Spatial Regulation of floating head Expression in the Developing Notochord

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ABSTRACT The zebrafish homeobox gene floating head (flh) is essential for notochord development and is one of the earliest genes to be expressed in notochord precursors. To understand how flh is regulated during notochord development, we compared the wild-type flh expression pattern to that in embryos mutant for flh and no tail (ntl), the zebrafish homologue of Brachyury. In the early gastrula, the pattern of flh expression is not affected in either flh or ntl mutants, implying that the initial establishment of a gastrula notochord domain is independent of the function of these genes. However, flh RNA is expressed at lower levels in flh mutants, suggesting that flh positively regulates its own expression. During gastrulation, flh mutants show an abrupt loss of flh expression in cells which have involuted and entered the hypoblast, while the rest of the expression pattern appears normal, thus flh+ function is specifically required to maintain flh expression in hypoblast cells. The anterior-most part of the notochord rudiment differentially maintains flh expression in both wild types and flh mutant embryos, suggesting that there is unique regulation of flh in this region of the developing notochord. In ntl mutants, the spatial pattern of flh expression is altered as early as the late gastrula stage, becoming broad and diffuse. We hypothesize that ntl+ is required for the proper convergence movements of flh-expressing cells. Dev. Dyn. 209:156–165, 1997.
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INTRODUCTION

In vertebrate embryos, the dorsal side of the gastrula has been defined as the organizer region, a source of signals that establish and pattern the body axis (Spermann and Mangold, 1924; Oppenheimer, 1936; Gerhart et al., 1991; Beddington, 1994; Shih and Fraser, 1996). Fate mapping studies have revealed that a principal tissue derivative of the organizer is the notochord (Keller, 1976; Kimmel et al., 1990; Lawson et al., 1991; Selleck and Stern, 1991; Shih and Fraser, 1995; Melby et al., 1996). The notochord itself is important for the patterning of the adjacent somitic and neural tissue, as indicated by mutational analysis (Dietrich et al., 1993; Halpern et al., 1993; Koseki et al., 1993; Ang and Rossant, 1994; Weinstein et al., 1994) and heterotopic transplantation experiments (Placzek et al., 1990; Hirano et al., 1991; Yamada et al., 1991; Pourquié et al., 1993). Homologues of the Drosophila signaling factor hedgehog are expressed in the notochord (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994; Ekker et al., 1995), and have been shown to mediate inductive patterning of the neural tube (Roelink et al., 1995) and somites (Fan and Tessier Lavigne, 1994; Fan et al., 1995). Therefore, notochord formation is a key step in the proper organization of the vertebrate body axis.

We are interested in characterizing the processes involved in notochord specification and development. Here, we examine the regulation of expression of floating head (flh), a homeobox gene recently identified in zebrafish (Talbot et al., 1995) and homologous to the Xenopus gene Xnot (Gont et al., 1993; von Dassow et al., 1993) and the chick gene Cnot/Gnot (Knezevic et al., 1995; Stein and Kessel, 1995). flh and its homologues are the earliest known genes to be specifically expressed in notochord precursors. In zebrafish, the early gastrula expression domain of flh corresponds at the cellular level with the fate map domain for notochord (Melby et al., 1996). flh continues to be expressed in notochord precursors during gastrulation, as they involute and form the notochord rudiment in the axial hypoblast. During the segmentation period following gastrulation, flh expression is progressively lost anteriorly, but continues to be expressed in tailbud cells in the axis that presumably contribute to posterior notochord (Talbot et al., 1995).

Several lines of evidence indicate that flh+ activity is critical for the specification of notochord. Overexpression of Xnot in Xenopus leads to increased development of notochord tissue (Gont et al., 1996). In zebrafish flh mutants, no notochord develops, and instead, muscle cells occupy the position normally occupied by notochord (Talbot et al., 1995). Fate mapping in flh mutants has shown that notochord precursors, which originally

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express flh RNA, develop as muscle (Melby et al., 1996). flh mutants show inappropriate expression of paraxial mesoderm markers in the axial midline during gastrulation (Halpern et al., 1995), suggesting that notochord precursors are respecified toward a paraxial fate. Together, these results indicate that flh acts not only to properly specify notochord development, but also to repress muscle development.

Notochord development in zebrafish also requires the activity of the no tail (ntl) gene (Halpern et al., 1993). ntl is the zebrafish homologue of the widely conserved Brachyury gene (Schulte Merker et al., 1994) first identified in mouse (reviewed by Beddington et al., 1992; Herrmann and Kispert, 1994). Ntl/Brachyury protein is found in the nucleus (Schulte Merker et al., 1992; Kispert and Herrmann, 1994), and presumably acts as a transcription factor (Kispert and Herrmann, 1993; Kispert et al., 1995). In ntl mutant embryos, at least some notochord precursors develop as mesenchymal cells that resemble neither notochord nor muscle (Halpern et al., 1993; Melby et al., 1996). These cells are located in the axial midline and express sonic hedgehog (shh), in a similar fashion to wild-type axial cells (Krauss et al., 1993). In addition to lacking a differentiated notochord, ntl mutants also fail to develop a tail (Halpern et al., 1993). ntl is expressed in all presumptive mesoderm cells at the margin in the late blastula and gastrula periods, and in the tailbud during segmentation. Specific expression in axial mesoderm arises only after cells have inviolated (Schulte Merker et al., 1992; Schulte Merker et al., 1994). Overexpression studies of the ntl homologue Xbra in Xenopus indicate that it can cause cells to differentiate as mesoderm (Cuniliffe and Smith, 1992; Isaacs et al., 1994). Studies in mice have suggested that loss of Brachyury function disrupts mesodermal development, possibly by affecting mesodermal morphogenesis (Yanagisawa et al., 1983; Hashimoto et al., 1987; Wilson et al., 1993, 1995). Therefore, ntl may act at several stages in notochord development—in the initial specification of mesoderm, and then later in the axial mesoderm, and in the tail.

In order to understand flh regulation, we have characterized its expression in wild-type embryos, and compared this to expression in flh and ntl mutant embryos. We find that spatial and temporal subsets of the flh-expressing cells in the notochord domain are differentially affected in flh and ntl mutants, thus defining important transitions involved in notochord specification. In addition, our results support the hypothesis (Beddington et al., 1992) that flh functions in controlling early mesodermal cell movements.

**METHODS**

**Maintenance of Fish**

Embryos were obtained from matings of either wild-type fish or fish heterozygous for flh+/ntl+/b195 (Talbot et al., 1995), ntl+/b195, and ntl+/b195 (Schulte Merker et al., 1994) are all putative null alleles. Groups of 25–50 stage-matched embryos were set aside during the early cleavage period for later fixation. Embryo stages are according to Kimmel et al. (1995).

**In Situ Hybridization**

Embryos were fixed overnight or for several days at 4°C in 4% paraformaldehyde dissolved in phosphate-buffered saline (PBS). Embryos were fixed in their chorions, and dechorionated after brief washing in PBS with 0.1% tween 20 (PBST). The in situ hybridization procedure was as described in Thissel et al. (1993) except for the following differences: embryos were not dehydrated in methanol prior to hybridization, but were put into hybridization solution for prehybridization after five brief washes in PBST. Alkaline hydrolysis of probes and proteinase K treatment of embryos were also eliminated. For plastic sectioning, embryos were overstained as whole mounts and then embedded in Epon (Westerfield, 1993).

For most experiments, we identified mutant embryos based on clear differences in the flh expression pattern, which were present in one quarter of a given batch of embryos derived from an intercross between heterozygotes. At the early gastrula stage, flh and ntl mutant embryos could not be unambiguously identified based on their flh expression pattern, so we determined the genotypes of individual embryos (after in situ hybridization) by PCR using allele-specific primers. Hybridized, stained embryos were cleared in glycerol, individually photographed and then rinsed and placed in 50 µl of embryo lysis buffer (Postlethwait et al., 1994). Embryos were boiled for 10 min, treated overnight with 10 mg/ml proteinase K at 55°C, boiled again for 10 min, and centrifuged. The supernatant was used for PCR reactions. For identification of flh+/+ embryos, we used the allele-specific primers described in (Talbot et al., 1995). For identification of ntl+/+ embryos, we used the following primers: #2284 (5′-AAGACTGACTGCTGAT-3′), #2285 (5′-CGGAGTTGTGGACCAAAT-3′), and #2286 (5′-GTGCCACCCCAAGATTGG-3′). #2284 and #2285 hybridize to the wild-type ntl sequence, while #2286 hybridizes to DNA from the insert present in the ntl mutant allele (Schulte Merker et al., 1994).

**Mounting and Photography**

Stained embryos were dehydrated in methanol and cleared in either methyl salicylate (2 changes, 5–10 min each, until the embryo sank) or a 1:1 mixture of benzyl alcohol:benzyl benzoate (about a 5 min incubation). Cleared, dehydrated embryos were mounted in Permount (Fisher) on triple-bridged glass slides, made by gluing a set of three size 1.5 square glass coverslips (VWR) on each end of a glass microslide. Embryos were photographed within a few hr after mounting, so that they could be re-positioned before the Permount had hardened, and were kept in the dark as much as possible to prevent the yolk from darkening. Embryos were photographed on slide film, using Nomarski optics on a Zeiss Universal microscope equipped with a mercury lamp.
RESULTS

The Gastrula Pattern of flh Expression is Established Independently of flh+ and ntl+ Function

We have shown previously that flh RNA is expressed in the region corresponding to the fate map domain for notochord in the early gastrula (Melby et al., 1996). Using flh expression as a marker for notochord precursors, we wanted to further elucidate the sequence of events involved in early notochord development by examining the regulation of flh expression in flh and ntl mutant embryos. In wild-type embryos, flh RNA is first expressed at dome stage in the late blastula period, in a complete ring of cells at the margin of the blastoderm (Talbot et al., 1995). flh expression rapidly clears ventrolaterally, so that by 30% epiboly (15–30 min later), expression is now confined to the presumptive dorsal side.

We wondered if either flh+ or ntl+ might function in establishing and/or maintaining the gastrula pattern of flh expression. At the earliest stages of flh expression in the late blastula period, we could not detect differences within clutches containing either flh or ntl mutants (data not shown). In the early gastrula period (shield stage, 5.5–6 hr), flh mutant embryos can be sorted from wild-type embryos based on lower levels of flh expression (Fig. 1A–C). Our sorting was confirmed by PCR analysis of embryo genotype: 15/15 embryos predicted to be wild-type by in situ hybridization had a wild-type analysis of embryo genotype. However, the spatial pattern of flh expression is unaffected. In both flh mutant and wild-type embryos, flh is expressed in a small arc centered on the dorsal midline (Fig. 1A–C). In a similar analysis of shield stage ntl mutant embryos, we found that neither the levels nor the spatial pattern of flh expression were affected by the loss of ntl+ function (Fig. 1D). These results indicate that other genes, besides flh+ and ntl+, are involved in initiating flh expression and refining its pattern through the early gastrula period.

flh+ is Required to Maintain the Level of flh Expression in the Axial Hypoblast

During gastrulation, notochord precursors involute and enter the axial hypoblast (Warga and Kimmel, 1990). flh RNA continues to be expressed in notochord precursors, so that by the late gastrula period, flh is expressed strongly in the notochord rudiment of the hypoblast (Talbot et al., 1995; see also Fig. 2A and C). flh is also expressed strongly in the axial epiblast and hypoblast of the tailbud, in a region termed the chordoneural hinge (Pasteels, 1943; Gont et al., 1993; Melby et al., 1996). During segmentation (Fig. 3A, see below), flh is transiently expressed in the polster or pillow (part of the anterior mesoderm which gives rise to the hatching gland; (Warga, 1996)) and shows maintained expression in a region of the brain that will give rise to the epiphysis (S. Wilson, personal communication).

As gastrulation proceeds, flh mutant embryos continue to express flh RNA in the same general pattern as wild types, but begin to specifically lose expression in the posterior axial hypoblast. In the midgastrula (70% epiboly), flh RNA is expressed strongly in the hypoblast and epiblast, but becomes undetectable in the notochord, which is down-regulated anteriorly in the notochord rudiment; but throughout the segmentation period, it continues to be expressed in the axial epiblast and hypoblast of the tailbud, in a region termed the chordoneural hinge (Pasteels, 1943; Gont et al., 1993; Melby et al., 1996). During segmentation (Fig. 3A, see below), flh is transiently expressed in the polster or pillow (part of the anterior mesoderm which gives rise to the hatching gland; (Warga, 1996)) and shows maintained expression in a region of the brain that will give rise to the epiphysis (S. Wilson, personal communication).

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epiboly), when flh mutant embryos can be easily sorted from wild types due to fainter expression, sectioning reveals that flh expression is much weaker in the hypoblast, with only a few cells faintly expressing flh (compare Fig. 2A to B). By late gastrulation (95% epiboly), flh is expressed in very few hypoblast cells, but continues to be expressed in the epiblast (Fig. 2D). The loss of flh expression is very abrupt: hypoblast cells near the margin that presumably have just involuted show no expression of flh. Similarly, during the segmentation period, flh is expressed in the epiblast of the tailbud of flh mutants, but is absent from the hypoblast (see Fig. 6C).

While loss of flh function specifically affects hypoblast expression of flh RNA, other elements of the flh expression pattern appear unchanged. The anterior extent of flh expression in the late gastrula epiblast is
Fig. 4. The ntl mutation affects the spatial pattern of flh expression in the late gastrula. Whole mount dorsal views, with the animal pole to the top, of a wild-type (A) and two ntl mutant (B, C) embryos at 90% epiboly (9 hr). A: flh is expressed in an axial stripe extending from the margin towards the animal pole. There is also faint expression in the forerunner cells (arrowhead) and bands extending laterally near the margin of the blastoderm (arrows). B: A ntl mutant sibling of the embryo shown in (A). Staining extends up the axis, but is fainter, and appears broader and more diffuse near the margin. Expression in the lateral bands is also more intense relative to the axial staining. Arrowhead indicates the forerunner cells. C: A ntl mutant showing a more severe disruption of flh expression. This embryo was from a different experiment than those shown in (A) and (B), and is overstained. Some clutches of embryos contained mutants with this more extreme broadened expression pattern during gastrulation, however, this phenotype was not seen consistently, and was probably due to genetic background effects.

Fig. 5. flh expression at the bud stage (10 hr) in wild-type and ntl mutant embryos. Dorsal-posterior (A, B) and side (C, D) views of wild-type (A, C) and ntl mutant (B, D) embryos. Anterior is to the top in (A, B). A: flh expression is confined to a narrow axial stripe in the tailbud of a wild-type embryo. B: In a ntl mutant, flh expression in the tailbud is diffuse and spread out laterally. The axis is also wider. There is a clearing in the posterior midline of expression, with the forerunner cluster visible in the midline (arrowhead). C: At this stage in a wild-type embryo, flh expression has not yet begun to down-regulate anteriorly. The embryo is just starting to show expression in the neural plate (arrows in C and D), and in the polster (triangles in C and D). D: The ntl mutation does not affect the flh expression in the polster (triangle) or anterior nervous system (arrowhead), but there is no staining anteriorly in the axis. There is expression posteriorly in the axis, including the posterior hypoblast.

Fig. 6. flh-expressing cells spread posteriorly in the tailbud of ntl mutant embryos. Side views of the tailbuds of a wild-type embryo (A), ntl (B), and flh (C) mutant embryos, at the 1–2-somite stage (10.3–10.7 hr). A: Higher power view of the same embryo shown in Figure 5A. The orientation is as in Figure 5A. In wild types (A) and flh mutants (C), flh expression in the epiblast respects a posterior boundary that is demarcated by the forerunner cluster (arrowheads in all three panels). The arrow in (A) points to flh expression in the notochord rudiment. In ntl mutants (B), epiblast cells expressing flh are located posteriorly to the forerunner cluster. Note the hypoblast expression apparent in ntl (B) vs. flh (C), which shows a gap between the forerunner cluster and expression in the overlying epiblast. The arrow in C points to flh expression in the epiblast; sectioning of flh mutants at this stage confirms that there is no flh expression in the tailbud hypoblast (data not shown).
comparable in flh mutants and wild types (Fig. 2C,D). Similarly, the later expression of flh RNA in the brain and transient expression in the polster resembles the wild type pattern (Fig. 3D and not shown). flh RNA is also expressed in the forerunner cells, a separate cluster of dorsal marginal cells in the gastrula that do not involute, but instead migrate vegetally on the yolk surface to the tailbud where they form Kupffer’s vesicle, a unique structure of teleost embryos involved in tail development (Cooper and D’Amico, 1996; Melby et al., 1996). The arrow in Figure 2A indicates flh expression in the forerunner cells, which is weaker than in adjacent axial mesoderm. flh continues to be expressed in the forerunner cells throughout gastrulation and the early segmentation period, and this expression is not affected in either flh or ntl mutant embryos (see Fig. 6).

Although flh+ is generally required to maintain flh expression in cells that will become axial hypoblast, we discovered that cells at the anterior tip of the notochord rudiment show differential regulation of flh expression. In wild types during the early segmentation period, when flh expression is down-regulated from anterior to posterior, flh expression is transiently maintained in a short stretch of the anterior axial hypoblast (Fig. 3A). To determine the antero-posterior location of this region of flh expression, we co-hybridized with flh and a probe for krox20, which marks cells forming rhombomeres 3 and 5 in the hindbrain (Oxtoby and Jowett, 1993). During the early segmentation period, the anterior tip of the notochord rudiment extends underneath the forming hindbrain and posterior midbrain (K. Hatta, unpublished observations). Figure 3B shows that flh expression in the axial hypoblast is centered below the stripes of krox20 expression, placing it in the region corresponding to the anterior tip of the notochord. Interestingly, flh mutants also show maintained flh expression in this region, although wild types (Fig. 3C) have more flh-expressing cells than do flh mutant embryos (Fig. 3D). ntl RNA expression has also been observed to persist in the anterior axis of flh mutant embryos (S.L. Amacher, unpublished observations). These results indicate that gene expression is differentially regulated in the region of the anterior notochord, and that unlike the rest of the axial hypoblast, maintenance of flh expression here does not require flh+ function.

ntl+ Regulates the Spatial Pattern of flh Expression in Both the Axis and Developing Tail

Unlike wild types and flh mutant embryos, where flh RNA is expressed in a tight axial stripe, ntl mutant embryos exhibit an altered pattern of flh expression during gastrulation. ntl mutant embryos can first be distinguished from wild types in the midgastrula (80% epiboly) based on lower apparent levels of flh expression (Talbot et al., 1995). Here we show that by the late gastrula stage (90% epiboly; Fig. 4), ntl mutants show a broadening of the flh expression domain near the margin with a slight dearing at the axial midline (Fig. 4B, arrowhead), and heavier expression laterally (Fig. 4A and B, arrows). Transverse sections of late gastrula stage wild-type and ntl mutant embryos (not shown) indicate that the broadening of flh expression is primarily in the epiblast. The degree to which these spatial differences are expressed in the late gastrula varies among mutant embryos (compare Fig. 4B to C). Following the end of gastrulation (i.e., bud stage and somite stages), the differences in flh RNA pattern between ntl mutants and wild types have become even more striking (Fig. 5A, B), and also more consistent among mutant embryos, with the flh expression domain becoming more diffuse and flh-expressing cells more widely scattered.

We have shown above that flh+ is required to maintain expression of flh RNA in the posterior axial hypoblast. In contrast, in ntl mutants we find that during gastrulation and the early segmentation period, cells in the axial hypoblast initially express flh RNA. Expression in the hypoblast can be seen in sectioned material (not shown) and also in whole mount side views (Figs. 5D and 6B). However, ntl mutants down-regulate the anterior axial expression of flh much sooner than wild-type embryos. In wild-type embryos, flh is only beginning to down-regulate in the anterior axis at the bud stage, but in ntl mutants, flh expression is limited to the posterior axis by this stage (compare Fig. 5C to D). Thus, ntl+ function is not required when cells first involute, but is required soon after to maintain flh expression.

An interesting characteristic of the tailbud expression pattern for flh (and its Xenopus homolog Xnot) is that expression is restricted to a distinct subset of cells within the tailbud, the cells of the chordoneural hinge (Gont et al., 1993; Melby et al., 1996). In Xenopus, there is a clear posterior boundary of Xnot expression, which corresponds to the wall of the neuroenteric canal, while the ntl homologue Xbra is expressed throughout the tailbud (Gont et al., 1993). In zebrafish embryos, the chordoneural hinge lies at the A-P level of the forerunner cluster (Fig. 6A). The forerunner cluster is particularly apparent in flh mutant embryos, which lack hypoblast expression but still show an abrupt posterior boundary of flh expression (Fig. 6C). In contrast, ntl mutant embryos do not show a distinct posterior boundary of flh expression; instead labeling of epiblast cells is spread posteriorly beyond the forerunner cluster (Fig. 6B). Note that the posterior spreading of flh-expressing cells occurs only in the epiblast, not the hypoblast of the ntl mutant, which shows reduced flh expression in the hypoblast compared to the wild-type embryo, but greater expression than the flh mutant. As development proceeds, the expression of flh RNA in the tailbud of ntl mutant embryos becomes more and more diffuse. Eventually, by the late segmentation period (17-somite stage),
flh expression disappears from the tailbud in ntl mutants, while expression in the brain persists (Fig. 7).

**DISCUSSION**

**flh** is Part of a Network of Genes That Specify Notochord Fate

We postulate that flh+ functions early in the specification of notochord fate since flh RNA is specifically expressed in the notochord fate map domain of the early gastrula (Melby et al., 1996). Cells in the notochord fate map domain of flh mutants develop as muscle, and this change in fate is apparent by the midgastrula stage when midline cells in flh mutants express markers of paraxial mesoderm such as snail1 and MyoD (Halpern et al., 1995). Our results show that there is lower flh expression in flh mutants by the early gastrula stage, suggesting that flh+ is required to maintain high levels of flh expression in notochord precursors. However, the early gastrula pattern of flh expression in the notochord domain is not affected, suggesting that other genes are involved in refining the broad domain of flh expression during the late blastula period. flh mutants also show appropriate patterns of expression for other midline genes such as shh (Talbot et al., 1995) and twist (Halpern et al., 1995) in the early gastrula period. These results suggest that flh+ is but one component of a network of genes that act to specify notochord fate in the early gastrula. In the absence of flh+ function, other members of the network are capable of turning on flh and other midline genes in the appropriate pattern at the dorsal midline. Based on mutational analysis in mice, a likely component of this network is the forkhead-domain gene HNF-3β (Ang and Rossant, 1994; Weinstein et al., 1994), whose zebrafish homologue is the gene axial (Strähle et al., 1993).

Spatial and Temporal Regulation of flh Expression in Notochord Precursors

Although flh expression can initiate independently of flh+ and ntl+ function, cells require flh+ and ntl+ activity at different steps to maintain flh expression as the notochord develops. In wild-type embryos, cells in the gastrula notochord domain that initially express flh RNA continue to express it once they have involuted and entered the axial hypoblast. In flh mutants, hypoblast expression of flh RNA is lost, indicating that flh+ function is required to maintain flh expression in cells that have involuted (with the exception of a discrete group of cells in the anterior notochord rudiment; see below). The lack of flh expression in the hypoblast appears to represent a distinct spatial requirement for flh+ function since staining appears normal in the epiblast adjacent to the margin, but disappears abruptly in the newly involuted hypoblast. ntl+ is also required in hypoblast cells to maintain flh expression. In ntl mutants, flh RNA is initially expressed in the hypoblast, but turns off anteriorly much more quickly than in wild-type embryos. ntl RNA expression in the hypoblast depends on both ntl+ and flh+ activity (Schulte Merker et al., 1994; Talbot et al., 1995), thus these two genes form a positive feedback loop for gene expression in the axial hypoblast. Our results indicate that flh+ is required at an earlier stage than ntl+ to maintain flh expression in the hypoblast (compare Fig. 6B and C), suggesting that the autoregulation of flh expression occurs independently of ntl+ function, possibly through the direct action of flh on its own promoter.

Other genes have been identified in zebrafish that show specific expression in the hypoblast, for instance the receptor tyrosine kinase gene rtk-1 (Xu et al., 1994); or that show a different pattern in the hypoblast versus the epiblast; such as ntl (Schulte Merker et al., 1992;
Schulte Merker et al., 1994). Differential regulation and expression of genes between the epiblast and hypoblast layers is intriguing since during the gastrula period, cells at the blastoderm margin move from the epiblast to the hypoblast, and thus the same cells inhabit both layers at different times. Cells which are destined to involute may be controlled by a special intrinsic program of development that requires flh activity to maintain flh expression in an autoregulatory loop. Another possibility is that cells receive signals from the extracellular environment of the hypoblast that then influence gene expression. During segmentation, notochord precursors probably do not continue to translocate from epiblast to hypoblast, since recent fate map analysis of the zebrafish tailbud (J.P. Kanki and R.K. Ho, 1997) has revealed that notochord precursors are only found in the hypoblast by the bud stage. Thus, the absence of flh expression in the tailbud hypoblast of flh mutants during the segmentation period probably reflects an earlier failure to maintain flh expression during gastrulation.

Our data indicate that the anterior-most portion of the developing notochord is regulated differently than the rest of the notochord. In the anterior notochord rudiment of wild-type embryos, flh expression is transiently maintained, while in the remainder of the axis except the chordoneural hinge posteriorly, expression has downregulated. In flh mutants, a few cells in the anterior axis express flh and also ntl RNA (S. Amacher, unpublished data), supporting the notion that gene expression in this region is at least partially independent of flh activity. In general, the flh mutant phenotype is less severe anteriorly: axial genes are initially expressed appropriately, and cell types that are presumably involved by notochord, such as floor plate (Halpern et al., 1995; Talbot et al., 1995) and adaxial cells (E. Melancon et al., in preparation), are present anteriorly. The anterior axis in flh mutants, then, may be specified by other genes in the notochord-specifying network, which partially compensate for the loss of flh function. However, the differential maintenance of flh expression in wild types suggests that different A-P regions of the notochord are under distinct molecular control.

ntl \(^+\) May Control the Morphogenesis of Mesodermal Precursors

In contrast to the decreased levels of expression seen in flh mutant embryos, the most striking change in flh expression in ntl mutant embryos is in the spatial pattern. As early as the late gastrula stage, the marginal epiblast expression of flh is broadened laterally, and during segmentation, flh-expressing cells are spread both laterally and posteriorly in the tailbud. These changes in pattern could represent ectopic expression of flh, implying that ntl activity is involved in directing appropriate flh expression. This seems unlikely since ntl is expressed throughout the blastoderm margin during gastrulation and throughout the tailbud during segmentation. A possibility that we favor is that the change in flh pattern results from misdirected cell movements. In wild-type embryos, an initially broad notochord domain becomes long and narrow through the movements of convergent-extension (Warga and Kimmel, 1990; Shih and Keller, 1992). The broadened flh expression domain that we observe is consistent with a model whereby ntl activity is required for proper convergence.

Several lines of evidence support the hypothesis that ntl/Brachyury is involved in controlling mesodermal morphogenesis. Analysis of the Brachyury mutation in mice has suggested that the abnormal posterior morphology of these mutants is a consequence of disrupted cell movements. Brachyury mutants show a higher ratio of ectoderm to mesoderm (Yanagisawa et al., 1981); and in chimeras between wild type and Brachyury, mutant cells accumulate in the primitive streak and tailbud (Wilson et al., 1993; 1995). In culture, cells from the primitive streak of Brachyury mutant mice show slower migration than wild-type cells do on an extracellular matrix substrate (Hashimoto et al., 1987). These results have been interpreted to mean that loss of Brachyury function causes a defect in cell adhesion such that mutant cells fail to migrate properly through the primitive streak (Wilson et al., 1995).

Brachyury homologues in zebrafish, frog and mouse are expressed in mesoderm prior to gastrulation (Smith et al., 1991; Schulte Merker et al., 1992; Kispert and Herrmann, 1994), and in Xenopus, overexpression of Xbra leads to greater mesodermal differentiation (Cunliffe and Smith, 1992), implicating Brachyury genes in mesodermal specification. Xbra can regulate expression of eFGF (Isaacs et al., 1994; Schulte Merker and Smith, 1995), a growth factor which promotes convergence-extension (Isaacs et al., 1994; Griffin et al., 1995). Furthermore, blockade of FGF-signaling by expression of a dominant negative FGF receptor causes a phenotype similar to ntl, with severe truncation of posterior development (Amaya et al., 1993; Griffin et al., 1995). These results imply that Brachyury genes function in mesodermal specification and may affect cell movements via a mechanism that depends upon FGF signaling. This hypothesis can be tested in zebrafish by direct observation of cell movements in ntl mutants and in ntl\(^-\)/wild-type mosaic embryos.

**CONCLUSION**

The regulation of flh expression by flh\(^+\) and ntl\(^+\) has revealed important steps in the development of the notochord. Initially, a domain of cells fated to become notochord is set aside in the gastrula. flh\(^+\) may have a role in establishing the notochord domain, however other genes can establish the notochord domain in the absence of flh function. flh\(^+\) probably maintains specification to a notochord fate by maintaining high levels of flh expression either directly or indirectly in an autocatalytic loop. The specific loss of flh expression in the hypoblast indicates that involute is an important transition: once notochord precursors involute, they
require flh\(^+\) function to maintain flh expression. Soon after involution, ntl\(^+\) function is also required to maintain expression of flh and presumably other genes important for notochord differentiation. Thus flh\(^+\) and ntl\(^+\) interact in a regulatory loop to specify notochord cell fate. Which gene predominates depends upon the development context. In the hypoblast, flh would appear to act upstream of ntl\(^+\) in that its function is required earlier to maintain proper gene expression. However, weaker flh expression and the altered pattern seen during gastrulation in ntl mutants suggest that ntl also functions upstream of flh, probably in its role as a general specifier of mesoderm. flh/ntl double mutants more closely resemble ntl single mutants, further supporting the hypothesis that ntl can act upstream of flh (Halpern et al., submitted). The ntl mutation may affect morphogenesis, causing fewer notochord precursors to arrive in the appropriate location, and this may contribute in part to the disruption of tail patterning and extension.

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