Promoting notochord fate and repressing muscle development in zebrafish axial mesoderm

Sharon L. Amacher* and Charles B. Kimmel

Institute of Neuroscience, University of Oregon, Eugene, OR 97403-1254, USA

*Author for correspondence (e-mail: amacher@uoneuro.uoregon.edu)

Accepted 2 February; published on WWW 18 March 1998

SUMMARY

Cell fate decisions in early embryonic cells are controlled by interactions among developmental regulatory genes. Zebrafish floating head mutants lack a notochord; instead, muscle forms under the neural tube. As shown previously, axial mesoderm in floating head mutant gastrulae fails to maintain expression of notochord genes and instead expresses muscle genes. Zebrafish spadetail mutant gastrulae have a nearly opposite phenotype; notochord markers are expressed in a wider domain than in wild-type embryos and muscle marker expression is absent. We examined whether these two phenotypes revealed an antagonistic genetic interaction by constructing the double mutant. Muscle does not form in the spadetail floating head double mutant midline, indicating that spadetail function is required for floating head mutant axial mesoderm to trans fate to muscle. Instead, the midline of spadetail floating head double mutants is greatly restored compared to that of floating head mutants; the floor plate is almost complete and an anterior notochord develops. In addition, we find that floating head mutant cells can make both anterior and posterior notochord when transplanted into a wild-type host, showing that environmental signals can override the predisposition of floating head mutant midline cells to make muscle. Taken together, these results suggest that repression of spadetail function by floating head is critical to promote notochord fate and prevent midline muscle development, and that cells can be recruited to the notochord by environmental signals.

Key words: floating head, spadetail, Xnot, not genes, no tail, Brachyury, Genetic mosaic, Floor plate, Myogenesis

INTRODUCTION

In 1924, Spemann and Mangold demonstrated that the dorsal blastopore lip of an amphibian gastrula can induce an ectopic embryonic axis when grafted to the ventral side of a host embryo (Spemann and Mangold, 1924). This dorsal tissue defines a region of the gastrula known as the organizer, as it recruits surrounding host cells into the mesoderm and ectoderm of the secondary axis. Organizer cells themselves are fated to become dorsal mesodermal tissues, including the notochord, a midline derivative of axial mesoderm (Keller, 1976; Kimmel et al., 1990; Lawson et al., 1991; Selleck and Stern, 1991; Shih and Fraser, 1995; Melby et al., 1996). The notochord is an important signaling center and is thought to play a critical role in the induction of floor plate and motoneuronal cells of the overlying spinal cord in tetrapods (reviewed in Placzek, 1995) and in patterning the adjacent somitic tissue in tetrapods (reviewed in Cossu et al., 1996) and in teleosts (Halpern et al., 1995). Previous studies have demonstrated that flh and ntl interact in a complex way, with each being required to maintain the expression of the other (Talbot et al., 1995, Melby et al., 1996, Halpern et al., 1997). In this work, we examine the genetic interaction between flh and another gene that affects mesodermal cell fate, spadetail (spt).

The zebrafish flh gene encodes a homeodomain transcription factor homologous to Not genes in Xenopus and chick (von Dassow et al., 1993; Gont et al., 1993; Talbot et al., 1995, Knezevic et al., 1995; Stein et al., 1996) and is expressed in the organizer in a domain that correlates well with the gastrula notochord fate map domain (Melby et al., 1996). flh mutant embryos entirely lack notochord and instead have muscle in the midline (Halpern et al., 1995; Talbot et al., 1995). Lineage analysis has shown that cells in the notochord fate map domain of flh gastrula become muscle instead of notochord (Melby et al., 1996). Analyzing the expression of molecular markers in flh mutants reveals that this change in fate is evident as soon as midgastrulation (Halpern et al., 1995); earlier in gastrulation, expression of molecular markers reveals that
midline cells have normal axial mesodermal character (Halpern et al., 1995; Talbot et al., 1995). Overexpression of Xnot, a Xenopus gene related to flh, leads to excess notochord formation (Gont et al., 1996). Thus, flh acts to promote notochord development and repress muscle development (see also Melby et al., 1997).

Zebrafish spt mutant embryos have a nearly opposite phenotype to that of flh mutants. spt mutants fail to form trunk somites and, correspondingly, there is a large deficiency of trunk muscle later in development (Kimmel et al., 1989). Trunk somitic precursors move inappropriately during gastrulation and many of them end up in the enlarged tailbud (Ho and Kane, 1990). These abnormal cell movements can be visualized in part using molecular markers (Hammerschmidt et al., 1993, 1996a; Thisse et al., 1993). In contrast to flh− embryos, which express the muscle marker myoD inappropriately in the midline (Halpern et al., 1995), spt− embryos fail to express myoD at all during gastrulation and instead express it in a reduced domain during later stages (Weinberg et al., 1996). Other derivatives of lateral and ventral mesoderm, such as blood, pronephros and pectoral fin are also deficient (Kimmel et al., 1989; Warga, 1996). A prominent notochord forms in spt− embryos; the notochord primordium is especially wide near the end of gastrulation but seems to regulate during segmentation stages (Thisse et al., 1995; Hammerschmidt et al., 1996a; S. L. A. and C. B. K., unpublished observations). Therefore, spt controls both mesodermal morphogenesis and cell fate.

We investigated whether spt and flh interact by constructing the double mutant. We show that spt−;flh− embryos resemble spt single mutant embryos in many respects. They lack midline muscle cells that are normally found in flh− embryos, indicating that spt function is required for flh− axial mesoderm to translocate to muscle. Additionally, differentiated notochord forms anteriorly in spt−;flh− embryos, demonstrating that loss of spt function can bypass the requirement for flh function in notochord development. We also show that flh− cells can form notochord in genetic mosaics, by mixing small numbers of flh− cells among wild-type neighbors. Our results suggest that flh blocks muscle formation and promotes notochord development in axial mesodermal cells by antagonizing spt function, and that cells can be recruited to the notochord by environmental signals.

MATERIALS AND METHODS

Maintenance of fish and mutant lines

Fish were reared at 28.5°C and cared for as described (Westerfield, 1995). Homozygous mutant embryos were obtained from natural matings of carriers heterozygous for mutant alleles. Embryos were collected and sorted into embryo medium (Westerfield, 1995) during early cleavage stages and maintained at 28.5°C until the desired developmental stage. Embryos were staged according to Kimmel et al. (1995; h, hours postfertilization).

The flh allele used in these studies is flh−1, a spontaneous null allele that results from a 2 bp deletion in the coding sequence upstream of the homeobox (Talbot et al., 1995). The origin of the spt allele used, spt−104, is unknown; it was originally discovered among the haploid progeny of a single female with a complex genetic background (Kimmel et al., 1989). To learn whether spt−104 might be a null allele, we tested it against a deficiency allele, spt<sup>−322</sup>, spt<sup>−333</sup> is a gamma-ray-induced allele that eliminates polymorphic DNA markers located near the spt gene (S. L. A., data not shown). The early phenotype of spt<sup>−322</sup>/spt<sup>−333</sup> homozygotes and spt<sup>−322</sup>/spt<sup>−333</sup> transheterozygotes is nearly identical to spt<sup>−333</sup>/spt<sup>−322</sup> homozygotes. Thus, we conclude that spt<sup>−104</sup> is a null or near-null allele. The flh and spt loci are mapped and are unlinked (Postlethwait et al., 1994; Talbot et al., 1995; and S. L. A., data not shown). Both spt and flh mutant alleles used in this study produce recessive lethal phenotypes.

Carriers heterozygous for flh and spt mutations were obtained by crossing fish heterozygous for one mutation to fish heterozygous for the other and raising their progeny. Doubly heterozygous carriers were identified among these offspring by sibling crosses and were then intercrossed to produce homozygous double mutant embryos. At 24 hours postfertilization (h), the progeny of two doubly heterozygous carriers can be sorted into four phenotypic classes in the ratio expected for two independently assorting mutations (wild type: flh−; spt−: flh−; spt−: flh− = 8.90: 3.02: 0.99, n=4,342, χ<sup>2</sup>=1.044, P>0.70). When the progeny of two double mutant carriers are sorted during early segmentation stages (11-14 h), the number scored as spt/flh double mutants is lower than expected for Mendelian segregation of independently assorting alleles (WT: flh−; spt−: flh−; spt− = 8.89: 2.85: 3.67: 0.59, n=623, χ<sup>2</sup>=12.70, P<0.01). We suspected that a significant number of spt−;flh− embryos have a seemingly intact notochord primordium at this stage that makes them indistinguishable from spt mutant singles. We confirmed this by observing the same embryo later in development (24 h) when double mutant embryos can be reliably identified on the basis of their distinct body shape (see Fig. 1). We also confirmed the presence or absence of the flh<sup>e</sup> allele in all spt− and spt−;flh− embryos by DNA genotyping using wild-type flh and mutant flh<sup>e</sup> allele-specific primers in PCR reactions as described previously (Halpern et al., 1997). Upon DNA genotyping, mutant embryos are found in the expected ratios (WT: flh−; spt−; flh−; spt− = 8.89: 2.85: 3.39: 0.87, n=623, χ<sup>2</sup>=2.92, P>0.30).

In situ hybridization

Embryos were processed for whole-mount in situ hybridization as described (Melby et al., 1997). Digoxigenin-labeled RNA probes were synthesized using T7 polymerase (Boehringer-Mannheim) from a HindIII-linearized sonic hedgehog (shh) template (Krauss et al., 1993), an Xbal-linearized myoD template (Weinberg et al., 1996) and an XhoI-linearized no tail (ntl) template (Schulte-Merker et al., 1992), and using T3 polymerase (Boehringer-Mannheim) from a PsI-linearized krox-20 template (Oxtoby and Jowett, 1993). Following probe detection, most embryos were mounted in Permout (Halpern et al., 1997), either between coverslips or on triple-bridged glass slides (Melby et al., 1997). In other cases, embryos were taken through a glycerol series and mounted in 80% glycerol between coverslips separated by a thin application of vaseline. Embryos were photographed as described (Halpern et al., 1997). In some cases, individual spt−;flh− embryos were genotyped by PCR after in situ hybridization to confirm the presence of the flh<sup>e</sup> mutant allele.

Cell transplantation

Donor embryos were uniformly labeled as described (Halpern et al., 1993) by injecting lineage tracer dye (a mixture of 3% tetramethylrhodamine dextran and 3% lysine fixable biotinylated dextran in 0.2M KCl) at the 1- to 4-cell stage. Four or more cells (sometimes up to 40) were transplanted heterochronically from older donor blastulae to younger unlabeled host blastulae; the heterochronic technique greatly increases the percentage of host embryos in which donor cells contribute to axial structures such as floor plate, notochord and hypochord (see Halpern et al., 1995). Because transplantations were performed before the mutant phenotype is distinguishable, donor embryos were kept alive to score phenotype later by morphology and to genotype by PCR. In every case, DNA genotyping confirmed that the donors had been scored correctly. In other transplantation experiments, cells were transplanted isochronically between the shield
As muscle tissue does not differentiate in the Continuous floor plate forms in spt results show that the ability of in mesoderm flanking the ectopic expression in the is strong expression in the myotomes of wild-type embryos, similar to those described above for earlier stages. There somites have formed, we see excluded from the midline (Fig. 2D). By the time that tail strikingly resembles that in S. Eisen and M. Westerfield, unpublished data). In contrast, slow muscle cell fate (E. Melançon, S. L. A., S. H. Devoto, J. Methods). In contrast to using putative null alleles at both loci (see Materials and Methods), the seemingly opposite phenotypes of flh studies have shown that to appear fused (Fig. 2B, Halpern et al., 1995). Dye labelling is not expressed during gastrulation in to become the most superficial muscle cells (Devoto et al., 1996). This population of slow muscle precursors initially lies adjacent to the developing notochord, but subsequently migrates through the somite to become the most superficial muscle cells (Devoto et al., 1996). Additionally, myoD is expressed ectopically in midline mesoderm, causing the adaxial cell rows to appear fused (Fig. 2B, Halpern et al., 1995). Dye labelling studies have shown that flh- midline mesodermal cells adopt a slow muscle cell fate (E. Melançon, S. L. A., S. H. Devoto, J. S. Eisen and M. Westerfield, unpublished data). In contrast, myoD is not expressed during gastrulation in spt- embryos and, later, variable numbers of myoD-expressing cells flank the notochord are detected during segmentation stages (Fig. 2C, Weinberg et al., 1996). myoD expression in spt-;flh- embryos strikingly resembles that in spt single mutant embryos and is excluded from the midline (Fig. 2D). By the time that tail somites have formed, we see myoD expression patterns (Fig. 2E-H) similar to those described above for earlier stages. There is strong expression in the myotomes of wild-type embryos, ectopic expression in the flh- midline and irregular expression in mesoderm flanking the spt- and spt-;flh- midline. These results show that the ability of flh- midline cells to express muscle markers and make muscle is spt-dependent.

Continuous floor plate forms in spt-;flh- embryos

As muscle tissue does not differentiate in the spt-;flh- midline, we investigated the character of midline cells using molecular markers for other cell types. To do this, we first examined sonic hedgehog (shh) expression, a marker of developing notochord and floor plate (Krauss et al., 1993). During early segmentation stages, shh is expressed in wild-type and spt- embryos in both of these tissues (Fig. 3A,C). In contrast, shh is not expressed in midline mesoderm and is detected only discontinuously in the ventral neural tube of flh- embryos (Fig. 3B and sections, data not shown). shh expression in spt;flh double mutants is substantially greater than that in flh mutants (Fig. 3D). In addition, sections reveal patchy shh expression in anterior midline mesoderm (data not shown). As expected due to variability in spt-;flh- notochord length (Fig. 1 and Materials and Methods), the extent of shh expression in spt-;flh- midline mesoderm is also variable (see Fig. 3 legend). At 25 h, when notochord and floor plate differentiation are well underway, we see the same results (Fig. 3E-H). In wild-type embryos at this

---

**RESULTS**

**Midline mesoderm in spt-;flh- embryos does not differentiate as muscle**

The seemingly opposite phenotypes of spt and flh mutants suggested that these two genes interact in a genetic hierarchy with one gene normally antagonizing the activity of the other. To examine this, we constructed the spt;flh double mutant using putative null alleles at both loci (see Materials and Methods). In contrast to flh- mutants, myotomal muscle was not observed in the spt-;flh- double mutant midline (Fig. 1).

As a more sensitive assay for muscle development, we asked whether developing spt-;flh- midline mesodermal cells express the muscle marker, myoD, as they do in flh mutants (Fig. 2). During early segmentation in wild-type embryos, myoD is expressed in two domains (Fig. 2A). First, myoD is expressed in adaxial cells in two bilateral rows of segmental plate mesoderm (Weinberg et al., 1996). This population of slow muscle precursors initially lies adjacent to the developing notochord, but subsequently migrates through the somite to become the most superficial muscle cells (Devoto et al., 1996). Additionally, myoD is expressed dynamically in each somite (Weinberg et al., 1996). In flh- embryos, myoD is expressed ectopically in midline mesoderm, causing the adaxial cell rows to appear fused (Fig. 2B, Halpern et al., 1995). Dye labelling studies have shown that flh- midline mesodermal cells adopt a slow muscle cell fate (E. Melançon, S. L. A., S. H. Devoto, J. S. Eisen and M. Westerfield, unpublished data). In contrast, myoD is not expressed during gastrulation in spt- embryos and, later, variable numbers of myoD-expressing cells flanking the notochord are detected during segmentation stages (Fig. 2C, Weinberg et al., 1996). myoD expression in spt-;flh- embryos strikingly resembles that in spt single mutant embryos and is excluded from the midline (Fig. 2D). By the time that tail somites have formed, we see myoD expression patterns (Fig. 2E-H) similar to those described above for earlier stages. There is strong expression in the myotomes of wild-type embryos, ectopic expression in the flh- midline and irregular expression in mesoderm flanking the spt- and spt-;flh- midline. These results show that the ability of flh- midline cells to express muscle markers and make muscle is spt-dependent.

**Continuous floor plate forms in spt-;flh- embryos**

As muscle tissue does not differentiate in the spt-;flh- midline,
stage, shh is expressed in the floor plate, a single-cell row in the ventral spinal cord, and in the posterior notochord (Fig. 3E; Krauss et al., 1993). In flh mutants, floor plate development is disrupted; shh expression is patchy in the trunk and greatly reduced in the tail (Fig. 3F; Talbot et al., 1995; Halpern et al., 1995; Odenthal et al., 1996). spt- embryos, like wild-type embryos, express shh in both posterior notochord and floor plate, although frequently we observe pattern distortions such as bifurcations and widening, as well as the presence of shh-expressing cells ventral to the notochord (Fig. 3G). In spt-;flh- embryos, the floor plate is nearly continuous, demonstrating that loss of spt function corrects the floor plate defect of flh mutants (compare Fig. 3F and 3H).

Anterior notochord forms in spt-;flh- embryos

The expression of shh in spt-;flh- midline mesoderm reveals that this cell population retains notochord marker expression, at least during early notochord development. We also observe differentiated notochord tissue in the spt;flh double mutant midline (Fig. 4). In spt-;flh- embryos examined by light microscopy at or after 24 h, we find morphologically identifiable stretches of differentiated, well-vacuolated notochord cells (Fig. 4I). The notochord length is variable, but generally is found in one or two contiguous stretches in the anterior of the embryo, underlying the hindbrain and extending posteriorly through the anterior trunk (see Fig. 4 legend). We rarely detect notochord in the posterior trunk and have never detected notochord in the tail of spt-;flh- embryos. This clearly demonstrates that flh- cells can form notochord when spt function is also lost.

The no tail (ntl, Brachyury homolog) gene is essential for notochord development (Halpern et al., 1993; Schulte-Merker et al., 1994). ntl expression is not maintained in flh- axial mesoderm during midgastrulation (Talbot et al., 1995), consistent with the requirement for ntl for notochord development. To examine whether the spt-;flh- notochord develops by utilizing the normal, ntl+-dependent genetic pathway, we examined ntl expression during early (12 h) and late (20 h) segmentation stages (Fig. 4). Our results show that ntl expression is maintained in the region of the spt-;flh- midline where notochord develops. Wild-type embryos express ntl strongly in undifferentiated notochord and tailbud at 12 h and in the developing notochord at 20 h (Fig. 4A,E). At 12 h, ntl expression in spt-;flh- embryos is approximately normal in the midline with an expanded domain in the tail (Fig. 4C). At 20 h, ntl expression frequently reveals distortions and kinks in the spt- notochord (Fig. 4G; see also Kimmel et al., 1989; Hammerschmidt et al., 1996a). Midline ntl expression in flh-
expression are observed in ntl expression reveals a wide, kinked notochord. Patches of anterior expression in approximately 1-6 cells ntl extensive shown; however, a significant number (8/20, 40%) have more each containing 2-5 cells, and the remaining double mutants (2/12) rhombomere 5) or more posteriorly at midtrunk levels. A few double anteriorly (as shown in H, see small clump just anterior to these embryos have additional smaller clumps located either more midline cells underlying the posterior half of the hindbrain; three of these embryos have additional smaller clumps located either more anteriorly (as shown in H, see small clump just anterior to rhombomere 5) or more posteriorly at midtrunk levels. A few double mutants (2/12) have two small patches of ntl-expressing midline cells each containing 2-5 cells, and the remaining double mutants (2/12) show no ntl expression at this stage. (I) 30 h Nomarski view of spr\textsuperscript{-};flh\textsuperscript{-} notochord cells. Scale bars, 100 \textmu m (A-H), 50 \textmu m (I).

**Fig. 4.** Anterior spr\textsuperscript{-};flh\textsuperscript{-} midline cells express the essential notochord gene, ntl. (A-D) Dorsal views of ntl expression during early segmentation stages (4-6 somites, 11.3-12 h, anterior to the top). ntl is expressed in wild-type embryos (A) in the developing notochord and in the tailbud (Schulte-Merker et al., 1992, 1994) and, in these overstained embryos, in the more laterally located presumptive pronephric ducts. In flh\textsuperscript{-} embryos (B), midline ntl staining is conspicuously missing. In about half the flh\textsuperscript{-} embryos, there is anterior midline ntl expression in approximately 1-6 cells (not shown), ntl expression in spr\textsuperscript{-} embryos (C) is approximately normal (Hammerschmidt et al., 1996a), except the presumptive pronephros domain is reduced or absent. Midline ntl expression is observed in all spr\textsuperscript{-};flh\textsuperscript{-} embryos (D), typically in one large anterior patch, and occasionally in a few additional smaller patches, like those shown here. Many spr\textsuperscript{-};flh\textsuperscript{-} embryos (10/20, 50%) look as shown; however, a significant number (8/20, 40%) have more extensive ntl expression, some of which were recovered from the spr\textsuperscript{-} class after staining. A few spr\textsuperscript{-};flh\textsuperscript{-} embryos have only about 10 ntl-positive cells (2/20, 10%). The observed number of spr\textsuperscript{-};flh\textsuperscript{-} embryos was as expected (20/316). (E-H) Dorsal views of krox-20 and ntl staining during later segmentation stages (22 somites, 20 h, anterior to the left) when notochord differentiation is well underway. krox-20 is expressed in a broad band at the extreme left of each photograph that marks a hindbrain segment, rhombomere 5, in all embryos (Oxtonby and Jowett, 1993). In wild-type embryos (E), ntl is expressed in the entire notochord, beginning at the level of rhombomere 4 or 5 and extending posteriorly. Expression is beginning to fade in the anteriormost notochord. No midline ntl expression is observed in any flh\textsuperscript{-} embryos (F). In spr\textsuperscript{-} embryos (G), ntl expression reveals a wide, kinked notochord. Patches of anterior ntl expression are observed in spr\textsuperscript{-};flh\textsuperscript{-} embryos (H). Most spr\textsuperscript{-};flh\textsuperscript{-} embryos (8/12) show expression in a patch containing about 20-60 midline cells underlying the posterior half of the hindbrain; three of these embryos have additional smaller clumps located either more anteriorly (as shown in H, see small clump just anterior to rhombomere 5) or more posteriorly at midtrunk levels. A few double mutants (2/12) have two small patches of ntl-expressing midline cells each containing 2-5 cells, and the remaining double mutants (2/12) show no ntl expression at this stage. (I) 30 h Nomarski view of spr\textsuperscript{-};flh\textsuperscript{-} notochord cells. Scale bars, 100 \textmu m (A-H), 50 \textmu m (I).

**Fig. 5.** flh\textsuperscript{-} mutant cells can make notochord when transplanted into a wild-type environment. Blastula transplantations were performed as described in the Materials and Methods. (A-D) Lateral views of transplants at 24-28 h; shh-expressing cells are shown in blue and the transplanted cells are shown in brown. (A) A control transplant showing transplanted wild-type cells contributing to wild-type host notochord (concave arrowheads) and floor plate (arrowheads). (B) A transplant showing flh\textsuperscript{-} cells contributing to wild-type host notochord and floor plate. (C) A transplant showing contribution of flh\textsuperscript{-} cells to wild-type host floorplate, but not to the notochord. (D) The same embryo as in C, but a different focal plane to show the large number of flh\textsuperscript{-} donor muscle cells (asterisks). (E-H) Images of wild-type (E,F) and flh\textsuperscript{-} (G,H) transplanted cells (shown in red) in live wild-type hosts. Embryos were photographed several times during the segmentation period, beginning at the 3-somite stage (11 h) (E,G) and ending at the 20-somite stage (19 h) (F,H). Scale bars, 50 \textmu m (A-D), 25 \textmu m (E-H).
Transplants that contain notochord also contain only a few mesodermal transplants. The other two notochord in two-thirds (6/9) of the \textit{-}flh (Halpern et al., 1995). Thus, although observation is consistent with results reported previously majority of \textit{-}flh where transplanted mutant cells can make notochord, the \textit{-}flh all the derived muscle cells (Table 1). In these two transplants, almost mesodermal transplants because they also have 35-45 donor-notochord cells (Fig. 5B), but are classified as large different behavior of \textit{-}flh and muscle cells they contain at 24 h. Our transplants reveal a different behavior of \textit{-}flh cells than reported previously. Among our \textit{-}flh- to wild-type transplants, eight wild-type hosts contain \textit{-}flh- notochord cells (Table 1, Fig. 5B). Six of these are classified as small mesodermal transplants, and contain between 1 and 4 \textit{-}flh- notochord cells. Thus, \textit{-}flh- cells make notochord in two-thirds (6/9) of the \textit{-}flh- to wild-type small mesodermal transplants. The other two \textit{-}flh- to wild-type transplants that contain notochord also contain only a few \textit{-}flh- notochord cells (Fig. 5B), but are classified as large mesodermal transplants because they also have 35-45 donor-derived muscle cells (Table 1). In these two transplants, almost all the \textit{-}flh- muscle cells are located anteriorly, many body segments away from the \textit{-}flh- tail notochord cells. Hence, as in the transplants with small cell numbers, the \textit{-}flh- donor notochord cells in these two embryos were effectively entirely surrounded by wild-type cells.

In contrast to \textit{-}flh- to wild-type small mesodermal transplants, where transplanted mutant cells can make notochord, the majority of \textit{-}flh- to wild-type large mesodermal transplants do not contain mutant notochord cells and instead have large numbers of \textit{-}flh- muscle cells (8/10, 80%) (Table 1; Fig. 5C,D). The latter observation is consistent with results reported previously (Halpern et al., 1995). Thus, although \textit{-}flh- cells can make notochord in wild-type hosts, they tend to do so when transplanted in small numbers and/or when isolated from other \textit{-}flh- mesodermal neighbors. When transplanted in large numbers, \textit{-}flh- cells tend to make muscle. These results greatly contrast with control transplants which demonstrate that transplanted wild-type cells can form notochord in large and small numbers (Table 1; Fig. 5A). The size of the transplant does not appear to affect the ability of wild-type mesodermal cells to contribute to wild-type host notochord, as small and large mesodermal transplants containing only notochord or containing mixed notochord and muscle are obtained at approximately the same frequency (70% versus 59% and 30% versus 40%, respectively; Table 1). Additionally, only one of the 90 control transplants contained only muscle and no notochord, compared to 11/19 for the \textit{-}flh- to wild-type transplants.

In addition to the 8 examples in which \textit{-}flh- cells make notochord (Table 1), we have also watched the behavior of 7 \textit{-}flh- cells that eventually formed notochord in an additional mosaic embryo (Fig. 5G,H). When compared to a similar staged wild-type to wild-type control (Fig. 5E,F) over an 8 hour period during segmentation, \textit{-}flh- notochord cells begin the vacuolation process, a characteristic notochord cell behavior, at the appropriate time relative to wild-type cells. Thus, \textit{-}flh- cells can make notochord cells that appear to undergo proper morphogenetic behaviors (Fig. 5). We have not followed the fate of these cells past 24-28 h.

In the above experiments, we transplanted cells heterochronically from older donor blastulae to younger host blastulae, as this method increases the percentage of host embryos in which transplanted cells contribute to notochord and floor plate (Halpern et al., 1995). This permitted us to construct and analyze a large number of genetic mosaics. To show that the ability of a wild-type environment to rescue notochord development of \textit{-}flh- cells is not due to the heterochronous technique, we have also transplanted cells isochronically (see Materials and Methods). Using isochronous transplantation, we have observed that small numbers of \textit{-}flh mutant cells can be recruited to wild-type notochord as late as the gastrula stage (\textit{n}=2 hosts, data not shown).

### DISCUSSION

Embryos require \textit{-}flh function for notochord development; otherwise, muscle forms in the midline (Halpern et al., 1995, Talbot et al., 1995). We show that loss of \textit{spt} function rescues many aspects of the \textit{-}flh mutant phenotype. \textit{spt} \textit{-}flh- embryos have an anterior notochord, have a nearly complete floor plate and do not form ectopic midline muscle. Thus, \textit{-}flh- cells can make anterior notochord if the embryos also lack \textit{spt} function, which is normally required for proper morphogenesis and fate specification of trunk somitic muscle cells (Kimmel et al., 1989, Ho and Kane, 1990). These data support a model in which \textit{-}flh normally antagonizes \textit{spt} function in developing midline mesoderm, thereby promoting notochord formation and blocking muscle development (Fig. 6). We also show \textit{-}flh- cells can contribute to notochord at all body levels when

<table>
<thead>
<tr>
<th>Donor to host</th>
<th>Small mesodermal transplants†</th>
<th>Large mesodermal transplants‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Noto only</td>
<td>Noto + Musc</td>
</tr>
<tr>
<td>WT to WT§</td>
<td>9/13 (70%)</td>
<td>4/13 (30%)</td>
</tr>
<tr>
<td>flh\textsuperscript{-} to WT¶</td>
<td>4/9 (44%)</td>
<td>2/9 (22%)</td>
</tr>
</tbody>
</table>

*All transplants containing floor plate cells [90/252 wild-type (WT) to WT control and 19/64 flh\textsuperscript{-} to WT] are included. Since notochord (noto) and muscle (musc) are by far the major trunk and tail mesodermal tissues to which transplanted cells contribute, we have used the number of cells in these two tissues to subdivide the floor plate-containing transplants into two classes.
†Notochord and muscle cells totalled 15 or fewer.
‡Notochord and muscle cells totalled 16 or greater.
§In small mesodermal transplants, the notochord cell number ranged between 1 and 15 cells and the muscle cell number between 1 and 5 cells; in large mesodermal transplants, the notochord cell number ranged between 1 and 85 cells and the muscle cell number between 1 and 60 cells.
¶In mixed transplants with 1-15 muscle cells, the notochord and muscle cells were sometimes mingled in the same body region (6/14) or sometimes located far apart (8/14).
**Almost all the muscle cells in these transplants were located many body segments away from the tail notochord cells.
by antagonizing notochord development. Previous work has function either indirectly by promoting myogenesis or more directly. This, combined with other studies, has led to the hypothesis that Merker et al., 1994) and is expressed in anterior notochord cells of the anterior/posterior axis. The indicating that notochord development is differentially regulated along the anterior/posterior body axis, perhaps by local cellular interactions and signals. We discuss the model presented in Fig. 6 in more detail above.

**Fig. 6.** Model for the genetic interaction among spt, flh and ntl genes in the zebrafish midline. Ectopic muscle does not form in the spr"-flh"-midline, revealing that spt function is required for flh" cells to transfect to muscle (as assayed by myoD expression) and suggesting that flh" normally represses spt activity at all body levels, perhaps by preventing spt expression in developing midline mesoderm. Notochord formation is rescued anteriorly in spr"-flh" embryos, indicating that notochord development is differentially regulated along the anterior/posterior axis. The ntl gene (Brachyury homolog) is essential for notochord development (Halpern et al., 1993; Schulte-Merker et al., 1994) and is expressed in anterior notochord cells of the spr"-flh"-midline at times when it is no longer expressed in flh single mutants. This suggests that, at anterior body levels, spt" normally antagonizes ntl function. As discussed in the text, spt" may block ntl function either indirectly by promoting myogenesis or more directly by antagonizing notochord development. Previous work has established a complex regulatory interaction between flh" and ntl"; this, combined with other studies, has led to the hypothesis that ntl" may be involved in repressing floor plate fate (Halpern et al., 1997; see text). Finally, our genetic mosaic experiments demonstrate that wild-type environmental signals can promote notogenesis of transplanted flh" cells; we indicate that these signals act to maintain or augment ntl activity, but these signals may act at other levels of the pathway as well. Analysis of notochord development in spr"-flh"-embryos suggests that these signals may be differentially regulated along the anterior/posterior body axis, perhaps by flh" itself (see text).

transplanted in small numbers into wild-type hosts, showing that flh" cells can respond to wild-type notochord-promoting signals. We discuss the model presented in Fig. 6 in more detail below.

**Local cellular interactions and signals promote notochord cell fate**

Our data show that flh" cells can make either notochord or muscle when transplanted into wild-type hosts. The choice appears to correlate with the number of mesodermal cells in the transplant (Table 1). In Halpern et al. (1995) and in most of our genetic mosaics classified as having large mesodermal transplants, flh" cells do not contribute to wild-type host notochord, but instead give rise to large numbers of muscle cells. In contrast, in our genetic mosaics classified as having small mesodermal transplants, flh" cells contribute to wild-type host notochord. Hence, our results extend the previous study by showing that when isolated or small groups of flh" cells are transplanted to midline positions, they can be recruited by surrounding wild-type cells into the host notochord. Interestingly, a similar observation has been made in grafting experiments in Xenopus. When labeled gastrula cells from prospective notochordal, somitic or epidermal regions are placed within the notochord territory of an unlabelled host, the gifted cells can adopt behaviors typical of their new location and differentiate as notochord; cells appear more likely to incorporate into the host notochord when grafted in several small clumps instead of one large clump (Domingo and Keller, 1995).

The results in Xenopus and our results in zebrafish suggest signalling occurs at short range and that, even as late as the gastrula stage, cells that normally adopt non-notochordal fates are plastic and can be recruited into the notochord. We propose that these results indicate the presence of notochord-promoting signals or cell interactions (Fig. 6). It is intriguing to speculate on the nature of such signal(s); one potential candidate is eFGF, a secreted molecule that can induce Brachyury expression (Isaacs et al., 1994). Alternatively, it is possible that dispersing flh" cells among wild-type neighbors disrupts muscle 'community effect' (Gurdon, 1988), thus preventing mutant cells from becoming muscle. Although disrupting mutant cell-cell interactions might explain why dispersed flh" cells often fail to form muscle, it is difficult to explain why they adopt the notochord fate without invoking the presence of notochord-promoting signals or local cell-cell interactions. Our transplants suggest that environmental signals and cell-cell interactions promote notochord and muscle cell fate choices; we have incorporated these ideas into our model to explain how loss of spt function rescues many aspects of the flh mutant phenotype (see below).

**Ectopic midline muscle formation in flh mutants requires spt function**

spt has mainly been described as a regulator of cell movements (Kimmel et al., 1989; Ho and Kane, 1990), although other observations, such as the lack of myoD expression in spr"-gastrulae, have suggested that spt may also regulate myogenic specification (Weinberg et al., 1996; S. L. A., unpublished observations). How does loss of spt function prevent ectopic muscle formation in the flh mutant midline? The simplest model based upon the epistatic relationship of spt and flh is that flh-expressing axial mesoderm cells normally repress spt activity in the notochord domain and thereby prevent improper activation of myogenesis in the midline (Fig. 6). Improper midline spt activity might promote myogenesis either by promoting muscle community effect or by directly influencing muscle specification or by a combination of both mechanisms. Alternatively, spt" may promote community effect in all regions of the developing mesoderm, and flh" is required dorsally to block or modify such interactions. It is interesting to note that sometimes there are a few myoD-expressing midline cells in the spr"-flh"-tail (Fig. 2H). This suggests that flh" may also repress the activity of additional genes that activate myoD expression caudally. spt mutants have relatively normal tail muscle development (Kimmel et al., 1989), suggesting that other genes either replace or are redundant with spt in the tail (K. J. P. Griffin, S. L. A., C. B. K. and D. Kimelman, unpublished data).
Anterior and posterior notochord development are differentially regulated

Notochord cells form only anteriorly in spr<sup>−</sup>:flh<sup>−</sup> embryos, indicating that notochord development is differentially regulated along the anterior/posterior axis. There are also other mutations that regionally disrupt notochord development (Hammerschmidt et al., 1996b; Odenthal et al., 1996). For example, anterior notochord forms in zebrafish chordino mutants, but is often truncated posteriorly (Hammerschmidt et al., 1996b; Schulte-Merker et al., 1997), a phenotype similar to that observed in spr/flh mutants. What distinguishes anterior notochord from posterior notochord? Expression analysis has shown that flh is expressed in the anteriormost notochord primordium at a time when it is no longer detected more posteriorly; this suggests that flh (and perhaps other genes) is differentially regulated in the notochord (Melby et al., 1997). Intriguingly, a few flh<sup>−</sup> anterior midline cells maintain flh and ntl expression longer than more posterior cells (Melby et al., 1997; Fig. 4). Although notochord does not differentiate in flh mutants, such expression patterns may identify anterior midline cells as being particularly sensitive to conditions that favor notochord development. We now consider the possible mechanisms by which loss of spr function rescues notochord development in flh<sup>−</sup> embryos.

In the absence of spr function, flh<sup>+</sup> is not required for anterior notochord development

In spr<sup>−</sup>:flh<sup>−</sup> embryos, ntl (Brachyury homolog) is expressed in axial mesodermal cells and anterior notochord develops (Fig. 4). This demonstrates that ntl expression and the notochord fate can be maintained in flh<sup>−</sup> anterior midline mesodermal cells if spr function is also lost. We indicate in our model that spr<sup>+</sup> normally functions to repress ntl activity; this would explain how loss of spr function permits axial mesodermal cells to maintain ntl expression (and the notochord fate) in a flh<sup>+</sup>-independent manner (Fig. 6). The mechanism of this repressive interaction is unclear, and we consider three possible explanations.

One possible explanation for the antagonistic action of spr<sup>+</sup> on ntl expression is that spr<sup>+</sup> may only be needed to repress spr<sup>+</sup>, and thus myogenesis, in anterior midline cells. A difference between our genetic mosaic results and the double mutant analysis is that flh<sup>−</sup> cells can make notochord along the entire axis of wild-type hosts, but notochord formation in spr<sup>−</sup>:flh<sup>−</sup> embryos occurs only anteriorly. This difference may be resolved if notochord-promoting signals are themselves dependent upon flh<sup>+</sup> function, and if this requirement is much stronger posteriorly than anteriorly (Fig. 6), an idea consistent with the observation that axial mesoderm markers are initially present in flh<sup>−</sup> gastrulae, but fail to be maintained except in a few of the anteriormost cells as gastrulation proceeds (Halpern et al., 1995; Talbot et al., 1995; Odenthal et al., 1996; Melby et al., 1997; Beattie et al., 1997). In more posterior regions of the trunk and tail, flh not only blocks muscle formation but also would be required to promote notochord development, consistent with the observation that overexpression of Xnot, the Xenopus flh homolog, causes ectopic notochord formation (Gont et al., 1996). An alternative hypothesis is that notochord-promoting signals are not differentially regulated along the anterior/posterior axis but, instead, flh<sup>−</sup> cells are unable to respond to such signals unless they are dispersed in small numbers among wild-type cells, as they are in our transplantation experiments. We would argue that muscle community effect among flh<sup>−</sup> midline cells would be disrupted either by dispersing flh<sup>−</sup> cells into wild-type hosts, or by loss of spr function. Thus, spr<sup>−</sup>:flh<sup>−</sup> embryos would form notochord anteriorly, in the region of the embryo most affected by the spr mutation.

A second explanation for the antagonistic action of spr<sup>+</sup> on ntl expression is that spr<sup>+</sup> may play a more direct role in repressing notochord development, not just in promoting myogenesis. Fate mapping studies of the early gastrula show that spr<sup>−</sup> notochord cells arise from the normal dorsal domain as well as from lateral domains (Kimmel et al., 1989; Warga, 1996) that are well outside the normal wild-type notochord domain boundaries (Shih and Fraser, 1995; Melby et al., 1996). We have observed that ntl staining in the blastoderm margin of spr<sup>−</sup> gastrulae is more intense than in wild-type embryos (S. L. A., data not shown). Thus, although ntl is expressed in all mesodermal progenitors in the epiblast prior to involution, spr may be required to restrict ntl expression to notochord progenitors of the hypoblast after involution. Misexpression of ntl in hypoblast cells outside the notochord domain might predispose them to a notochord fate. However, it has long been known from Xenopus studies that overexpression of Brachyury (ntl homolog) does not induce notochord unless other genes such as noggin or pintallavis are also overexpressed (Cunliffe and Smith, 1992, 1994; O’Reilly et al., 1995). Therefore, if notochord-promoting signals are indeed graded in an anterior-to-posterior manner in flh<sup>−</sup> embryos, then anterior axial mesodermal cells lacking both spr and flh, but expressing ntl, may be uniquely able to adopt a notochord fate.

A third, but related, explanation for the antagonistic action of spr<sup>+</sup> on ntl expression may be that spr<sup>+</sup> is required in anterior axial mesoderm (i.e., in cells fated to become non-notochordal head mesoderm) in order to prevent inappropriate notochord development. A role for spr<sup>+</sup> in this region has been proposed since expression of a head mesendodermal gene, snail2, is reduced in spr<sup>−</sup> embryos (Thiess et al., 1995). This model would predict that when head mesendodermal cells and anterior notochord progenitors are closely associated, as at the beginning of gastrulation, some spr<sup>−</sup> head mesendodermal progenitors might inappropriately join the notochord population. Thus, in spr<sup>−</sup>:flh<sup>−</sup> embryos, notochord would be present only anteriorly. Although this mechanism may contribute cells to the rescued spr<sup>−</sup>:flh<sup>−</sup> notochord, we suspect that it is probably not the only mechanism for two reasons. First, mesendodermally derived head structures in spr<sup>−</sup>:flh<sup>−</sup> embryos are often truncated anteriorly, in the region of the embryo most affected by the spr mutation.

An interacting gene network controls notochord development

The possible mechanisms that we discuss to explain the spr<sup>−</sup>:flh<sup>−</sup> phenotype are not mutually exclusive and it is likely that a combination of them is at work. Indeed, the model is already more complex when we consider the genetic interaction between the flh and ntl genes (Halpern et al., 1997).
Although neither gene is required to initiate expression of the other in notochord precursors, flh and ntl each regulate the maintenance of the others expression in that domain, beginning during midgastrulation (Talbot et al., 1995; Melby et al., 1997; Odenthal et al., 1996). Reflecting this complex genetic interaction, flh−ntl− embryos mostly resemble ntl single mutants, but sometimes resemble flh single mutants in mid-body regions (Halpern et al., 1997). Analysis of flh−ntl− embryos, combined with other genetic and expression data (Halpern et al., 1997; T. Wu, S. Ekker and M. E. Halpern, personal communication), has lead to the hypothesis that ntl+ acts in axial mesodermal cells to prevent floor plate development. On the contrary, flh+ acts within the same cell population to repress muscle development (Halpern et al., 1995; Talbot et al., 1995; Melby et al., 1996). With this in mind, our model (Fig. 6) is consistent with the flh−ntl− phenotype; it predicts that lack of ntl function results in notochord precursors adopting the floor plate fate. Alternatively, ectopic spt+ activity in the flh−ntl− midline can direct these same cells toward the muscle fate. The cells usually chose the first alternative; they make floor plate. Although highly speculative, it may be that posterior axial mesodermal cells in spt−flh− embryos that do not express ntl are diverted to a floor plate fate, and thus contribute to the nearly continuous spt−flh− floor plate.

**Does the spt:flh genetic interaction share similarities with dorsal/ventral patterning pathways?**

The organizer has long been thought to be a source of active dorsalizing factors that pattern the adjacent tissues. However, this idea has recently been challenged by the discovery of secreted ventralizing factors such as BMP-4 and Xwnt-8. When these ventralizing factors are overexpressed, they override the activity of the organizer and, when their activity is blocked, ventral fates are lost (Harland, 1994; Christian and Moon, 1993; Hoppler et al., 1996). The organizer is protected from these secreted ventralizing signals as it produces and secretes factors that antagonize their activity. Noggin and Chordin are typically localized factors, bind and thereby inactivate BMP-4 (Piccolo et al., 1996; Zimmerman et al., 1996), and frzb similarly antagonizes the function of Xwnt-8 (Leys et al., 1997; Wang et al., 1997). Both these interactions prevent the ventralizing factors from binding their respective receptors and shield the organizer region from their influence. We show that flh and spt also interact antagonistically, and we suggest that the dorsally located flh-encoded transcription factor acts to repress the activity of spt. Future work will establish what signaling molecules function in the notochord and muscle domains, and how they impinge upon the regulatory pathway that we have described.

We thank Cecilia B. Moens for help with genetic mosaic experiments and Judith S. Eisen, Marnie E. Halpern, William S. Talbot and members of the Kimmel laboratory for helpful comments on the manuscript. We also thank Rachel M. Warga, Tammy Wu and Marnie E. Halpern for sharing data prior to publication. We gratefully acknowledge the Oregon Zebrafish Facility for their excellent fish care and John H. Postlethwait and members of his laboratory for sharing advice and resources in genetic mapping experiments. This work was supported by American Cancer Society Fellowship PF-4087 (to S. L. A.) and by NIH grant HD22486.

**REFERENCES**


Promoting notochord fate in zebrafish 1405