos are consistent with such a functional interdependence of the two genes: both are expressed in the organizer, notochord, prechordal plate mesoderm and subsequently also in the midline of the neuroectoderm. The neuroectodermal expression of both genes is also dependent on the *cyclops (cyc)* gene (Krauss *et al.*, 1993; Strähle *et al.*, 1993); mutations in *cyc* block the formation of the floor plate and ventral forebrain by interfering with neuroectodermal reception of inducing signal(s) from the mesoderm (Hatta *et al.*, 1991, 1994).

Here we extend our previous expression analysis to larval stages and investigate the relationship between *axl* and *shh* more closely. We find that both *axl* and *shh* are expressed in a dynamic fashion in overlapping but not identical domains along the body axis in tissues derived from all three germ layers. We have also analyzed midline deficient mutants in order to identify other genes that affect *axl* and *shh* expression. Both *no tail* (*ntl*, zebrafish *Brachyury*; Halpern *et al.*, 1993; Schulte-Merker *et al.*, 1994) and *floating head* (*flh*, zebrafish *Xnot1*, Talbot *et al.*, 1995) are required for correct *axl* and *shh* expression in the neural tube of the trunk and tail but not the brain.

Results

axl expression in wild type embryos

At 24 h of development, *axl* is expressed along the ventral midline of the neural tube. Anteriorly, expression terminates at the mid-diencephalic boundary (Fig. 1A; see also MacDonald *et al.*, 1994) with two horn-like expression domains extending dorsally towards the epiphysis (Fig. 1B). The broad expression in the midbrain narrows to a one to three cell-wide stripe caudally which comprises the floor plate and cells immediately lateral to it, the latter being hereafter referred to as floor plate lateral or FPL cells (Fig. 1C to F). Expression of *axl* in FPL cells of the trunk and tail is stronger than in floor plate cells (arrows Fig. 1C and D). Furthermore, *axl*-expressing FPL cells are distinguished from floor plate cells by their elongated cell shape when viewed laterally and by the accumulation of alkaline phosphatase reaction product at both the apical and basal poles, in contrast to its basal localization in floor plate cells (see also Fig. 4G and H). Unlike at earlier stages when *axl* is expressed strongly in the notochord (Strähle *et al.*, 1993), expression is barely detectable in the notochord at 24 h, with the exception of the tail bud (Fig. 1D). By 36 h of development, expression in the notochord has ceased entirely, whereas expression in the neural tube persists beyond 60 h of development (Fig. 2A to E).

High levels of *axl* expression are also detectable in the anterior endoderm in 24 h embryos (Figs. 1E,G and 2). To investigate the origin of these endodermal cells we investigated earlier stages using a more sensitive *in situ* hybridization protocol. We observed weak expression of *axl* in isolated cells lateral to the midline (Fig. 1H, arrowheads), in addition to the previously reported expression in the nascent axis of gastrula stage embryos (Strähle *et al.*, 1993). Consistent with an endodermal fate, these scattered cells are evident in the hypoblast around the entire blastoderm margin at the onset of gastrulation. As gastrulation continues, expression can no longer be detected at the blastoderm margin; instead expressing cells are present in a band at a distance from the margin that broadens to cover more anterior regions dorsally than ventrally (Fig 1I). Lateral *axl* expressing cells are not apparent during somitogenesis stages; instead weakly expressing cells are found underlying the anterior body axis (data not shown).

Expression in the anterior endoderm increases dramatically between 24 h and 36 h. High levels of transcript are detectable in the endoderm from the head back to the level of the pectoral fin buds (Fig. 2B and data not shown) whereas more posteriorly in the endoderm of the hindgut, expression is barely detectable (Fig. 2C). *axl* transcripts are present in a subset of cells within the cell mass overlying the yolk syncytium at the level of the pectoral fin buds. By 48 h, the mass of endoderm has undergone cavitation and developed a lumen lined by an epithelium which strongly expresses *axl* (Fig. 2D). At 60 h, *axl* is expressed in the endodermal lining of the oral cavity, the branchial arches, the anlage of the swim bladder, in the intestine and the liver (Fig. 2E and data not shown). The hindgut appears to be devoid of *axl* expression at 60 h of development.

axl and shh display distinct but overlapping patterns of expression

Previous studies in fish as well as in other species have suggested that the transcriptional regulation of *axl/HNF-3β* and *shh* may be mutually dependent, *axl* activating *shh* in the notochord which in turn induces *axl* transcription in the neural plate (Echelard *et al.*, 1993; Krauss *et al.*, 1993; Ang and Rossant, 1994; Sasaki and Hogan 1994; Weinstein *et al.*, 1994; Ruiz i Altaba *et al.*, 1995b; P.B., U.S. and P.W.I. unpublished). To investigate how wide-

Fig. 1. Whole-mount embryos hybridized to *axl* digoxygenin antisense probe. (A) *Lateral view of head of 24 h old embryo. Expression terminates anteriorly at the forebrain/midbrain boundary (arrowhead) and extents posteriorly through the ventral midbrain and hindbrain all along the ventral spinal cord.* **(B)** *Dorsolateral view of 26 h embryo showing the two horns of axl expression (arrowheads) extending dorsally in the lateral walls of the neural tube at the forebrain/midbrain boundary.* **(C)** *Lateral view of trunk of a 24 h old embryo. axl is expressed weakly in the floor plate and more strongly in cells immediately lateral to the floor plate (thin arrows).* **(D)** *Tailbud of 22 h old embryo. In addition to expression in the ventral neural keel the posterior tip of the notochord (n) still expresses axl at this stage. Notochordal expression that is detectable during earlier stages (Strähle et al., 1993) has ceased anteriorly.* **(E and F)** *Transverse section through 24 h old embryos at the level of the hindbrain posterior to the otic vesicle and at the level of the hindgut, respectively. axl expression is broader than the floor plate (arrowheads) which is one cell wide in the trunk. Arrows in* **E** *indicate axl expression in the endoderm.* **(G)** *Dorsal view of the hindbrain and anterior trunk of 24 h old embryo. In addition to expression in the ventral neural tube (white arrowhead), strong axl expression is seen in the endoderm (arrows) (endodermal expression is not seen in* **A** *and* **B** *because it has been dissected away together with the yolk).* **(H and I)** *60% and 90% epiboly stage embryos, respectively. Besides the strong expression of axl in the nascent axis (open arrow) scattered cells in the hypoblast are detected around the blastoderm margin at 60% epiboly (arrowheads). By 90% epiboly, these cells are found predominantly at the dorsal side of the embryo.* **(I)** *The yolk has been removed in embryos shown in* **A** *to* **D**. *Orientation of embryos is: anterior left, dorsal up (A to D); dorsal up (E and F); anterior up and view onto dorsal (G); dorsal right, anterior up (H): dorsal down, view onto animal pole (I). Bar: 60 μm (A to D), 30 μm (E, F). (e, eye; n, notochord).*

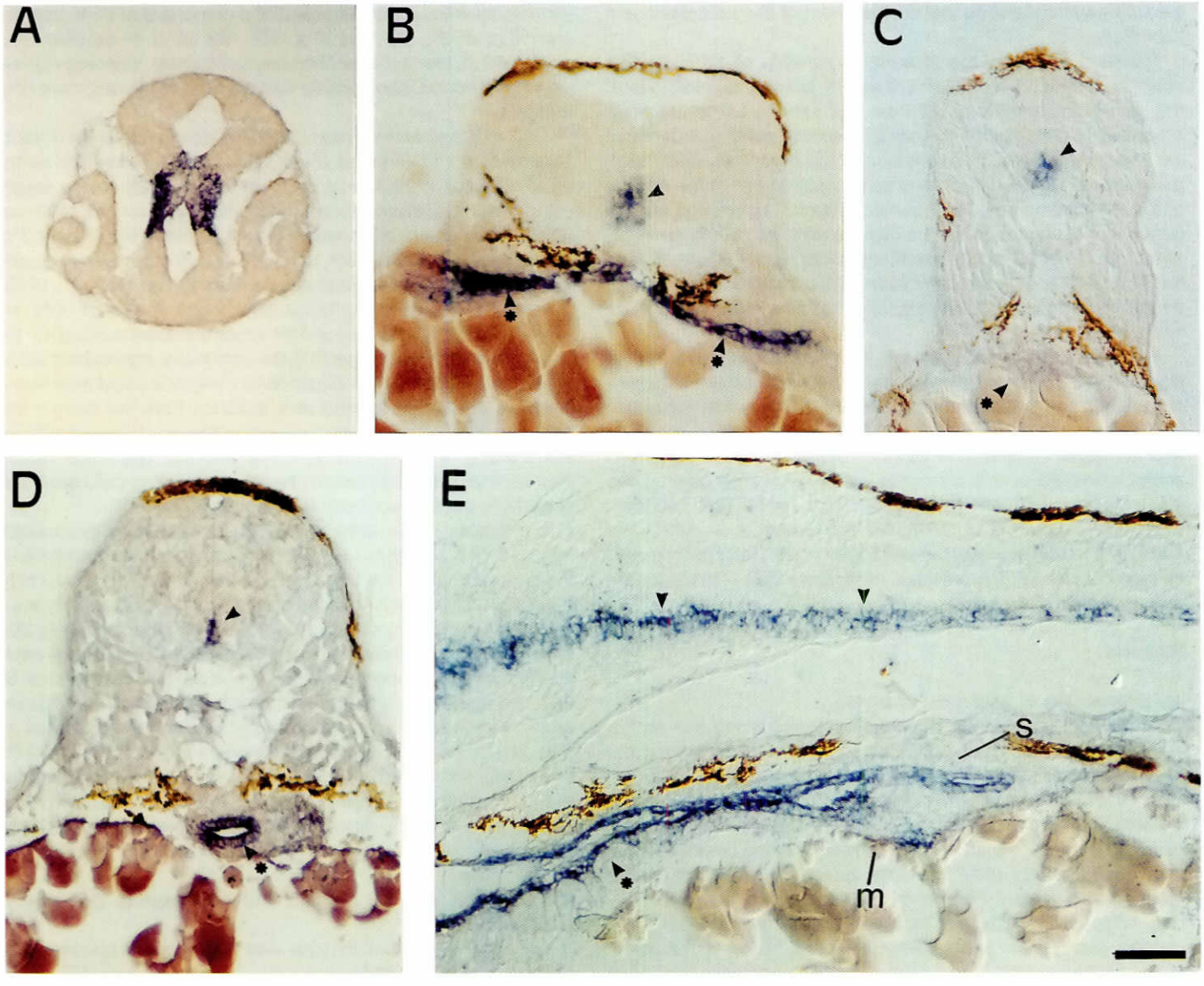

Fig. 2. Expression of *axl* in postsomitogenesis stage embryos. *Cryostat sections were hybridized to axl antisense DIG probe.* **(A)** *Section through midbrain/forebrain boundary of 36 hour stage embryo.* **(B and C)** *Transverse sections through the hindbrain and posterior trunk, respectively, of a 36 h embryo. Whereas strong axl staining is evident in the endodermal sheet giving rise to the pharynx and branchial arches (arrowheads with asterisks in* **B***) only weak axl expression is detectable in the hindgut (arrowhead with asterisks in* **C***). Expression in the ventral neural keel is indicated (arrowheads without asterisks).* **(D)** *Transverse section through the trunk of a 48 h embryo. By 48 h of development a gut lumen has formed that is lined by an epithelium strongly expressing axl (arrowhead with asterisk).* **(E)** *Sagittal section through a 60 h embryo showing expression of axl in the pharynx, branchial arches (arrowhead with asterisk), swim bladder primordium (s) and midgut (m). The ventral neural tube still expresses axl at this stage (arrowheads) Orientation of sections: dorsal up (A to E), anterior to the right (E). Bar: 120 μm (A), 30 μm (B to E).*

spread such a regulatory relationship may be, we compared the expression patterns of *axl* and *shh* throughout the embryo at different developmental stages. In 24 h embryos, *shh* is expressed in the floor plate along the ventral neural tube, but in contrast to *axl*, this expression extends beyond the mid-diencephalic boundary to occupy the floor of the anterior diencephalon (compare Figs. 3A and 1A). To compare the expression domains of the two genes along the neural tube, 24 h old embryos were stained with either the *axl* or *shh* probe and in addition with the zn12 antibody (Trevarrow *et al.*, 1990). zn12 recognizes the HNK1/L2 epitope that marks, among other structures, the medial longitudinal fascicles (MLF) which run along the ventrolateral aspects of the neural tube (Metcalfe *et al.*, 1990; Trevarrow *et al.*, 1990). Whereas expression of *shh* is confined to the ventral most cells of the hindbrain, *axl* expression is broader, with its lateral boundaries coinciding with the MLF (Fig. 3B and C). At trunk level, *axl* is expressed in cells lateral to the floor plate, whereas *shh* is expressed only in the floor plate (data not shown).

shh mRNA disappears from the notochord in an anterior to posterior progression in a manner similar to, though slightly delayed relative to, that of *axl* (Krauss *et al.*, 1993; Yan *et al.*, 1995; and our unpublished observations). At 48 h, neural expression of

shh persists whereas expression in the notochord is no longer detectable (Fig. 3F). At this stage, expression of *shh* is apparent in the endoderm (Fig. 3D to C) though in contrast to *axl* which at 48 h is not detectable in the hindgut, *shh* is expressed along the entire length of the endoderm (Fig. 3E and F).

axl expression in cyclops mutants

The *cyclops*^{b16} *(cyc)* mutation in zebrafish causes fusion of the eyes, lack of ventral structures in the anterior brain and lack of floorplate (Hatta *et al.*, 1991, 1994; Hatta, 1992). *axl* expression is absent in the neural keel of 8-somite stage embryos homozygous for the *cyc* mutation, but is unaffected in the notochord (Strähle *et al.*, 1993); *shh* is similarly affected (Krauss *et al.*, 1993). At 24 h, *axl* expression is absent in the brain of *cyc* embryos with the exception of a small patch of cells in the dorsal aspect of the mid-diencephalic boundary (Fig. 4C compare with Fig. 1A). In contrast to earlier stages, *axl* expression can also be detected in isolated cells along the midline of the trunk of *cyc* embryos at this stage (Fig. 4A and B). The morphology of the majority of these cells corresponds to FPL cells in wildtype embryos (Fig. 4F to I). The notion that these *axl*-positive cells are indeed FPL cells is further supported by their regular spacing along the entire spinal cord as seen with FPL cells in wildtype embryos. Furthermore, *shh* and other floor plate marker genes which are expressed at low levels typically in only a few cells in the tail neuroectoderm of *cyc* embryos at 24 h (Hatta *et al.*, 1994; Krauss *et al.*, 1993; Yan *et al.*, 1995) do not show this regular pattern of expression. Only occasionally are *axl* expressing floor plate cells found at 24 h in *cyc* mutants, whereas more *axl* positive cells with floor plate morphology are present at later stages (data not shown). *axl* expression, like that of *shh*, appears to be unaffected in the endoderm of *cyc* embryos (Fig. 4D and E and data not shown).

Expression of axl and shh in no tail mutant embryos

The *no tail (ntl)* gene encodes the zebrafish ortholog of the murine transcription factor *brachyury*, (Schulte-Merker *et al.*, 1992; Kispert *et al.*, 1995). Mutations in the *ntl* locus result in embryos that have defects in tail development and lack notochords but have clearly identifiable notochord precursor cells (Halpern *et al.*, 1993). The expression domains of *axl* and *ntl* overlap in the organizer and

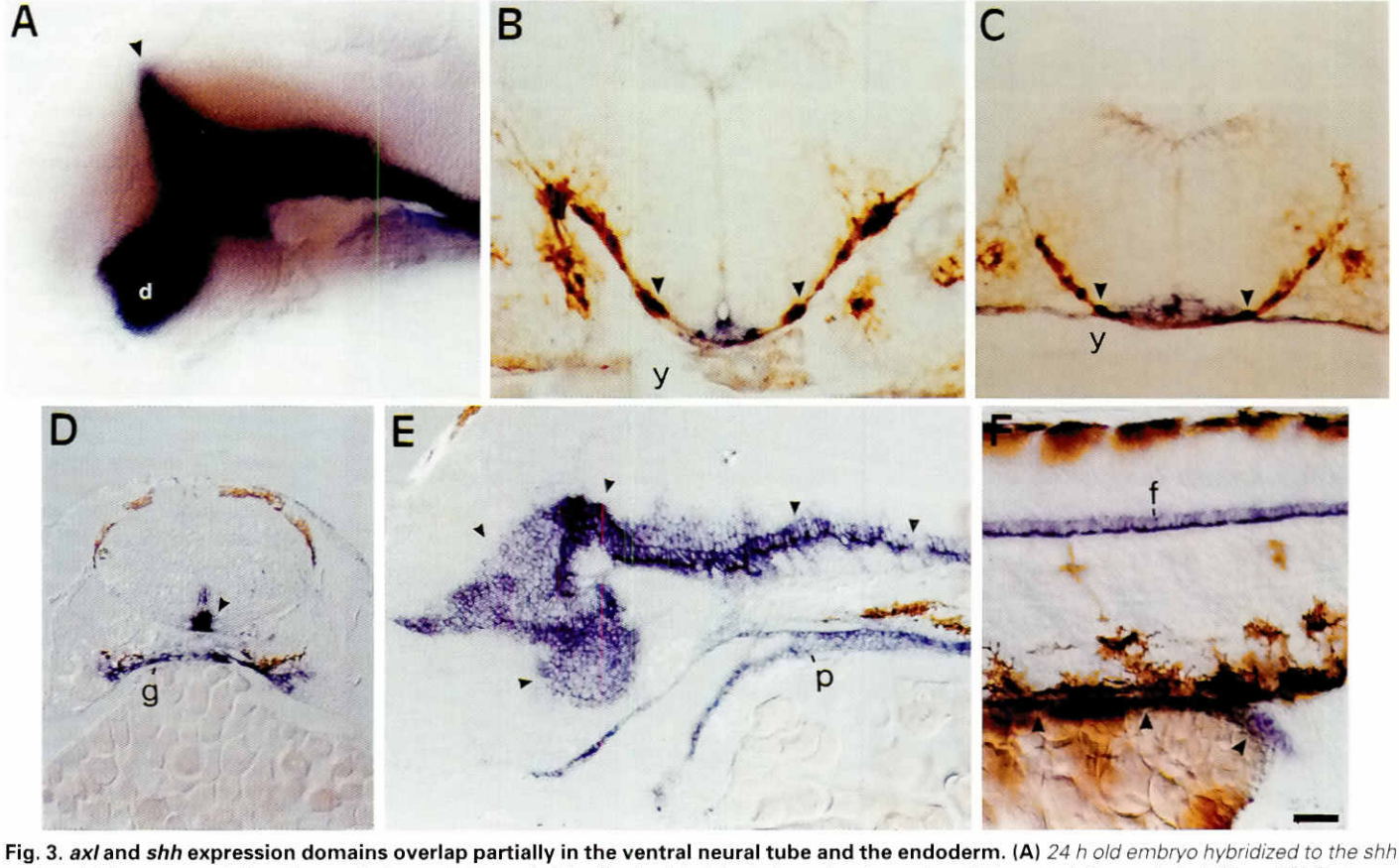

Fig. 3. *axl* and *shh* expression domains overlap partially in the ventral neural tube and the endoderm. (A) *24 h old embryo hybridized to the shh probe. shh is expressed along the ventral neural tube and at the forebrain/midbrain boundary (arrowhead) (d, ventral diencephalon).* **(B and C)** *Embryos hybridized to shh* **(B)** *and axl* **(C)** *probes (blue stain) were subsequently subjected to immunohistochemistry with the zn12 antibody (brown stain) which recognizes the HNK1/L2 epitope; embryos were sectioned transversely through the hindbrain. The zn12 antibody detects among other structures the medial longitudinal fascicles running along the ventrolateral aspects of the neural tube (indicated by arrowheads). Whereas axl expression spans the entire ventral aspects between the two fascicles, shh expression is confined to the ventral-most cells.* **(D and E)** *Transverse section through hindbrain* **(D)** *and sagittal section through 48 h embryos hybridized to the shh probe. Expression in floor plate and anterior brain is indicated by arrowheads. In addition, strong shh expression can be detected in the anlage of the gut (g).* **(F)** *48 h old whole-mount specimen showing expression of shh in the hindgut (arrows). Embryos are oriented dorsal up and anterior to the left (A,E and F). (d, ventral diencephalon; f, floor plate; g, gut; p, pharyngeal endoderm; y, position of yolk lost during sectioning). Bar: 50 μm (A,D,E,F) and 25 μm (B, C).*

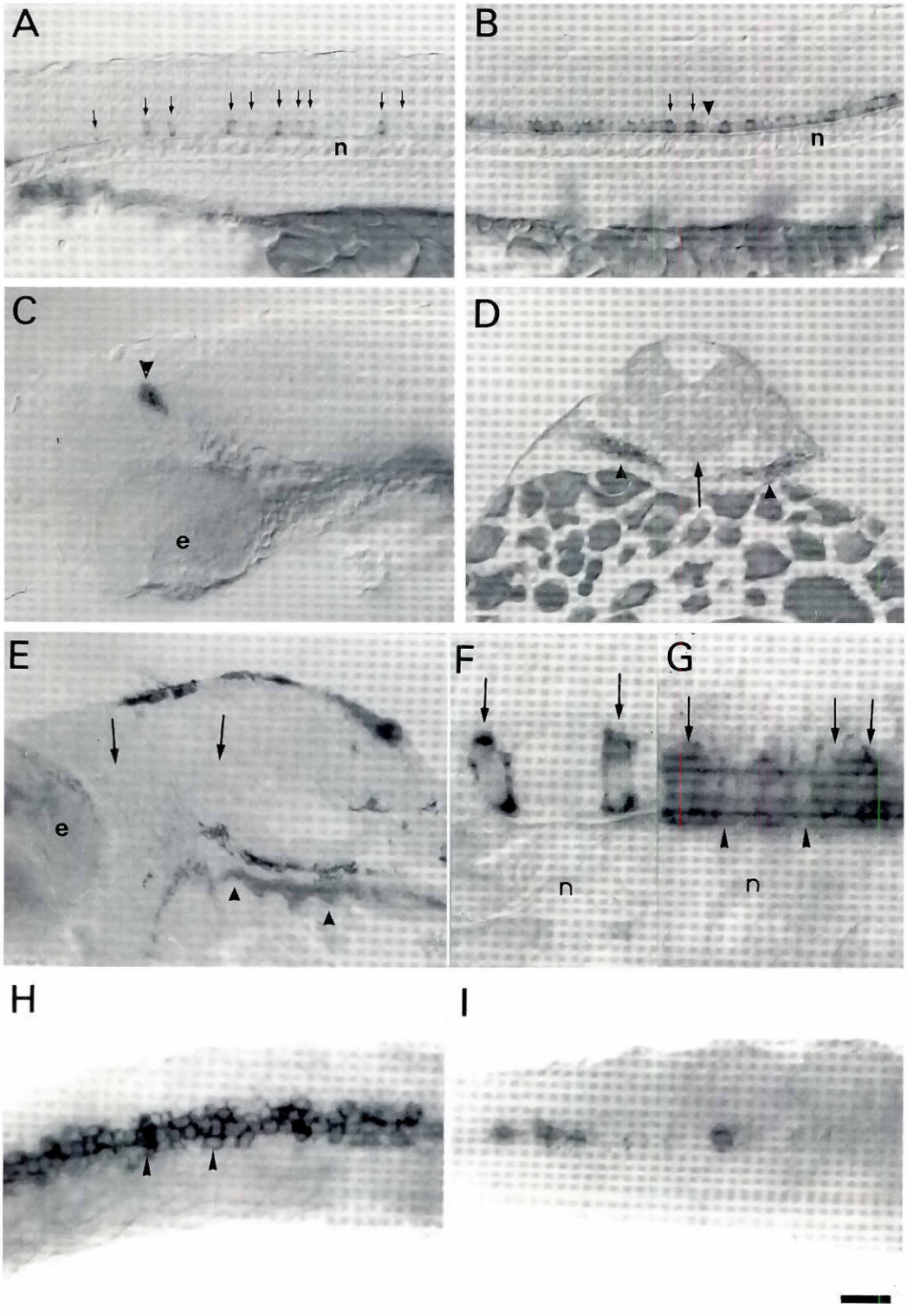

Fig. 4. Expression of *axl* in *cyclops*^{b16} (*cyc*) mutant embryos. **(A and B)** *axl expression in a cyc mutant embryo* **(A)** *and a wildtype sibling* **(B)** *at 24 h. Isolated, regularly spaced cells with a morphology of floor plate lateral cells (small arrows) express axl in the cyc mutant. (floor plate in the wildtype embryo is indicated by arrowhead in* **B***).* **(C)** *The anterior neural tube of a cyc mutant embryo at the 24 h stage is devoid of axl expression with the exception of a small number of cells at the dorsal aspects of the diencephalon (arrowhead).* **(D and E)** *Transverse section through the hindbrain of a 36 h cyc embryo* **(D)** *and sagittal section through the head of a 48 h cyc embryo* **(E)** *were hybridized to axl probe. Endodermal expression of axl is unaffected by the cyc mutation (arrowheads), whereas neuroectodermal expression is missing (arrows).* **(F)** *FPL cells (arrows) in cyc embryos stained with axl.* **(G)** *FPL cells (arrows) and floor plate cells (arrowhead) stained with axl probe. axl expressing FPL cells, in contrast to floor plate cells, are more elongated, frequently spindle-like with accumulation of alkaline phosphatase reaction product in the basal and apical pole.* **(H and I)** *Dorsal view onto wildtype and cyc neural tube. Arrowheads point at FPL cells. Orientation of embryos is dorsal up (A to G), and anterior to the left (with the exception of D); H and I: dorsal view, anterior left. (n, notochord; e, eye).*

the notochord (Schulte-Merker *et al.*, 1992; Strähle *et al.*, 1993) and as initiation of *ntl* expression precedes that of *axl* (Schulte-Merker *et al.*, 1992; Strähle *et al.*, 1993) it is possible that *ntl* activates *axl*transcription. To investigate this possibility, we analyzed *axl* expression in embryos homozygous for the loss of function *ntl*^{b160} allele (Halpern *et al.*, 1993; Schulte-Merker *et al.*, 1994). The distribution of *axl* transcripts appears normal in all early gastrula stage embryos from crosses between parental fish heterozygous for *ntl*^{b160} (Fig. 5A to C). At 90% epiboly, approximately 25% of embryos have impaired *axl* expression at the blastoderm margin (arrow in Fig. 5D) and show a slightly broadened expression domain caudally. In addition, *axl* expressing cells in the posterior

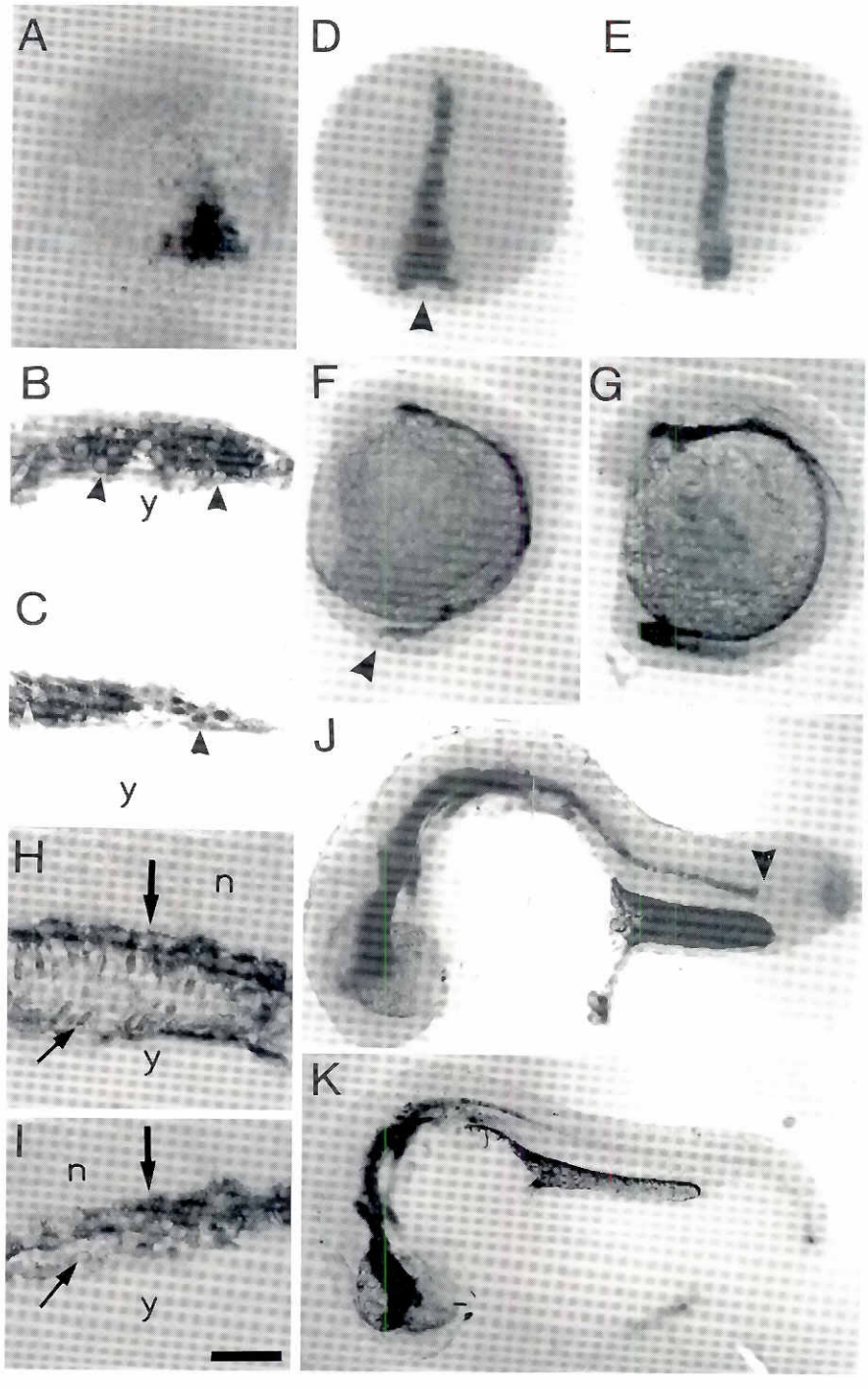

Fig. 5. *axl* expression in *no tail*^{b160} *(ntl)* mutant embryos. (A) *50% epiboly stage ntl embryo stained with axl probe. (Dorsal to the front, animal pole up). The genotype of the embryo shown in* **A** *was verified by immunohistochemistry with the anti-Ntl antibody and sectioning.* **(B and C)** *Sagittal sections through the embryonic shields of mutant and wildtype sibling, respectively. Ntl negative cells with unstained nuclei but axl mRNA staining in the cytoplasm are pointed out by arrowheads in* **B**. *Arrowheads in* **C** *highlight cells with nuclear Ntl staining.* **(D and E)** *show 90% epiboly stage ntl embryo and wildtype sibling, respectively, hybridized to axl probe. axl expression in a ntl mutant embryo is slightly broader in the posterior axis and expression is impaired at the blastoderm margin (arrowhead in* **D**). **(F and G)** *ntl embryo* **(F)** *and wildtype sibling* **(G)** *at the 10 somite stage stained with axl probe. Expression of axl in the tail bud is impaired by the mutation (arrowhead in* **F**). *Embryos are oriented anterior up and dorsal to the left.* **(H and I)** *Sagittal section at the midtrunk level through 10-somite stage wildtype* **(H)** *and ntl embryo* **(I)** *double-labeled with axl probe and anti-Ntl antibody. Thick arrows indicate axl expression in the midline of the neural keel (n). Thin arrows point out mature notochord cells with Ntl-positive nuclei in the wildtype embryo* **(H)** *and undifferentiated mesenchymal cells of the mutant not expressing Ntl* **(I)**. *Orientation of the sections is dorsal up, anterior left.* **(J and K)** *ntl mutant* **(J)** *and wildtype* **(K)** *embryos at 24 h. axl expression is missing in the tail rudiment of ntl embryos (arrowhead in* **J**), *but axl expression in the trunk of the mutant is stronger than in wildtype. Embryos are oriented anterior left and dorsal up. The position of the yolk lost during cutting of sections in panels B, C, H and I is indicated (y). Bar, 30 μm (B,C,H,I).*

axis of such embryos do not form as coherent a stripe as in wildtype; rostrally *axl* expression is indistinguishable from that of a wildtype embryo (Fig. 5E). Homozygous *ntl*^{b160} embryos, which lack Ntl protein, were confirmed by re-staining with the anti-Ntl antibody (Schulte-Merker *et al.*, 1992). Consistent with the early effect, expression of *axl* is impaired in the tail bud and tail at the 10-somite stage (compare Fig. 5F and G) and in 24 h old *ntl* embryos (Fig. 5 J and K). At the 10-somite stage, *axl* expression in the tail bud of *ntl* mutants does not extend as far into the tail bud nor does it form the wedged-shaped expression domain comprising the notochord anlage and the overlying neuroectoderm in the wildtype. More anteriorly, *axl* is strongly expressed in the neuroectoderm (Fig. 5H and I). Expression of *shh* is affected in a similar manner in 10-somite stage embryos (data not shown, Krauss *et al.*, 1993).

At 24 h, expression of *axl* and *shh* in both the endoderm and the anterior neural tube of *ntl* embryos is indistinguishable from that in wildtype. Interestingly, *axl* expression in the neuroectoderm of the trunk of mutant embryos appears stronger and slightly broader than in wildtype embryos (Compare Fig. 5F with G and J with K). Similarly, *shh* is expressed in a broader band of cells (3-4 cells

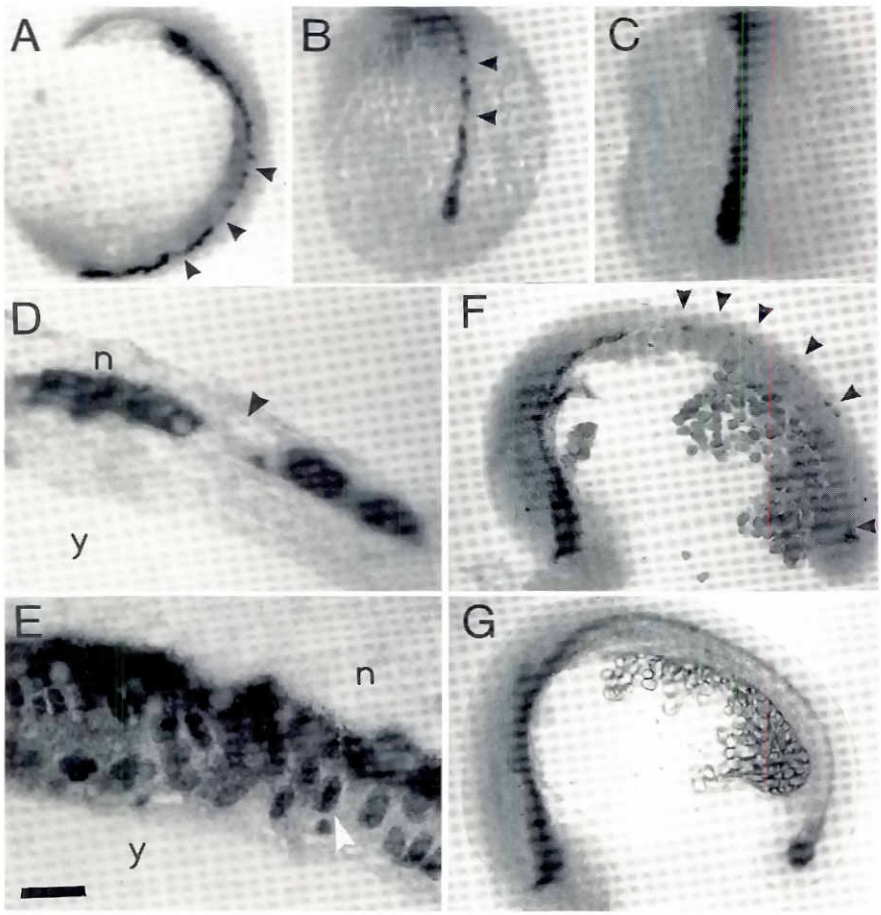

Fig. 6. *axl* expression in *floating head* (*flh*) embryos. (A) *Eight-somite stage flh embryo hybridized to axl probe. axl expression in the trunk neural keel is discontinuous. Gaps of expression are indicated by arrowheads. Orientation is dorsal right and anterior up.* **(B and C)** *show axl expression in the tail bud of a flh embryo and a wildtype sibling at the 8 somite stage, respectively. In contrast to ntl mutants, expression of axl in the tail bud is only marginally affected by the flh mutation at this stage of development.* **(D and E)** *Sagittal sections through flh* **(D)** *and wildtype* **(E)** *8-somite stage embryos double-stained for axl transcript and the Ntl protein. Sections are oriented dorsal up and anterior left. The position of the yolk (y) and the neural keel (n) is indicated. The white arrowhead* **(E)** *highlights a Ntl-positive nucleus in the notochord. Weak axl expression is present in the notochord of the wildtype embryo but not the flh mutant. Orientation of sections is dorsal up and anterior to the left.* **(F and G)** *flh embryo and wildtype sibling at the 20-somite stage, respectively. Few scattered cells in the neural keel posterior to the hindbrain express axl in the flh embryo at this stage (arrowheads). Orientation of embryos is dorsal up and anterior to the left. Bar, 20 μm (D,E).*

wide) than the 1-cell wide stripe typical of wild type (Fig. 7C and D). In agreement with the broader floor plate indicated by the expanded *shh* expression domain, *axl*-expressing floor plate cells are more abundant in *ntl* embryos, while the number of FPL cells appears unaffected (data not shown).

Expression of axl and shh in floating head mutant embryos

The *floating head (flh)* gene encodes the zebrafish homolog of *Xenopus XNot1* , a homeodomain protein that is expressed in the organizer and notochord (Talbot *et al.*, 1995). Embryos homozygous for *flh* mutations are phenotypically similar to *ntl* homozygotes; in contrast, however, the notochord precursor cells present in *ntl* embryos are replaced in *flh* embryos by somites fused at the midline in the trunk suggesting a change in the specification of midline mesoderm (Halpern *et al.*, 1995; Talbot *et al.*, 1995). To investigate whether *flh* is required for *axl* expression, embryos derived from crosses between flh^{n1}/+ parents were hybridized with the *axl* probe. At the 50% epiboly stage, all embryos show a normal pattern of *axl* expression. Slightly later, at 80% epiboly, expression in the nascent axis is discontinuous in approximately 25% of the embryos while expression at the blastoderm margin appears normal (data not shown). Thus, in the absence of *flh* activity, *axl* expression is activated in cells ingressing at the blastoderm margin but is not maintained in midline mesoderm. This dependence of *axl* expression on *flh* is more obvious at the 8-10 somite stage; *flh* homozygotes show discontinuous expression of *axl* in the neural keel of the trunk and no expression in the underlying mesoderm (Fig. 6). At the 20-somite stage *axl* expression appears normal in the head; but, in the neural tube of the trunk and tail, only scattered cells express *axl* (Fig. 6F and G). In contrast to *cyc* embryos, expression of *axl* is affected in both floor plate and FPL cells in *flh* embryos; the remaining patches of *axl* expression consist of both floor plate and FPL cells (data not shown).

Like *axl*, expression of *shh* is unaffected in the brain of *flh* mutant embryos (data not shown) whereas expression is impaired in the neuroectoderm of the trunk and tail (Fig. 7A and B).

Discussion

The *axl* gene is expressed in derivatives of all three germ layers along the axis of the zebrafish embryo in a highly dynamic manner. Whereas strong expression in the notochord is rapidly down regulated during somitogenesis, endodermal expression increases during post-somitogenesis stages. In the ventral neural keel, transcription is first detectable towards the end of gastrulation and persists, like the endodermal expression, for at least 72 h of development. On the basis of its expression in early embryos and by comparison of its sequence with other *forkhead/HNF3* family members, we previously suggested that *axl* is the zebrafish homolog of mammalian *HNF-3β* (Strähle *et al.*, 1993). This suggestion is supported by the analysis of *axl* expression at later developmental stages reported here. Although *axl* expression exhibits clear similarities to the pattern of *HNF-3β* expression in other vertebrate groups (Ang *et al.*, 1993; Monaghan *et al.*, 1993; Ruiz i Altaba *et al.*, 1993b; Sasaki and Hogan, 1993) there are, however, some differences. In *Xenopus HNF-3β* expression is not detectable in the notochord (Ruiz i Altaba *et al.*, 1993b), whereas *axl*, like *HNF-3β* in mouse and rat, is transiently expressed in this tissue. In contrast to comparable stages in mouse embryos (Ang *et al.*, 1993; Monaghan *et al.*, 1993; Ruiz i Altaba *et al.*, 1993b; Sasaki and Hogan,

1993), the hindgut of zebrafish embryos only ever expresses low levels of *axl*. The variations in expression pattern between the different vertebrate classes may be due to functional compensation by related factors. For example, the closely related *pintallavis* has been suggested to substitute for *HNF-3β* expression in the notochord of *Xenopus* embryos (Ruiz i Altaba *et al.*, 1993a).

Fate mapping in zebrafish embryos has shown that the endoderm is derived from cells that invaginate early during gastrulation (Kimmel *et al.*, 1990; Warga and Kimmel, 1990). The position of the cells that express *axl* weakly in the hypoblast around the blastoderm margin from the onset of gastrulation suggests that these cells enter the hypoblast early in gastrulation. Towards the end of gastrulation, these cells predominantly occupy dorsolateral and anterior position and, thus, appear to follow the convergence and involution movement characteristic of zebrafish gastrulation (Kimmel *et al.*, 1990; Warga and Kimmel, 1990). This redistribution to anterior and dorsal coordinates in the late gastrula would be in agreement with the later strong expression of *axl* in the anterior endoderm. We did not find, however, an accumulation of these cells at the dorsal side at the end of gastrulation; instead, expressing cells remain evenly spaced. It is not entirely clear whether these cells are the precursor cells of the anterior endodermal cells that express *axl* strongly at later stages as in early somitogenesis stages, only few cells in the prechordal plate mes/endoderm express *axl*. In contrast to *cyc*, mutations in *one-eyed pinhead*, which has a neural tube phenotype very similar to *cyc*, causes lack of endoderm (Schier *et al.*, 1996; Strähle *et al.*, submitted). *one-eyed pinhead* mutant embryos do not show *axl* expression in paraxial cells in the hypoblast of the gastrula. It is possible therefore that *axl*-expressing paraxial cells are the precursors of the anterior endoderm but that *axl* expression is transient in these cells during gastrulation and is re-activated in the endoderm during later stages.

In 24 h embryos, endodermal expression of *axl* is not affected by mutations in *cyc*, *ntl* and *flh* in contrast to its expression in the neuroectoderm. Neuroectodermal expression of *axl* is subject to modification during somitogenesis stages, suggesting it is under complex regulatory control. Whereas at early neurula stages *axl* is expressed along the entire length of the neural plate including the anlage of the hypothalamus (Strähle *et al.*, 1993), at post-somitogenesis stages, expression extends only as far as the mid-diencephalon at the level of the epiphysis; the hypothalamus no longer expresses *axl* (see also MacDonald *et al.*, 1994). Posterior to the mid-diencephalic boundary, expression of *axl* is detectable in the floor plate and subsequently in cells immediately lateral to the floor plate that are not apparent during early somitogenesis stages. Early somitogenesis stage embryos homozygous for the *cyc* mutation do not express *axl* in the neuroectoderm suggesting that *cyc* function is required for neural expression of *axl* at these early stages (Strähle *et al.*, 1993). *axl*-expressing FPL cells are however present, albeit in slightly reduced number in *cyc* embryos at 24 h, indicating that this expression is independent of that in floor plate precursor cells. Thus, expression of *axl* in the neuroectoderm appears to be established by at least three distinct regulatory mechanisms: first, expression is established in the midline of the early neurula, a process dependent upon *cyc* activity; second, expression is down-regulated in the ventral diencephalon; third, *axl* expression is activated in FPL cells in a process that appears to be independent of *cyc* function. In addition to *axl* expression in FPL cells of the *cyc* neural tube, a

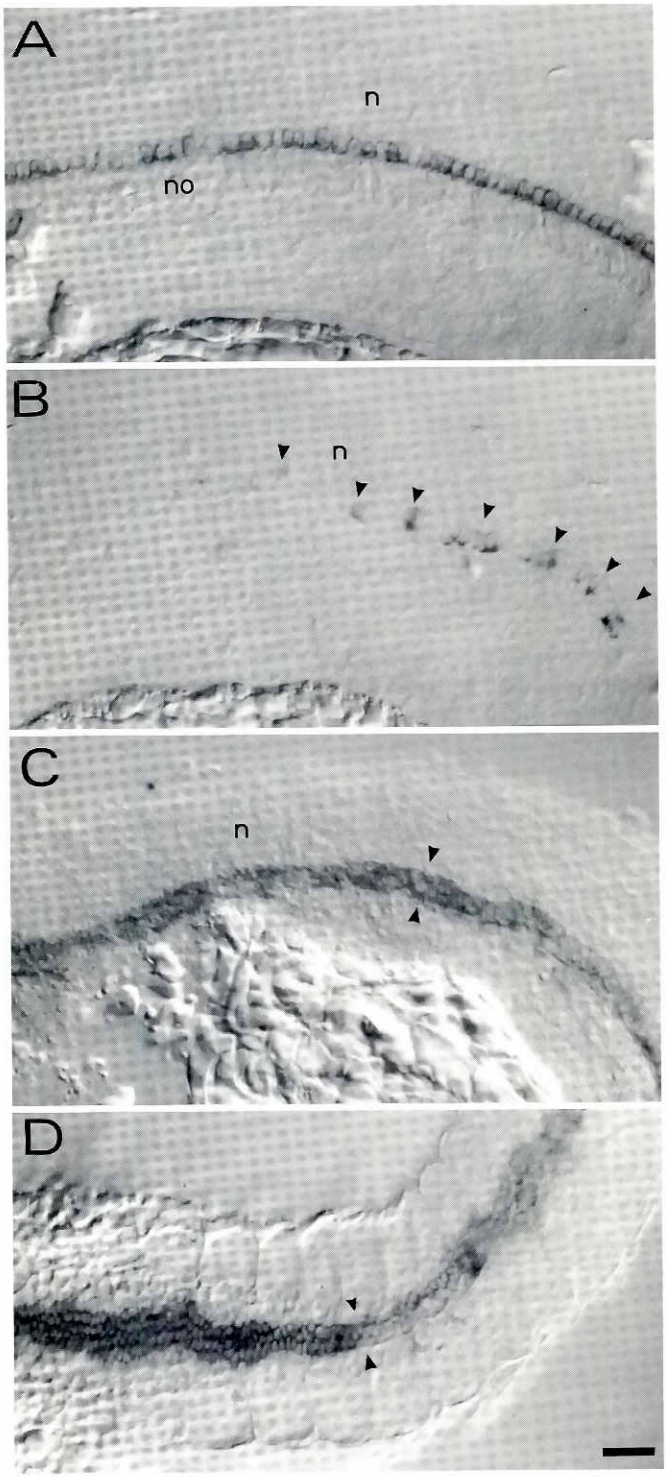

Fig. 7. *shh* expression in *ntl* and *flh* mutant embryos. (A) *shh expression in a wildtype embryo at 20 h (Lateral view of trunk).* **(B)** *flh embryo (20 h stage) hybridized to shh probe. Lateral view of trunk. Arrowheads indicate residual shh staining.* **(C)***ntl embryo (20 h stage) and wild type sibling hybridized to shh probe. (Lateral view on trunk). shh expressing cells are not as regularly arranged as in the wildtype and form a slightly thicker layer (arrowheads).* **(D)** *Dorsal view on trunk of ntl embryo of 24 h development showing broadened shh expression in the ventral neural tube (arrowheads). Embryos are oriented dorsal up (A to C), anterior right (A to D). n, neural tube; no, notochord. Bar, 50 μm.*

small group of cells expresses *axl* mRNA in the dorsal aspect of the mid-diencephalic boundary in the *cyc* brain. The fate of this dorsal diencephalic group of cells as well as of *axl* positive FPL cells is unclear. Intriguingly, however, Kolmer-Agdur neurons are located immediately lateral to the floor plate as *axl* expressing FPL cells and are only marginally reduced in number in *cyc* mutants (Bernhardt *et al.*, 1992).

From our analysis of *ntl* mutant embryos, it is clear that establishment of *axl* expression in the embryonic shield does not require *ntl* function. Lack of *ntl* activity does however affect *axl* expression in a region-specific manner during later stages. The vestigial tails of post-somitogenesis stage *ntl* embryos lack *axl* expression entirely, both in the mesoderm as well as in the neuroectoderm. Mutations of *ntl* in the zebrafish and its homolog *Brachyury* in the mouse and the effects of ectopic expression of *Xenopus Xbra (Xenopus Brachyury)* show that besides being required for notochord development, these molecules also control the development of posterior mesoderm (Cunliffe and Smith, 1992; Herrmann, 1992; Halpern *et al.*, 1993; Schulte-Merker *et al.*, 1994). Thus, the effects of mutation in *ntl* on expression of *axl* in the tail are likely to be the consequence of impaired development of posterior mesoderm.

The expression domains of *axl* is broader in the neural tube in *ntl* embryos when compared with that of wildtype embryos. One explanation for this effect could be that the floor plate inducing midline mesoderm is broader in *ntl* mutants. Although the expression domain of *axl* is slightly broadened in the posterior axis of late gastrula stage *ntl* embryos, *shh* and *axl* expressing midline mesoderm is not broader in early somitogenesis stage *ntl* embryos (data not shown, Krauss *et al.*, 1993). As *ntl* encodes a nuclear transcription regulator and as its expression is entirely confined to the notochord in the trunk (Schulte-Merker *et al.*, 1992; Kispert and Herrmann, 1993; Kispert *et al.*, 1995), broadened *axl* expression in the neural tube of the trunk may be an indirect, non-cell autonomous effect.

Expression of *axl* appears normal in early gastrula stage *flh* embryos suggesting that *flh*, like *ntl*, is also not required for establishment of *axl* expression in the organizer. At later stages, however, the effects of *flh* contrast dramatically with those of *ntl*. While expression of *axl* in the brain is unaffected, only short patches of cells express *axl* in the ventral neural tube of the trunk. These patches presumably correspond to the short stretches of floorplate reported to differentiate in *flh* mutants and which also express *shh* (Talbot *et al.*, 1995 and our unpublished observations). As *flh* function is required in the mesoderm but not in the neuroectoderm (Halpern *et al.*, 1995), the absence of *axl* expression from most of the ventral neural tube is probably an indirect consequence of lack of *flh* activity. Those cells that do express *axl* in the neuroectoderm may have been specified irreversibly early, prior to the shift from axial to paraxial development of midline mesoderm in *flh* mutants (Halpern *et al.*, 1995).

The expression of *axl* and *shh* is similarly affected in *ntl*, *flh* and *cyc* mutants (this report; Krauss *et al.*, 1993; Strähle *et al.*, 1993). Several lines of evidence suggest that *axl/HNF-3β* and *shh* may also be functionally interdependent with *axl/HNF-3β* acting both upstream and downstream of *shh* (Echelard *et al.*, 1993; Krauss *et al.*, 1993; Ang and Rossant, 1994; Roelink *et al.*, 1994; Weinstein *et al.*, 1994; Ruiz i Altaba *et al.*, 1995b; Tanabe *et al.*, 1995). In agreement, *axl* and *shh* expression coincide along the nascent axis of gastrula stage zebrafish embryos (Krauss *et al.*, 1993; Strähle *et al.*, 1993) and ectopic expression of either *axl* or *shh* leads to ectopic activation of the other gene in the anterior neural tube (Krauss *et al.*, 1993, P.B., U.S. and P.W.I., unpublished). The differences in the patterns of transcription of the two genes which we describe here indicate that they are also expressed independently from one another: for example, *shh* expression is not seen in paraxial cells of the gastrula nor in the FPL-cells of late somitogenesis stage embryos, *shh* is strongly expressed in the ventral diencephalon and in the hindgut at stages when *axl* expression can no longer be detected or in the finbuds, in which *axl* is never expressed. Levels of Axl protein may be crucial for *shh* activation; the absence of *shh* expression in paraxial cells during gastrulation may be due to their low levels of *axl* expression. It is also possible that the responsiveness of cells to *axl* expression as well as their ability to receive *shh* signal may differ due to cell specific factors modulating the responses. In *Xenopus*, it has been shown that the competence of the neuroectoderm to respond to ectopic expression of *HNF-3β* varies both spatially and temporally (Ruiz i Altaba *et al.*, 1995b). In the same way, cells lateral to the floor plate in the zebrafish may contain factors that prevent Axl from activating *shh* expression; such a scenario would provide a mechanism to restrict the homeogenetic inducing capabilities of floor plate (Hatta *et al.*, 1991; Placzek *et al.*, 1993; Ruiz i Altaba *et al.*, 1995b).

Materials and Methods

Fish stocks

Wildtype zebrafish were purchased from the Goldfish Bowl Oxford. *cyclops*^{b16}, and *no tail*^{b160} strains were a gift from K. Hatta, M. Halpern and C. Kimmel. *floating head*^{N1} were kindly provided by T. Jowett. Fish were bred and maintained as described (Westerfield, 1995).

In situ hybridization and immunohistochemistry

Digoxygenin whole-mount *in situ* hybridization was carried out as described (Strähle *et al.*, 1993) with the following modification: embryos were digested after acetone treatment with 10 μg/ml proteinase K in phosphate buffered saline (PBS), 0.1% Tween 20. The duration of proteinase treatment was varied according to stages (1 min for gastrula-stage, 4 min for 10-somite stage embryos and 15 min for 24 h and older embryos). Embryos were refixed in BT-Fix (Westerfield, 1993) at room temperature for 30 min, washed twice in PBS, 0.1% Tween 20 and once in PBS, 0.2 % BSA, 0.1% Tween 20 before hybridization. *In situ* hybridization on cryostat sections was carried out as described (Strähle *et al.*, 1994).

Embryos stained by *in situ* hybridization were treated with the rabbit anti-Ntl antibody (Schulte-Merker *et al.*, 1992) or the monoclonal antibody zn12 (Trevarrow *et al.*, 1990) as described (Strähle *et al.*, 1993). Bound antibody was detected using the Vector ABC system (Vector Labs). The horse radish peroxidase reaction was monitored under the dissecting microscope and terminated by transfer of embryos into cold PBS, 10 mM EDTA, 0.2% sodium azide, 0.2% Tween 20. Double-stained embryos were pre-embedded in low melting point agarose (BRL, 1% in PBS) for orientation prior to embedding in paraffin and 8 μm sections were cut as described (Godsave *et al.*, 1988).

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