Notochord induction of zebrafish slow muscle mediated by Sonic hedgehog

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The patterning of vertebrate somitic muscle is regulated by signals from neighboring tissues. We examined the generation of slow and fast muscle in zebrafish embryos and show that Sonic hedgehog (Shh) secreted from the notochord can induce slow muscle from medial cells of the somite. Slow muscle derives from medial adaxial myoblasts that differentiate early, whereas fast muscle arises later from a separate myoblast pool. Mutant fish lacking *shh* expression fail to form slow muscle but do form fast muscle. Ectopic expression of *shh,* either in wild-type or mutant embryos, leads to ectopic slow muscle at the expense of fast. We suggest that Shh acts to induce myoblasts committed to slow muscle differentiation from uncommitted presomitic mesoderm.

All vertebrates have two classes of muscle fibers: slow and fast. Slow fibers have low-force, long-duration contractions because they express myosin isoforms that are specialized for slow contraction and an oxidative metabolism. Fast fibers have distinct fast myosins and glycolytic metabolism, ideal for high-force, short-duration contractions. Each muscle has a specific mix and spatial array of fast and slow fibers from its formation early in development. How such arrays are patterned is unknown, but evidence for two contrasting models exists. In one view, proliferative myoblasts are intrinsically committed to form either fast or slow fibers and accumulate in appropriate regions of the embryo. Clonal cell analysis in chicks shows that myoblasts are heterogeneous before their differentiation: Some are specialized to form slow muscle, whereas others form fast (Miller and Stockdale 1986; Schafer et al. 1987; DiMario et al. 1993). Alternatively, naive myoblasts could be instructed by their environment to express specific isoforms of muscle proteins at the time of differentiation, as occurs in postnatal rodent muscles (Hughes and Blau 1992). A resolution of this issue is suggested by studies in *Drosophila* where local extrinsic signals induce commitment of muscle founder myoblasts to the formation of a particular type of muscle in each location (Baylies et al. 1995). In this paper, we examine vertebrate muscle patterning in the zebrafish somite. We show that the

secreted glycoprotein Sonic hedgehog (Shh) regulates the decision between fast and slow muscle formation and we suggest this decision involves induction of a specifically slow myoblast type.

Muscles are formed by the differentiation of mononucleate proliferative myoblasts into post-mitotic myocytes that subsequently fuse to form multinucleate muscle fibers. In amniotes, muscle fibers differentiate in two waves: The first-formed primary fibers are generally slow, whereas later secondary fibers, which form in close association with primary fibers, are fast (Kelly and Rubinstein 1980). These two fiber types are not separated spatially and, as they are formed over a considerable time period, the fate of individual cells as they mature has not been followed. In zebrafish, by contrast, somitic muscle fibers form in two temporally separated waves. The early differentiating cells are formed medially near the notochord and migrate laterally during late somitogenesis to become slow muscle (van Raamsdonk et al. 1978; Devoto et al. 1996). However, most somitic cells differentiate later and become fast muscle. How spatial information in the early somite generates this pattern is unclear.

The differentiation of somites is central to vertebrate mesoderm development. Somites are epithelial balls of mesoderm that arise from a mesenchymal mass of proliferative paraxial tissue in a rostro–caudal order. Once formed, somites differentiate rapidly into a ventral sclerotomal mesenchymal compartment and a dorsal epithelial structure, the dermomyotome. In lower vertebrates, such as fish, in which the sclerotome is small (Morin-Kensicki and Eisen 1997), the somite mainly gives rise to muscle. In amniotes, the dermomyotome contributes to trunk dermis and to several distinct popu-

lations of muscle cells. The dorsomedial lip of the dermomyotome, which is located next to the neural tube, forms differentiated muscle of the myotome that arises between the sclerotome and dermomyotome.

Signals from adjacent tissues regulate somitic muscle differentiation (for review, see Lassar and Munsterberg 1996). However, the precise source, nature, and roles of these signals is unclear. Axial structures (neural tube and notochord) are important as their removal leads to cell death and somite regression (Teillet and Le Douarin 1983; Rong et al. 1992), and they can enhance both myogenesis and chondrogenesis (Kenny-Mobbs and Thorogood 1987). Notochord can induce myogenesis in some assays of myogenic induction (Buffinger and Stockdale 1995; Gamel et al. 1995; Stern et al. 1995; Pownall et al. 1996), although ectopically positioned notochords in chick embryos can induce sclerotome at the expense of myogenic tissue (Pourquie et al. 1993; Bober et al. 1994; Fan and Tessier-Lavigne 1994; Goulding et al. 1994). Shh is a signaling molecule expressed in notochord at times when this tissue can influence muscle differentiation (Echelard et al. 1993; Krauss et al. 1993; Johnson et al. 1994; Roelink et al. 1994; Marti et al 1995). Shh can substitute for notochord in various assays of both sclerotome and muscle induction (Fan et al. 1995; Munsterberg et al. 1995), and induce ectopic muscle markers in vivo (Johnson et al. 1994; Concordet et al. 1996; Hammerschmidt et al. 1996; Weinberg et al. 1996). Moreover, mice homozygous for a targeted deletion of the *shh* gene have deficits in sclerotome and myotome precursor cell markers (Chiang et al. 1996). These data suggest that Shh may mediate notochord-dependent signals that induce myogenesis. However, two lines of evidence argue against this simple view. First, both the MyoD and Myf-5 muscle-specific transcription factors are still expressed in $shh^{-/-}$ mouse somites, although *Myf-5* mRNA is reduced (Chiang et al. 1996). As Myf-5 and MyoD are myoblast markers in amniotes, this suggests that the myogenic program can be initiated in the absence of Shh. Second, ablation of all axial structures has little effect on limb and body wall muscle development although somitic myogenesis is reduced, partly attributable to regression of the somite (Rong et al. 1992). Therefore, although notochord-derived Shh is a strong candidate for a regulator of myotomal muscle formation, its precise role in myogenesis remains enigmatic.

A confounding factor in understanding myotomal muscle induction is the heterogeneity of myogenic cell populations within the somite (for review, see Cossu et al. 1996b). The phenotypes of mice with null mutations in members of the MyoD family of myogenic regulatory transcription factors (MRFs) suggest that several distinct populations of myogenic cells exist in different parts of the developing murine dermomyotome (Rudnicki et al. 1993; Tajbakhsh et al. 1997) and these populations appear to differ in their sensitivity to loss of Shh (Chiang et al. 1996). In addition to notochord, neural tube also contains inductive signals that can support somitic myogenesis (Teillet and Le Douarin 1983; Rong et al. 1992; Goulding et al. 1994; Buffinger and Stockdale 1995; Stern

and Hauschka 1995), and dorsal neural tube can induce myogenesis, an effect that can be mimicked by some Wnt proteins (Gamel et al. 1995; Munsterberg et al. 1995; Stern et al. 1995). Moreover, inhibitory signals from lateral plate mesoderm and surface ectoderm have been suggested to influence myogenesis (Fan and Tessier-Lavigne 1994; Pourquie et al. 1996). Therefore, although several distinct signals and muscle cell populations exist, what signals induce each cell population in vivo is unclear.

In the zebrafish, the somite gives rise mainly to muscle, which is probably the primary fate of paraxial mesoderm during early chordate evolution (Holland et al. 1995). Even in this simple system, however, three muscle cell populations can be resolved. Adaxial cells form next to the notochord, eventually giving rise to slow muscle (van Raamsdonk et al. 1978; Devoto et al. 1996) and are the first cells in the embryo to express the muscle transcription factors *myoD* and *mef2D, mef2A,* and *mef2C* (Ticho et al. 1996; Weinberg et al. 1996). A specialized subpopulation of adaxial cells, the muscle pioneers, form at the dorsoventral midline of each somite (Felsenfeld et al. 1991), express engrailed proteins (Hatta et al. 1991), and appear to be induced by two sequential signals from outside the somite (Currie and Ingham 1996). The majority of the somite forms the third muscle cell population that both expresses *myoD* and differentiates later (Devoto et al. 1996; Weinberg et al. 1996).

Here, we describe the development of slow and fast muscle in the zebrafish embryo. We show that, as in amniotes, slow muscle differentiates first and that fast muscle is formed later in close association with differentiated slow fibers. We demonstrate that notochord-derived signals are required for formation of slow, but not fast, muscle. We find that Shh, a molecule expressed and secreted by early notochord, can induce slow muscle ectopically at the expense of fast muscle. Taken together, our results suggest that slow primary muscle in zebrafish somites is induced by Shh from the notochord, but that neither notochord nor primary slow muscle is required to control the timing of secondary fast fiber differentiation.

Results

Early zebrafish embryos have distinct fast and slow muscle cell populations

To examine the patterning of muscle in zebrafish somites, we screened a series of anti-MyHC monoclonal antibodies for reactivity with 1- to 2-day zebrafish muscle tissue. Two antibodies detected all differentiated skeletal and heart muscle, whereas three antibodies detected specific subpopulations of cells within the somites of 24-hr (prim-5) embryos (Fig. 1). BA-D5, an antibody that specifically detects slow MyHC in muscle fibers of all ages of mammals and chicks examined (Schiaffino et al. 1989; C.S. Blagden and S.M. Hughes, unpubl.), detects a single layer of cells in the superficial
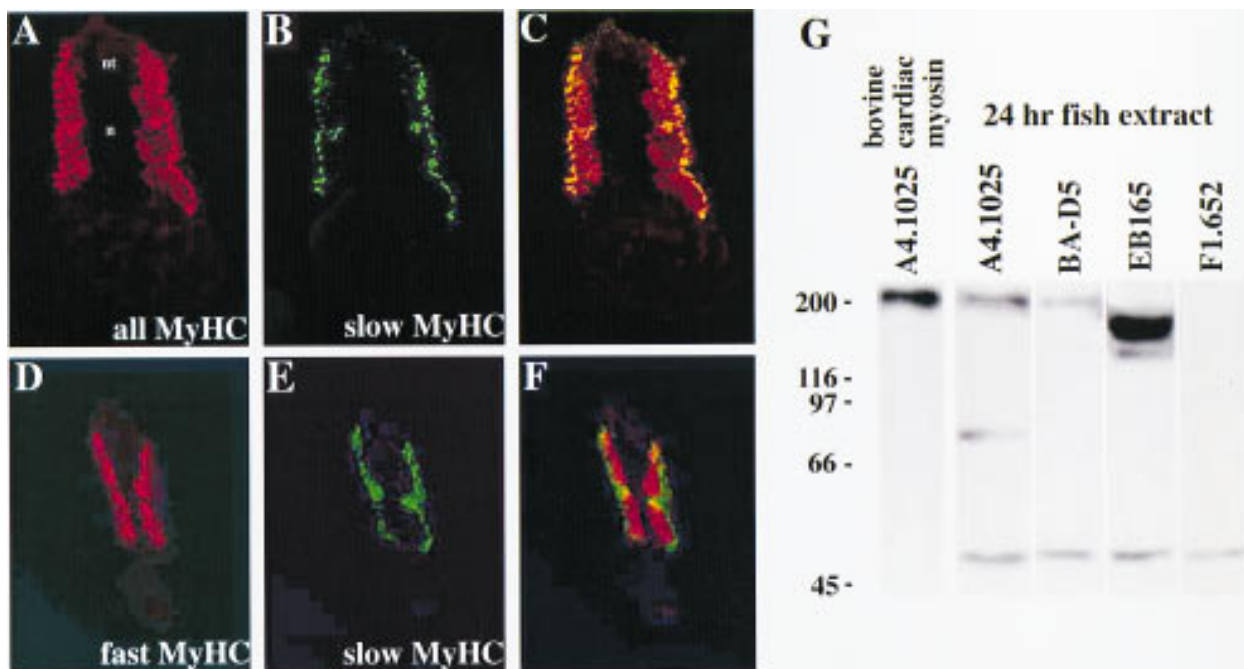
Figure 1. Slow and fast MyHC isoform-specific antibodies recognize discrete muscle fiber populations in developing embryonic 24-hr zebrafish. Slow BA-D5-reactive MyHC (green; *B,C*) is present in the outermost layer of A4.1025-reactive cells containing skeletal muscle MyHC (red; *A,C*) in a transverse cryosection through the trunk (*A–C*). A tail section (*D–F*) shows that fast EB165-reactive MyHC (red; *D,F*) was detected in medial fibers that do not express slow MyHC (green; *E,F*) so that muscle fibers at this stage can be divided into two subpopulations of slow lateral and fast medial cells (*C,F*). Western analysis (*G*) using protein extracted from 24-hr zebrafish embryos shows that A4.1025 and BA-D5 recognize major bands that migrate at the same rate as purified bovine cardiac muscle MyHC (~220 kD). EB165 detects two bands ~170 kD that could either reflect degradation of the fast MyHC or an unusual sized MyHC in zebrafish fast muscle. The lower ~50-kD band is nonspecific because it is also detected with antibody F1.652 to mouse embryonic MyHC that does not react with fish sections. The ~75-kD band in the A4.1025 lane reflects some degradation. (n) Notochord; (nt) neural tube.

region of 24-hr zebrafish somites at all anteroposterior positions within the body axis (Fig. 1B,C,E,F). In contrast, EB165, an antibody that detects fast fibers in embryonic and adult chicken muscle (Gardahaut et al. 1992), detects an adjacent nonoverlapping population of medial somitic muscle fibers in the 1-day zebrafish embryo (Fig. 1D,F). A third monoclonal antibody, A4.1025, which reacts with a conserved epitope near the ATP-binding site of all striated muscle MyHC isoforms examined in a wide variety of species (Dan-Goor et al. 1990), detects both the BA-D5$^+$ and EB165$^+$ populations of cells (Fig. 1A,C). All three antibodies reacted with muscle fibers in a striated pattern typical of sarcomeric myosin and Western analysis of 24-hr zebrafish extracts separated by SDS-PAGE demonstrated that all three antibodies detect protein bands at or just under M_r 200,000, the size of MyHC isoforms (Fig. 1G). Therefore, these anti-MyHC antibodies distinguish slow and fast differentiated muscle cells in the zebrafish embryo.

Slow muscle differentiates before fast muscle in zebrafish embryos

We determined the timing and location of slow and fast muscle differentiation throughout zebrafish somite de-velopment. New somites separate from the presomitic paraxial mesoderm in an anterior to posterior order about every half hour between 10.5 and 26 hrs of development at 28°C (Westerfield 1995). We observed that MyHC$^+$ adaxial cells appeared on each side of the notochord in an anterior-to-posterior order in each somite as it formed (Fig. 2A). Most, if not all, adaxial MyHC$^+$ cells also express slow MyHC (Fig. 2B). Fast MyHC was undetectable in 15 somite embryos (Fig. 2C). Therefore, the first population of muscle cells to differentiate in the zebrafish somite are the adaxial cells, and these cells express slow, but not fast, characteristics from their inception.

A recent study by Devoto et al. (1996) has elegantly shown that the differentiated adaxial cells of somites 16–20 in the gut extension region of zebrafish embryos migrate laterally through the somite 3–4 hr postsomitogenesis. Consistent with the results of Devoto et al. (1996), we find that before the 20-somite stage, adaxial slow MyHC$^+$ cells in all somites remain medial, but that the adaxial slow MyHC$^+$ cells of older somites appear to spread dorsally around the sides of the neural tube and ventrally past the hypochord to form a single layer of medial cells. At about the 20-somite stage the adaxial slow MyHC$^+$ cells of the most anterior somites appear to
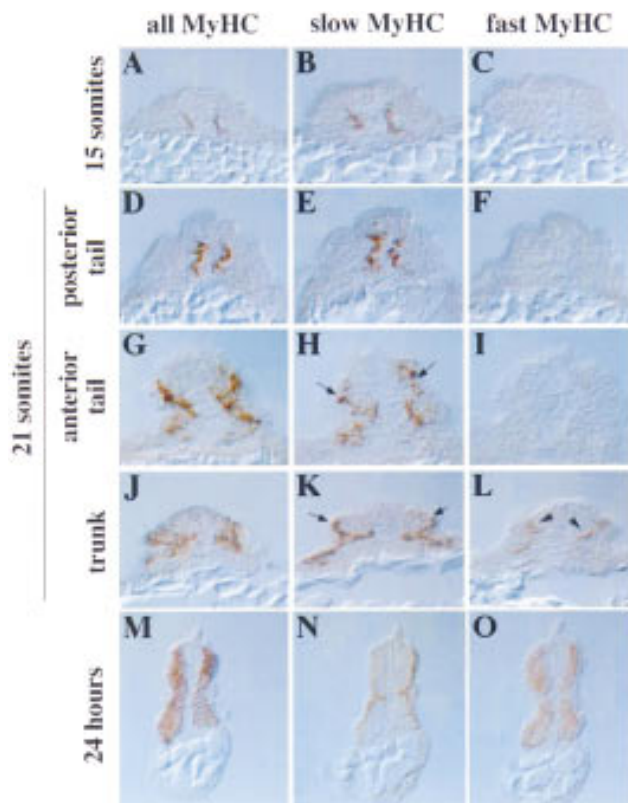
Figure 2. Slow muscle differentiates before fast in zebrafish embryos. (A–C) Serial transverse cryosections of a methanol-fixed 15-somite embryo show that muscle first differentiates next to the notochord (A), and expresses slow MyHC (B). Fast MyHC (C) is undetectable at any level of the embryo. (D–L) At the 21-somite stage, a developmental progression exists along the anteroposterior axis. Posteriorly, slow muscle cells are still medial (D,E) and fast MyHC is undetectable (F). However, more anterior sections of the same embryo show loss of slow MyHC⁺ cells medially and their appearance laterally (H,K; arrows). Somite cells medial to slow MyHC⁺ cells initiate differentiation (G,J) and fast MyHC expression (L; arrowheads). Therefore, medial fast muscle differentiates slightly before lateral in each individual somite. (M–O) By 24 hr, the slow muscle cells are aligned in a layer one cell thick at the lateral margin (N), and the remaining medial muscle expresses fast MyHC (O).

migrate laterally, through the undifferentiated somitic mesoderm. Although it is possible that this apparent migration represents a wave of fiber type conversion, we think this unlikely from the earlier findings of Devoto et al. (1996) that early adaxial cells migrate and form slow muscle. The wave of migration sweeps rapidly along the embryo from anterior to posterior so that by the 21-somite stage, slow muscle cells of the anterior somites are located at the lateral edge of the somite under the epidermis (Fig. 2J,K), whereas slow muscle cells of mid-body somites are found in the center of the somite (Fig. 2G,H) and the most posterior slow muscle cells, are still in the adaxial position (Fig. 2D,E). During the lateral migration of slow muscle cells the differentiation of fast muscle cells commences (Fig. 2F,I,L). No differentiated fast

muscle was observed lateral to migrating slow muscle cells. However, strikingly, fast muscle cells are detected medial to the slow muscle cells immediately after the migratory period in each somite (Fig. 2, cf. I and L). By the 26-somite stage, all slow muscle cells in somites 1–21 have migrated laterally (Fig. 2M,N; data not shown). At this stage fast muscle fills the medial bulk of the somite (Fig. 2O). Therefore, the differentiation of a distinct class of fast muscle cells rapidly succeeds the migration of the slow muscle cells past undifferentiated somitic cells.

Notochord defects correlate with lack of slow muscle differentiation

The formation of slow muscle next to notochord suggests that a notochord-derived signal induces slow muscle cells. To test this hypothesis we examined two mutant zebrafish strains that are defective in distinct stages of notochord development. Severely affected *bozozok* (*boz^{i2}*) fish do not have visible notochord, lack the notochord and floor plate marker Shh, and completely lack differentiated slow muscle (Fig. 3A–C). At 24 hr of development, when in wild-type embryos adaxial cells have differentiated, migrated, and express slow MyHC in all somites, no slow MyHC is detected in trunk or tail regions of *boz* embryos that lack notochord (six of six embryos sectioned; Fig. 3B), although unaffected sibling embryos appear wild type (data not shown). Mutation of the *boz* gene does not prevent muscle differentiation per se, because a single fused somite of differentiated muscle is present beneath the neural tube, and this expresses fast MyHC (Fig. 3A,C). Normal *boz* function is required for formation of slow muscle, rather than maintenance, as both severely and more mildly affected embryos from a *boz* heterozygote cross failed to express detectable slow MyHC at the 15-somite stage, whereas morphologically normal siblings showed normal slow MyHC expression (data not shown). Therefore, the absence of notochord in the *boz* mutant is accompanied by the specific loss of slow muscle.

Although *boz* function is required for notochord formation, it is possible that the wild-type gene might also be required in paraxial mesoderm to permit differentiation of slow muscle cells. We therefore examined *ntl* mutant embryos in which midline mesodermal cells are present but fail to differentiate into mature notochord cells. Previous studies have shown that *ntl* embryos also lack muscle pioneer cells, a subpopulation of the adaxial cells (Halpern et al. 1993). *ntl* is the zebrafish homolog of the *Brachyury* transcription factor and is expressed in notochord but not in adaxial cells at the time of their differentiation and therefore muscle defects are unlikely to be attributable to a cell-autonomous action of *ntl* in paraxial mesoderm (Schulte-Merker et al. 1994). In *ntl^{b160}* embryos, notochord precursors are present in anterior regions but absent posteriorly in the region beyond the yolk tube, which is severely truncated (Halpern et al. 1993; Odenthal et al. 1996; Fig. 3P). We examined *ntl^{b160}* fish for slow MyHC expression anticipating that the loss
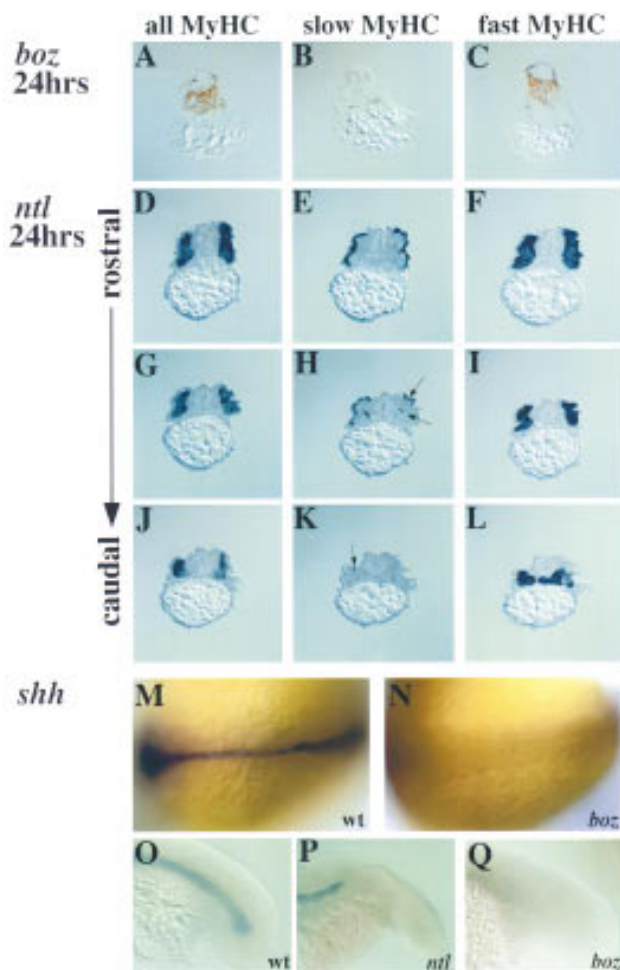
Figure 3. Severe midline defects correlate with loss of slow muscle formation. (A–C) Serial transverse sections of severely affected 24-hr *boz* embryos, which have no axial mesoderm from gastrulation, have no detectable slow MyHC (*B*). A single fused somite (*A*) expressing fast MyHC (*C*) extends under the neural tube (cf. Fig. 2, M–O). (D–L) Successive transverse sections of a 24-hr *ntl^b160* embryo. Anterior tail shows normal distribution of muscle fiber types (D–F), except for the lack of muscle pioneers at the dorsoventral midline (*E*). More posteriorly (G–I), slow cells are missing or migrate abnormally (arrows in *H,K*). Fast MyHC appeared unaffected (*F,I,L*). (M–Q) Whole-mount in situ mRNA hybridization for *shh* at 15 somites (M–O,Q) or 24 hr (*P*). Notochord and floorplate *shh* expression is completely absent in severe *boz* embryos (*N,Q*), compared with wild type (*M,O*). In contrast, only the most posterior *shh* expression is lacking in *ntl* mutants (*P*). (M,N) Dorsal views of the tail; (O–Q) lateral views of the tail.

of notochordal maturation might prevent slow muscle formation. Despite the absence of muscle pioneer cells at the dorsoventral midline, slow and fast muscle in anterior regions of *ntl* embryos appeared normal (Fig. 3D–F). Therefore, mature notochord is not required for slow muscle differentiation. However, more posterior regions of *ntl* embryos, in which axial mesoderm defects are more severe (Halpern et al. 1993), showed reduced slow

muscle formation and aberrant positioning (Fig. 3G–I). In rare embryos (1/16 serially sectioned) a complete absence of slow muscle was observed in the most posterior somite at 24 hr of development, even though extensive differentiated fast muscle was present (Fig. 3J–L). The remaining *ntl* embryos (15/16) showed regional slow deficits. Therefore, *ntl* mutant fish demonstrate that although mature notochord is not necessary for slow muscle formation, severe defects in notochord establishment in the tail correlate with loss of slow muscle differentiation.

Shh induces ectopic slow muscle differentiation

Examination of the muscle phenotype of *boz* and *ntl* mutant embryos suggested that notochord-derived signals may determine the slow muscle fate, reminiscent of the induction of floorplate and motoneurons by notochord-derived Shh protein (Ericson et al. 1996) and of muscle pioneer cells by notochord-derived hedgehogs (Currie and Ingham 1996). Consistent with this, *shh* mRNA is absent from those regions of both *boz* and *ntl* embryos that show defects in slow muscle formation (Fig. 3M–Q). To test the possibility that Shh might be a notochord-derived inducer of the slow muscle fate, we injected *shh* mRNA into two- or four-cell zebrafish embryos to create animals chimeric for *shh* overexpressing cells. Such injections lead to an easily detectable reduced retina phenotype (Krauss et al. 1993). In animals affected for retinal development, we observed an induction of slow MyHC expression across the entire width of the somite in each of 12 serially-sectioned 24-hr embryos (Fig. 4A–C). Strikingly, this expansion occurs at the expense of differentiated fast muscle (Fig. 4C). In those animals in which mosaic segregation of *shh* mRNA causes partial slow muscle induction, residual fast muscle is observed in regions not expressing slow MyHC (data not shown). When the same experiment was repeated using an equivalent amount of *echidna hedgehog* (*ehh*) mRNA, no defect was detected in any part of 10 embryos serially sectioned (data not shown). Therefore, Shh is a notochord-derived signal capable of inducing slow muscle at the expense of fast.

The wholesale conversion of large areas of somite to slow muscle by Shh has two possible explanations. Shh could induce somitic cells to differentiate as slow muscle prematurely. Alternatively, Shh might not affect the decision of when to differentiate, but simply determine what type of muscle is formed. To address this issue, we examined the effect of ectopic Shh on earlier stage zebrafish embryos. In 15-somite zebrafish embryos, Shh induces a wide region of ectopic lateral differentiated muscle within the somite (46/53 unselected injected embryos, Fig. 4G,H). Ectopic slow muscle differentiation occurred without premature induction of fast muscle tissue (Fig. 4D–F). The premature differentiation of lateral muscle tissue suggested that Shh might induce presomitic mesoderm to differentiate early. However, premature slow muscle differentiation before the normal time of adaxial cell differentiation was not observed in
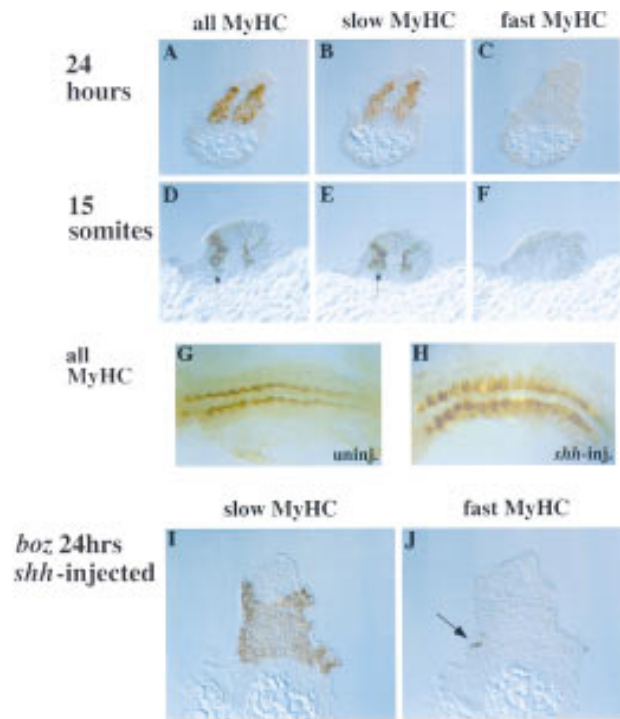
Figure 4. Ectopic overexpression of *shh* induces extra slow muscle cells in the myotome of wild-type and mutant embryos. (*A–C*) *shh* mRNA was microinjected into the zebrafish blastulae at the 2- or 4-cell stage and 24-hr embryos examined in transverse serial cryosections. All differentiated cells in both myotomes contained slow MyHC (*A,B*) and did not express fast MyHC (*C*). Some injected embryos showed ectopic slow MyHC and fast MyHC suppression on only one side, probably caused by mosaic expression of injected mRNA (data not shown). (*D–H*) Embryos injected with *shh* mRNA were analyzed at the 15-somite stage by whole-mount or cryosection immunohistochemistry. Compared with wild type (*G* and Fig. 2A), injected embryos showed bilateral (*H*) or unilateral (*D,* arrow) expansion of muscle differentiation. Ectopic muscle contained slow (*E,* arrow), but not fast, MyHC (*F*). Note that although ectopic lateral muscle is present in the anterior portion of *shh*-injected embryos (*H, left*), premature differentiation is not observed in presomitic mesoderm (*H, right*). (*I,J*) Embryos from *boz* mutant crosses were injected with *shh* mRNA and severely affected *boz* embryos (lacking eyes and notochord), were examined by serial cryosection immunohistochemistry. Widespread slow MyHC rescue (*I*) and supression of fast MyHC (*J*) was observed.

either presomitic mesoderm of any of 36 embryos examined at the 15-somite stage (Fig. 4H) or in any region of 22 embryos at tailbud stage (data not shown). Therefore, the earliest time somitic cells are competent to become slow muscle in response to Shh is when adaxial cells normally differentiate. However, at this stage, cells in all regions of the somite become competent.

The ability of Shh to induce slow muscle is consistent with the lack of slow muscle in regions of zebrafish mutants that lack midline *shh* expression. This correlation suggests strongly that the reason for the lack of slow muscle in the *boz*[i2] and the tail of *ntl*[b160] mutants is lack

of notochord-derived Shh (Concordet et al. 1996). However, the *boz* gene has not been cloned, so its expression is unknown, and the *ntl* gene is expressed transiently in presomitic mesoderm, as well as in notochord (Odenthal et al. 1996). This raises the possibility that the lack of slow muscle in these mutants reflects a need for a cell autonomous action of the respective genes in paraxial mesoderm. To address this issue, we overexpressed *shh* in embryos from *boz*[i2] mutant crosses and examined the resultant animals for slow MyHC expression. Five out of six severely affected *boz* mutants injected with *shh* mRNA showed induction of slow MyHC, and suppression of fast MyHC (Fig. 4I,J). Therefore, the *boz*[i2] mutation does not affect the ability of somitic tissue to respond to Shh and form slow muscle. Moreover, even in the complete absence of notochord Shh is sufficient for the formation of slow muscle.

A limited source of Shh is sufficient to rescue slow muscle

One limitation of overexpression of Shh by mRNA injection is that ectopic Shh is expressed in regions of the animal never normally exposed to Shh and at above normal physiological levels. This might perturb signals necessary for muscle development from other embryonic tissues. To examine slow muscle differentiation in response to localized lower levels of *shh* expression we took advantage of the *floating head* (*flh*) mutation. Animals homozygous for *flh* are defective in notochord maturation because of mutation in a homeobox-containing gene expressed chiefly in the notochord (Halpern et al. 1995; Talbot et al. 1995) and, like *ntl* embryos, lack notochords and muscle pioneers. However, unlike *ntl*, *flh* exhibits transdifferentiation of notochord tissue into muscle (Halpern et al. 1995). We examined *flh* embryos for muscle differentiation and found that it occurs in an altered location. Cells in the embryonic midline, not those in the adaxial position, are the first to differentiate in *flh* embryos (Fig. 5A,B). This differentiation is immediately beneath the presumptive floorplate that expresses Shh sporadically (Fig. 5C). Despite the unusual location of these muscle cells, they express slow, but not fast, MyHC (Fig. 5D–F), spread dorsally around the neural tube and ventrally in the midline, and appear able to undergo lateral migration to take up a normal position beneath the ectoderm by 24 hr of development (Fig. 5G–I). Therefore, in *flh* embryos, apparently normal slow muscle cells differentiate beneath the residual floorplate—the sole remaining location where somitic mesoderm abuts *shh*-expressing tissue.

The discontinuous location of *shh*-expressing cells in the floorplate of *flh* mutants (Fig. 5C) allows an examination of the relationship between the location of Shh and slow muscle differentiation. At the posterior limit of MyHC-containing cells in *flh* we found no correlation between the location of remaining floorplate *shh* expression and medial slow myoblast differentiation. Muscle differentiates both immediately beneath and between islands of *shh*-expression (Fig. 5C). This data suggested
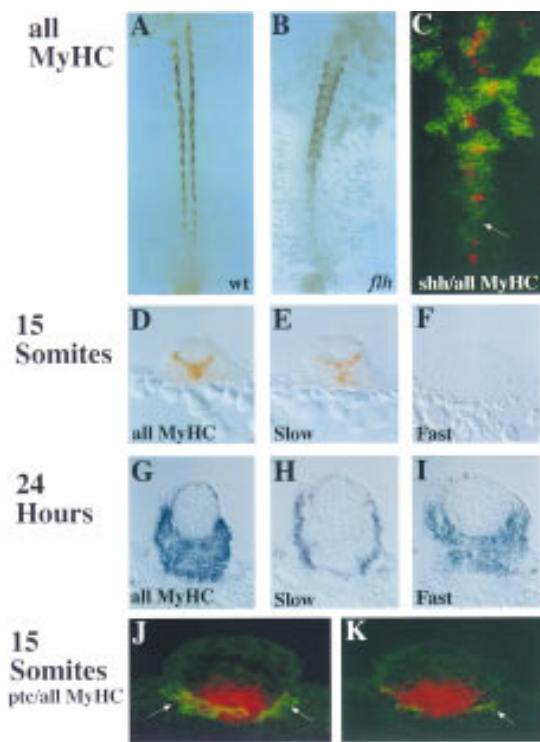
Figure 5. Slow muscle forms beneath the floorplate in *flh* embryos. (A–C) Differentiated muscle visualized by MyHC-staining in whole-mount 15-somite wild-type (A) or *flh* (B,C) embryos observed after flat-mounting. Dual labeling for *shh* mRNA (red; C) and MyHC (green; C) confirmed that the most posterior differentiated muscle was at the midline (arrow, C). (D–I) Transverse cryosections show that differentiated muscle in *flh* midline mesoderm expresses slow but not fast MyHC at 15 somites (D–F). By 24 hr, *flh* embryos have a single fused myotome (G), with lateral slow and medial fast muscle (H,I). Despite the ectopic location of formation of slow muscle cells in the midline the slow muscle cells appear to migrate normally so that the only detectable residual slow muscle defect is lack of muscle pioneers. (J,K) Dual labeling for MyHC (green) and *ptc1* mRNA (red) in the two most posterior MyHC-containing serial sections of a 15-somite *flh* embryo. Newly differentiating muscle cells close to the midline do not always express detectable *ptc1* (arrows), even though *ptc1* is expressed abundantly in the mesoderm underlying residual floor plate (K).

that terminal differentiation of slow muscle is not induced directly by Shh. Further evidence that muscle differentiation, per se, is not induced by Shh came from examining the up-regulation of *patched1* (*ptc1*) mRNA in *flh* mutant embryos. *ptc1*, a zebrafish homolog of *Drosophila patched,* is a Shh receptor (Stone et al. 1996), and is up-regulated adjacent to residual *shh* expression in *flh* embryos both at somitic and presomitic anteroposterior positions (Concordet et al. 1996). Therefore, mesodermal cells are first exposed to Shh long before muscle differentiation commences. Moreover, even the most recently differentiated muscle cells in *flh* embryos frequently do not express high levels of *ptc1* mRNA, despite adjacent mesoderm expressing *ptc1* abundantly (Fig. 5J,K). There-

fore, although Shh induces *ptc1* locally along the entire length of the *flh* embryo, there is a delay after Shh exposure before the appearance of differentiated slow muscle cells. In addition, by the time slow muscle differentiates, any spatial correlation between *shh* expression and myogenic cells has been lost. Taken together, these data suggest that Shh may initiate slow myoblast formation, but that continued exposure is not required to trigger the terminal differentiation of slow muscle fibers.

Discussion

Shh and slow muscle induction

Several lines of evidence show that adaxial slow muscle formation in zebrafish embryos is controlled by Shh. First, slow muscle differentiation occurs next to the notochord, which expresses *shh.* This observation confirms and extends the results of Devoto et al. (1996), who observed that adaxial cells give rise to slow muscle markers after they migrate laterally through the somite. Our data show that adaxial cells are already determined to form slow muscle as soon as they differentiate into skeletal muscle myosin-expressing myocytes. Second, a lack of slow muscle correlates with a lack of *shh* expression in mutant fish. Third, ectopic expression of *shh* can induce conversion of most, if not all, somitic cells to slow muscle, at the expense of fast muscle. Fourth, even in the absence of notochord, injection of *shh* mRNA can rescue formation of slow muscle. Fifth, in the absence of the normal Shh signal from notochord, a localized source of Shh correlates with induction of ectopic slow muscle cells, which then migrate in a similar fashion to slow muscle cells in wild-type embryos. These data are supported by the wild-type expression of a Shh receptor, *ptc1,* that is up-regulated in adaxial cells within presomitic mesoderm indicating that these cells are responding to hedgehog (hh) signaling (Concordet et al. 1996). Taken together, these findings make a strong case for notochord-derived Shh being the normal inducer of the differentiated slow adaxial muscle cell fate in the zebrafish.

Shh induces adaxial slow myoblasts

How might Shh induce the slow muscle fate in zebrafish? Muscle is formed in two steps—mesodermal commitment to the proliferative myoblast, followed by terminal differentiation into the postmitotic muscle fiber. Several lines of evidence suggest that Shh is responsible for induction of slow muscle precursor cells, rather than the terminal differentiation of slow muscle, per se. First, the muscle-specific transcription factor *myoD* is initially detectable in adaxial precursors located adjacent to *shh*-expressing cells within the embryonic shield several hours before their terminal differentiation at around the time of somitogenesis (Weinberg et al. 1996). This expression of *myoD* before terminal differentiation is also detected in adaxial cells at later stages when posterior somites arise from the tail bud. Second, zebrafish mutants like *boz* and *ntl* that lack slow muscle, also lack

the early adaxial *myoD* expression, and this correlates with a lack of axial Shh (Fig. 3; Concordet et al. 1996; Odenthal et al. 1996; Weinberg et al. 1996; Schier et al. 1997). Third, Shh signaling can induce premature *myoD* in lateral presomitic cells (Concordet et al. 1996; Hammerschmidt et al. 1996; Weinberg et al. 1996). We show that these ectopic *myoD*-expressing cells in lateral somites have other features, such as the direction and timing of their differentiation and sensitivity to additional hh signals (Currie and Ingham 1996), suggesting that the terminal differentiation of these cells into slow muscle is prefigured at the myoblast level. Fourth, our examination of the *flh* mutant suggests that adaxial myoblasts differentiate into slow muscle fibers independent of their proximity to residual *shh*-expressing floor plate cells, and independent of their exposure to Shh during the period of terminal differentiation, as assayed by *ptc1* expression (Concordet et al. 1996; Marigo and Tabin 1996). Therefore, at early stages MyoD may mark cells that, although not yet differentiated, have become committed to a slow myoblast lineage.

Previous data have suggested that the combined action of notochord-derived Shh and Ehh induces zebrafish muscle pioneer subset of the adaxial slow muscle cells (Currie and Ingham 1996). The data in the present paper demonstrate that Ehh is not required for production of the nonpioneer adaxial slow muscle cells. Ehh is not expressed in notochord of *ntl*[b160] (Currie and Ingham 1996), yet nonpioneer slow muscle cells form and migrate normally in the anterior of *ntl*[b160] embryos where Shh alone is expressed (Fig. 3). Similarly, in *flh* mutants, which lack notochord and *ehh* expression (Currie and Ingham 1996), apparently normal nonpioneer adaxial cells are formed ectopically close to residual floorplate

Shh. Moreover, Ehh does not appear able to substitute for Shh in the induction of nonpioneer adaxial cells as injection of *ehh* mRNA into wild-type embryos did not induce ectopic slow MyHC. These data support the hypothesis that in vivo Shh and Ehh serve distinct roles.

The finding that Shh induces slow myoblasts suggests a new view of the steps of muscle differentiation that contrasts with the traditional model in which somitic cells first become myoblasts and only subsequently specialize into one particular myoblast subclass. We suggest, as summarized in Figure 6, that the decision whether to form one type of muscle or another is made concurrently with myoblast commitment to the muscle lineage. This scheme concurs with conclusions from analysis of the *you*-type zebrafish mutants (van Eeden et al. 1996). Such a view also fits well with studies in *Drosophila* demonstrating that distinct extracellular signals serve to commit each founder myoblast to a particular muscle type (Baylies et al. 1995). However, it is possible that presomitic cells could be committed to myogenesis before *myoD* expression. Although MyoD is the first MRF to be expressed in birds, Myf-5 is the earliest MRF to appear at high levels in mammalian somites (Ott et al. 1991), and Pax-3 can induce myogenesis (Maroto et al. 1997; Tajbakhsh et al. 1997). Furthermore, whether all myoblasts are committed to form particular types of muscle from their inception is unclear. Whatever the case, our data show that Shh induces adaxial myoblasts that adopt a slow muscle fate.

Zebrafish fast muscle formation

Shh is not necessary for fast muscle formation. *boz* fish that lack Shh produce abundant fast muscle throughout

Figure 6. Three phases of zebrafish muscle development. A model in which presomitic mesoderm gains competence to respond to Shh early in development in wild-type zebrafish. The result of exposure to notochord-derived Shh at this stage is a slow myoblast phenotype involving *myoD* expression and up-regulation of *ptc1* (orange). However, a second event, either intrinsically timed within the slow myoblast, or in the form of a signal from outside of the somite (indicated by ? on the diagram, but unlikely to be either Shh or notochord-derived), is required for the slow myoblast to differentiate into a fiber (red). Simultaneously, the MyoD-positive myoblast state in lateral somitic cells arises independent of notochord-derived signals (yellow). Later, adaxial slow fiber migration and concurrent differentiation of lateral myoblasts into fast muscle (green) is timed by a third event(s). Fast fiber differentiation is independent of slow fiber migration, although the converse may not be true.
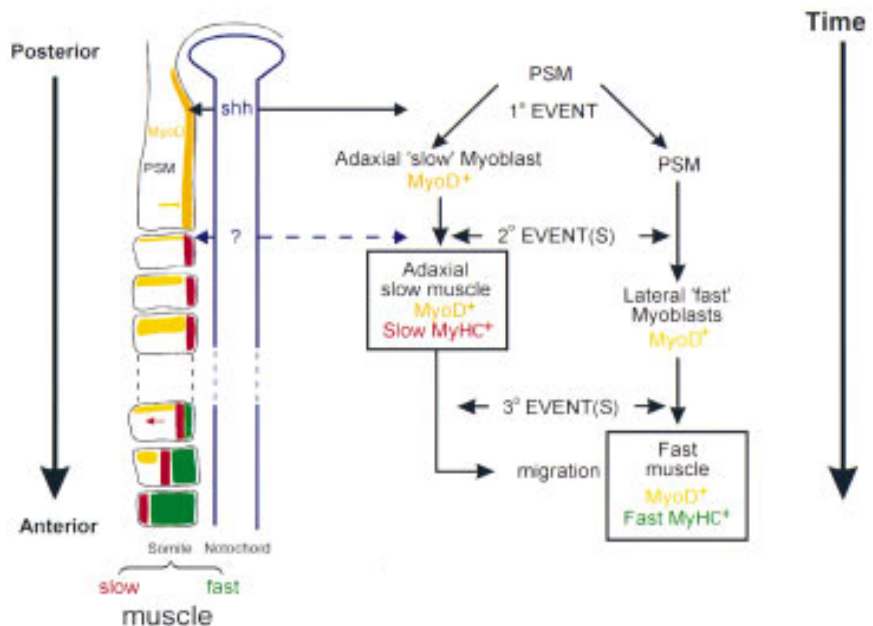
the somite. Moreover, the normal *myoD*-expressing myoblasts stripes across the posterior border of the somite form at the normal time just before somitogenesis in embryos that lack *shh* expression (Odenthal et al. 1996). Therefore, in zebrafish, MyoD may mark commitment to a myoblast fate irrespective of the type of myoblast formed. We find that cells that are initially lateral within the somite differentiate into fast muscle. They may be committed to formation of fast muscle from the inception of *myoD* expression.

Timing of myoblast differentiation

We show that ectopic Shh can induce premature muscle differentiation in the lateral somite. However, premature differentiation was slow, rather than fast, and was only observed at the normal time of slow adaxial cell differentiation. Therefore, no somite cells are competent to differentiate in response to Shh until around the time of somitogenesis, even though *myoD* is expressed earlier. This may explain why slow muscle does not appear earlier in development even though *shh* is expressed in the developing notochord from gastrulation onwards (Krauss et al. 1993), and is presumably secreted because *ptc1,* a marker of Shh exposure, is expressed highly in adjacent presomitic cells (Concordet et al. 1996). Two alternative models could explain the delay between MyoD and slow myosin expression (Fig. 6). In one model, the delay is caused by an intrinsically timed maturation of the somitic cells. Although cell division is not extensive in zebrafish somites (Kimmel and Warga 1987), Shh might induce myoblasts committed to division followed by differentiation as it can be a somitic mitogen (Fan et al. 1995). Mammalian myoblasts show such behavior in vitro (Quinn et al. 1985), which is reminiscent of the induction of division followed by terminal differentiation in *Drosophila* lamina ganglia neurons in response to retinal neuron-derived hh (Huang and Kunes 1996). Alternatively, in the second model, extracellular signals may control terminal differentiation. In amniotes, other signals can cooperate with Shh to regulate myogenesis (Munsterberg et al. 1995; Stern et al. 1995; Pourquie et al. 1996), and a variety of growth factors repress myoblast differentiation in culture. Ventral axial structures are unlikely sources of such signals as Shh is sufficient to induce slow MyHC in *boz* embryos that fail to form notochord or floor plate. Regardless of the mechanism by which the timing of terminal adaxial slow muscle differentiation is controlled, our data show that similar mechanisms can operate in the lateral somite to control ectopic slow muscle differentiation in response to Shh.

Evolutionary conservation of muscle patterning

We found that in fish embryos the first skeletal muscle fibers to form are slow from the time of their inception. Later, a second wave of fibers, which ultimately constitute the majority of all fibers, differentiate as fast muscle. In amniote limbs muscle fibers also form in two

waves, an early primary population that express slow (and embryonic) myosin and later secondary cells that, forming in close association with primary fibers, express fast (and embryonic) myosin from their inception (Kelly and Rubinstein 1980; Vivarelli et al. 1988; Cho et al. 1994). We suspect that zebrafish embryos may also express an embryonic myosin in both slow and fast fibers as the immunoreaction with our all-myosin antibody was stronger than with the specific slow and fast antibodies. Moreover, both adaxial and nonadaxial cells react from their inception with an antibody that detects embryonic myosin (Devoto et al. 1996). These analogies suggest that adaxial and nonadaxial somitic muscle cells in the zebrafish may be evolutionary homologs of amniote primary and secondary muscle fiber generations. Amniote secondary fibers form overlying the neuromuscular junctions of primary fibers and it has been suggested that signals from the forming neuromuscular junction region may be required to initiate secondary fiber formation (Duxson et al. 1989). This is not the case in the zebrafish as absence of differentiated slow primary fibers does not prevent differentiation of fast muscle despite the striking correlation between the lateral migration of slow fibers and the differentiation of fast fibers. The converse relationship, that fast fiber differentiation might cause slow fiber migration, remains a possibility. Nevertheless, the close similarities between fish and amniote fiber generation suggest that the common ancestor had two steps of muscle patterning: early fibers being slow and later fast.

There are further analogies between amniote and fish myogenesis. Amniote primary fibers are of several distinct fiber types that prefigure later muscle characteristics (Crow and Stockdale 1986), even though all express some form of slow MyHC (Kelly and Rubinstein 1980; Vivarelli et al. 1988; Page et al. 1992; Hughes et al. 1993). Slow adaxial cells in the zebrafish are also composed of two subpopulations, the muscle pioneer cells which express engrailed, and the nonpioneer adaxials. Engrailed proteins also mark a subpopulation of muscle cells in the jaw muscle of the zebrafish (Hatta et al. 1990). The data reported in the present paper, together with the previous findings that a second notochord-derived signal (Halpern et al. 1993), provided by Ehh (Currie and Ingham 1996), is responsible for regulating the formation of muscle pioneer cells, suggest that hh signaling molecules may regulate the diversity of muscle fiber types formed in the early fish embryo. *Banded hedgehog* is also expressed in particular regions of the *Xenopus* somite (Ekker et al. 1995). Whether similar signals control muscle patterning in amniotes remains to be determined.

Hedgehogs and vertebrate myogenesis

Secretion of Shh from notochord has been shown to induce floorplate markers in anterior (although not posterior) zebrafish central nervous system (CNS) and both floorplate and motoneurons in amniote neural tube (Echelard et al. 1993; Krauss et al. 1993; Roelink et al. 1994;

Ericson et al. 1996). The role of Shh in somite patterning has been less clear. In amniotes, in which much of the somite becomes sclerotome, either ectopic notochord or *shh*-expressing cells can induce extra sclerotome at the expense of dermomyotome markers (Fan and Tessier-Lavigne 1994). Conversely, $shh^{-/-}$ mice have deficits in sclerotomal derivatives (Chiang et al. 1996). On the other hand, in both chick and zebrafish, overexpression of *shh* induces ectopic *myoD* expression, suggesting a myogenic action (Johnson et al. 1994; Concordet et al. 1996; Weinberg et al. 1996). Moreover, $shh^{-/-}$ mice show defects in medial muscle formation (Chiang et al. 1996) and notochord can induce avian myogenesis (Pownall et al. 1996). So Shh may regulate formation of both ventral and more dorsal somitic tissues. Action of Shh at distinct concentrations or times (Ericson et al. 1996), or in collaboration with other factors (Munsterberg et al. 1995; Stern et al. 1995; Pourquie et al. 1996), could determine the outcome of Shh signaling.

Induction of distinct myoblast types and the subsequent control of their terminal differentiation may account for the numerous signals capable of influencing somite myogenesis. If equivalents of Shh-dependent adaxial cells exist in amniotes, we would expect that particular muscle markers are not distributed uniformly between distinct muscle cell types in the developing dermomyotome. In amniotes, MRFs are the earliest known definitive myogenic markers. Expression of at least one MRF is obligatory for myogenesis in mice (Rudnicki et al. 1993). MRFs are expressed at low levels in presomitic mesoderm, which has the capacity to form muscle in dissociated cell culture (George-Weinstein et al. 1994; Lin-Jones and Hauschka 1996). However, two myoblast populations arise with distinct temporal and spatial patterns within the dermomyotome: The first initially expresses *myf-5* in medial regions and the second *myoD* in lateral regions (Cossu et al. 1996a; Maroto et al. 1997; Tajbakhsh et al. 1997). That $shh^{-/-}$ mice have reduced expression of medial *myf-5* but no detectable change in lateral *myoD* expression (Chiang et al. 1996) suggests a role for Shh in induction of the medial population. Inhibitory signals, such as BMP4 (Fan and Tessier-Lavigne 1994; Cossu et al. 1996b; Pourquie et al. 1996), may function in vivo to suppress overt myogenic phenotypes in the lateral compartment that generates limb and body wall muscle and may have no homologous process in most zebrafish somites. So generation of further diversity within the dorsomedial myogenic compartment could be a role of Shh in amniote myogenesis. Distinct populations of slow and fast fibers may be present in amniote myotome (Dhoot 1994). In this paper, we have shown that in zebrafish, Shh regulates formation of myotomal slow muscle. Much slow muscle in amniote limbs is located near developing bone that expresses *indian hedgehog* (Bitgood and McMahon 1995; Vortkamp et al. 1996). Moreover, motoneurons, which strongly influence muscle development, can express *shh* (Bitgood and McMahon 1995; Stone et al. 1996), raising the possibility that diverse hh proteins may regulate muscle fiber diversification.

Materials and methods

Zebrafish lines and maintenance

Wild-type and heterozygote mutant breeding fish were maintained at 28.5°C on a 14-hr/10-hr light cycle. We obtained flh^{n1} from the University of Newcastle-upon-Tyne, ntl^{b160} from the University of Oregon, and boz^{i2} was isolated in the Ingham laboratory (P.D. Currie., T. Schilling, G. Bergemann, and P.W. Ingham, unpubl.). boz^{i2} fish exhibit a variable phenotype with defects ranging from reduced notochords to a severe lack of axial mesoderm at all rostro–caudal levels. Of 142 progeny of a heterozygous cross examined at F_2–F_4, 20 (13%) showed complete absence of eyes and notochord. A further 25 (17%) showed a partial phenotype with variable eyes and the anterior half of the notochord missing. These, and a number of other aspects of the phenotype, are strongly reminiscent of *boz* mutant fish (Solnica-Krezel et al. 1996). Complementation analysis by a cross of heterozygous boz^{i2} with boz^{m168} has shown reduced eyes and notochord in 3 out of 30 progeny. We therefore tentatively conclude that these genes are allelic. However, because of the incomplete penetrance of *boz,* definitive demonstration of allelism awaits the mapping of the mutation. Embryos were collected by natural spawning and staged by anatomical markers according to Westerfield (1995). *Prim-5* stage embryos are referred to as 24 hr.

RNA injection

RNA injections were performed as described (Currie and Ingham 1996).

Immunohistochemistry

The slow and fast MyHC antigens are destroyed by aldehyde fixatives, so embryos were fixed by incubating for 5 min each in graded methanols, rehydrated in 0.1% Tween 20, serially cryosectioned, and stained. However, preservation of younger embryos was better after staining in whole-mount, followed by post-fixation in 4% paraformaldehyde for 4 hr at 4°C before cryosectioning. Primary monoclonal antibody supernatants of A4.1025 (Dan-Goor et al. 1990) and BA-D5 (Schiaffino et al. 1989) were diluted 1:10. EB165 monoclonal ascites was used at 1:5000 (Gardahaut et al. 1992). First antibodies were detected with biotin-conjugated horse-derived anti-mouse IgG (Vector), Vectastain ABC Elite Peroxidase kit (Vector), and visualized using 0.5 mg/ml of diaminobenzidine with (black stains) or without (brown stains) 0.03% $CoCl_2$ enhancement. Cryosections for dual immunofluorescence had IgG first antibodies detected with Cappell goat anti-mouse IgG (γ-specific) Texas red. After a mouse IgG block, biotinylated BA-D5, prepared using Pierce NHS-Biotin reagent, was detected with Dako streptavidin-FITC. Sections were mounted in 150 mg/ml of polyvinyl alcohol, 30% glycerol PBS with DABCO antifade, and photographed by confocal microscopy.

Western blots

Embryos were dechorionated, deyolked, and homogenized manually on ice for 10 min in 63 mM Tris-HCl (pH 6.8), 10% glycerol, 5% β-mercaptoethanol, 3.5% SDS, 0.2 mM PMSF, 0.5 µM aprotinin, and 0.5 µM leupeptin. Samples were microcentrifuged for 5 min at 4°C, 0.01% bromophenol blue added to the supernatant, the equivalent of 10 embryos run on each lane of a 7.5% acrylamide denaturing gel at 200 mV for 30 min, and electroblotted onto nitrocellulose (Amersham). Purified bovine cardiac myosin was a kind gift of Dr. John Sleep (The Randall

Institute, London, UK). Nitrocellulose strips were blocked in 5% milk powder PBS/Az overnight, washed, and incubated with A4.1025 (1:10), BA-D5 (1:10), F1.652 [1:10; Webster et al. 1988)], or EB165 (1:250) for 2 hr at room temperature. After washing, primary antibody was detected with horseradish peroxidase-conjugated sheep anti-mouse IgG F(ab)$_2$ and an ECL kit (Amersham).

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