

# Identification of separate slow and fast muscle precursor cells in vivo, prior to somite formation

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## SUMMARY

We have examined the development of specific muscle fiber types in zebrafish axial muscle by labeling myogenic precursor cells with vital fluorescent dyes and following their subsequent differentiation and fate. Two populations of muscle precursors, medial and lateral, can be distinguished in the segmental plate by position, morphology and gene expression. The medial cells, known as adaxial cells, are large, cuboidal cells adjacent to the notochord that express *myoD*. Surprisingly, after somite formation, they migrate radially away from the notochord, becoming a superficial layer of muscle cells. A subset of adaxial cells develop into *engrailed*-expressing muscle pioneers. Adaxial cells differentiate into slow muscle fibers of the adult fish.

We have named the lateral population of cells in the segmental plate, lateral presomitic cells. They are smaller, more irregularly shaped and separated from the notochord by adaxial cells; they do not express *myoD* until after somite formation. Lateral presomitic cells remain deep in the myotome and they differentiate into fast muscle fibers. Thus, slow and fast muscle fiber types in zebrafish axial muscle arise from distinct populations of cells in the segmental plate that develop in different cellular environments and display distinct behaviors.

Key words: axial muscle, in vivo, cell fate, zebrafish, muscle fiber type, muscle pioneer, cell migration

## INTRODUCTION

The properties and distributions of different fiber types within vertebrate muscles have been well characterized, however, the mechanisms that regulate the initial development of fiber type differences are poorly understood. Vertebrate skeletal muscles contain muscle fibers of several types, which can be broadly classified as slow or fast fibers, on the basis of differences in contraction speeds, metabolic activities and motoneuron innervation. The first muscle fibers form in the embryo when proliferative myogenic precursors, known as myoblasts, cease dividing, fuse into myotubes and begin to express muscle-specific structural proteins (for review, see Hauschka, 1994). These embryonic muscle fibers have intrinsic fiber type properties (Butler et al., 1982; Thornell et al., 1984; Crow and Stockdale, 1986; Harris et al., 1989; Fredette and Landmesser, 1991a,b; Hughes et al., 1993). Transplantation experiments and in vitro clonal analyses have demonstrated that fiber type identities arise very early. Individual embryonic myoblasts can generate muscle fibers of a specific type (Miller and Stockdale, 1986a,b; Van Swearingen and Lance-Jones, 1995). However, the positions of fiber-type-specific myogenic precursor cells within the embryo are unknown and, thus, it is still unclear which cellular interactions might regulate this muscle cell fate decision.

In adult zebrafish, as in most fish, slow and fast muscle fibers occupy distinct regions of the body muscle (for review, see Bone, 1978). Fast muscle fibers (also known as white muscle),

comprise the deep portion of the myotome, which makes up most of the trunk musculature (van Raamsdonk et al., 1982b). Slow muscle fibers (red muscle) are segregated into a wedge-shaped region of the myotome at the lateral end of the horizontal myoseptum, which separates the hypaxial and epaxial muscle. Intermediate muscle fibers (pink muscle) are located between the slow and fast muscle fibers, within the deep region. As in other vertebrates, slow muscle fibers are small, darkly colored, more heavily vascularized, and contain more lipid and mitochondria than the large, pale fast muscle fibers (Hoyle, 1983). A fascia separates the slow muscle fibers from the intermediate and fast muscle fibers (Waterman, 1969).

Previous work suggested two contradictory temporal sequences for the generation of different types of muscle fibers in fish. Ultrastructural and histochemical analyses in trout (Nag and Nursall, 1972; Proctor et al., 1980) and zebrafish (Waterman, 1969) implied that deep myoblasts differentiate first, forming fast fibers, and that superficial myoblasts later differentiate into slow muscle fibers. In contrast, antibody staining has led to the proposal that zebrafish embryonic muscle fibers express slow muscle properties initially, and that a subset of them subsequently differentiates into the fast muscle fibers of the adult (van Raamsdonk et al., 1978, 1982a). Thus, these experiments leave unresolved questions of which fiber type arises first, or whether there are specific fiber type precursors.

We have identified the precursors of slow and fast muscle fibers in vivo, by injecting vital fluorescent dyes into single cells

of the zebrafish segmental plate and examining their subsequent development. We characterized two distinct populations of muscle precursors: adaxial cells, which are morphologically identifiable cells adjacent to the notochord (Thisse et al., 1993), and cells lateral to them, which we have named lateral presomitic cells. We followed the behaviors of both cell types in live, developing embryos and determined the fiber types of the muscles that they generate. As in earlier studies, we found that the adaxial cells differentiate very early into muscle fibers (Waterman, 1969; van Raamsdonk et al., 1978; Felsenfeld et al., 1991). However, the adaxial cells exhibit a surprising behavior not previously described for other vertebrate muscle cells. Adaxial cells elongate to span the length of the somite and then migrate radially through the somite. Following migration, they form a monolayer of superficial muscle cells that later differentiate into slow muscle fibers. In contrast, lateral presomitic muscle cells remain within the deep portion of the myotome where they give rise to fast muscle fibers. Our studies show that distinct slow and fast muscle precursors can be identified by morphological features *in vivo*, in the segmental plate, and that these cells exhibit distinct behaviors during their differentiation.

## MATERIALS AND METHODS

### Animals

Embryos were obtained from the \*AB line at the University of Oregon zebrafish colony. Embryos were staged by hours (h) or days (d) post-fertilization at 28.5°C (Kimmel et al., 1995; available at World Wide Web address: 'http://zfish.uoregon.edu'). Chorions were removed with watchmaker's forceps and embryos were maintained in Ringer's solution (Westerfield, 1995). Living embryos were mounted in agar (Eisen et al., 1989) and viewed with Nomarski (DIC) optics. Older embryos were anesthetized in a 0.6 mM solution of tricaine methyl-sulfonate (Sigma) to inhibit movement during observation.

### Cell labeling, imaging and photography

Adaxial or lateral presomitic cells were labeled by intracellular injection with lysinated rhodamine or fluorescein dextran (3 or 10×10<sup>3</sup> Mr, Molecular Probes) as described previously (Raible et al., 1992). Cells were monitored using an intensified CCD camera and low light level fluorescence microscopy. Images were recorded using AxoVideo (Myers and Bastiani, 1991; available from Axon Instruments), on a Macintosh IICI and an optical memory disc recorder (Panasonic). White-light and fluorescent images were combined using Photoshop (Adobe). With the exception of the removal of obvious dust specks, all image enhancements in Photoshop were executed on the entire image. The orientation of images was reversed, where necessary, to maintain the same orientation in all figures (in side views anterior to the left and dorsal to the top, in dorsal views anterior to the top; in sections dorsal to the top).

### Antibodies

F59 is an IgG1 monoclonal antibody raised against chicken myosin (Crow and Stockdale, 1986). F59 is specific for the fast isoforms of myosin heavy chain in chicken and other species, it recognizes myosin heavy chain in goldfish and electric ray (Miller et al., 1989). S58 is an IgA monoclonal antibody raised against chicken myosin. S58 is specific for the slow isoforms of myosin heavy chain in chicken (Crow and Stockdale, 1986). The specific isoforms of myosin heavy chain recognized by S58 and F59 in zebrafish are unknown. Tissue culture supernatants of F59 and S58 antibodies were used at a dilution of 1:10. 12/101 is an IgG1 monoclonal antibody raised against regenerating

newt limb (Kintner and Brockes, 1984). 12/101 is thought to recognize a membrane protein of the sarcoplasmic reticulum in newt muscle (Griffin et al., 1987). Tissue culture supernatant was purchased from The Developmental Studies Hybridoma Bank and used at a dilution of 1:10. 4D9 is an IgG1 monoclonal antibody generated against the *Drosophila invected* homeodomain (Patel et al., 1989). 4D9 recognizes Engrailed proteins in zebrafish (Hatta et al., 1991a; Ekker et al., 1992). Purified antibody was diluted 1:50. zn-5 is an IgG1 monoclonal antibody raised against zebrafish membranes (Trevarrow et al., 1990). zn5 recognizes a variety of neuronal and muscle cell populations (Hatta et al., 1991a). Purified ascites fluid was diluted 1:500. Peroxidase-conjugated sheep anti-fluorescein antibody (Boehringer) was diluted 1:1000. Secondary antibodies were from the following sources: FITC-labeled goat anti-mouse IgA (Sigma), FITC-labeled goat anti-mouse IgG1 (Southern Biotechnology Associates, Inc.) and peroxidase-conjugated donkey anti-sheep IgG (Sigma), and were diluted according to manufacturer's suggestions.

### Immunostaining, histology and RNA in situ hybridization

Anesthetized embryos or larvae were fixed in 4% paraformaldehyde in 0.05 M NaPO<sub>4</sub> buffer, pH 7.4 (PB), for 2-16 hours. They were washed in the same buffer, frozen and sectioned on a cryostat as previously described (Westerfield, 1995). Antibody labeling was done either as described previously (Raible et al., 1992) or with the following modifications.

The sections were rehydrated in PBS-Tw (PBS, 0.1% Tween20), non-specific staining was then blocked with PBS-Tw-B-N (PBS, 0.1% Tween20, 2% BSA, 5% goat serum) for 5 minutes. Sections were incubated in primary antibodies diluted in PBS-Tw-B-N for 1-16 hours, washed 5 times in PBS-Tw for 3 minutes each, then blocked again in PBS-Tw-B-N for 5 minutes. Secondary antibodies appropriate for the isotype of the primary antibody were used. For immunofluorescence, sections were incubated for 30-60 minutes in fluorescently conjugated secondary antibody diluted in PBS-Tw-B-N. After washing as above, some sections were incubated in Hoechst 33258 (1-10 µg/ml) to visualize nuclei, then further washed. Sections were cover-slipped in glycerol or Vectashield (Vector Labs) and observed by epifluorescence microscopy. With peroxidase-conjugated antibodies, after incubating in secondary antibody, the sections were reacted with diaminobenzidine (0.5 mg/ml in PBS-Tw with 0.004% H<sub>2</sub>O<sub>2</sub>), cover-slipped and viewed with Nomarski optics. In some experiments (e.g. that shown in Fig. 8C,D), the stained section was photographed, the coverslip was then removed and further antibody staining carried out as above. Non-specific staining was defined as the staining present when the primary antibody was excluded from the protocol.

Whole-mount *in situ* RNA hybridization was performed as previously described (Püschel et al., 1992).

For histochemical staining, embryos were fixed in Bouin's. Epon sections, 7.5 µm thick, were stained with methylene blue, azure A and basic fuchsin according to standard procedures (Humphrey and Pittman, 1974).

## RESULTS

Trunk skeletal muscle is derived from the segmental plate, a pair of thickened bands of mesoderm that flank the notochord and underlie the presumptive spinal cord. The segmental plate gives rise to somites, paired blocks of mesoderm, in an anterior to posterior sequence. In zebrafish, somite formation begins at about 10 h, and ends at 24 h, with one pair of somites forming approximately every one-half hour (Hanneman and Westerfield, 1989). Shortly after somite formation, the first elongated muscle cells in zebrafish are found adjacent to the notochord

(Waterman, 1969). We identified and characterized the cells in the segmental plate that give rise to these first muscle cells, as well as those giving rise to later developing muscle cells.

### Adaxial and lateral presomitic cells in the segmental plate

Two populations of cells can be distinguished in the zebrafish segmental plate, based on differences in their positions, morphologies and expression of *snail1* (Thisse et al., 1993) and *myoD* (Weinberg et al., 1996). Adaxial cells are the most medial cells in the segmental plate. They can be identified in living embryos and in histological sections, as larger and more regularly shaped than the more lateral segmental plate cells (Fig. 1A,B). Three to five rows of adaxial cells form an epithelial-like monolayer flanking each side of the notochord, extending from the most ventral-medial edge of the segmental plate to the region adjacent to the dorsal-most part of the notochord (Fig. 1B). At this stage, they are the only cells expressing abundant levels of *myoD* (Fig. 1C,D). They also express other muscle-specific genes, including *myogenin* and *tropomyosin* (data not shown; Thisse et al., 1993; Weinberg et al., 1996). Along the anterior-posterior axis, three to five dorsoventrally arranged columns of adaxial cells, a total of approximately 20 cells (Fig. 1E), become incorporated into each somite (data not shown; these cells are also shown in Fig. 7 of Waterman, 1969; Fig. 2 of Felsenfeld et al., 1991; and Fig. 1 of Hanneman, 1992).

In contrast, the other cells of the segmental plate are smaller and more irregularly shaped (Fig. 1A,B). We call all of the non-adaxial segmental plate cells, lateral presomitic cells. The lateral presomitic cells do not express abundant levels of *myoD*, or other muscle-specific genes before somitogenesis (Fig. 1C,D), although many express these genes after segmentation (Fig. 1C; Weinberg et al., 1996).

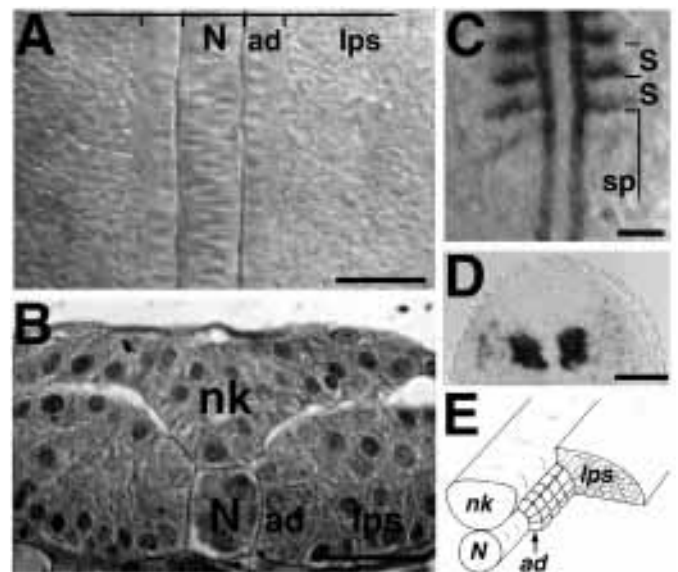
### Embryonic fates of individual adaxial and lateral presomitic cells

To learn whether adaxial cells and lateral presomitic cells have different fates, we injected single cells in the segmental plate with vital fluorescent dyes (Fig. 2A) and observed their subsequent differentiation. As the presomitic mesoderm segmented, injected adaxial cells were incorporated into somites. Adaxial cells elongated along the anterior-posterior axis until they spanned the length of the somite (Fig. 2B). Unexpectedly, injected adaxial cells then migrated laterally, between and past the uninjected, initially more lateral somitic cells. By 40 h, each injected adaxial cell became one of the most superficial muscle cells within the somite (Fig. 2C). The dorso-ventral position of an adaxial cell correlated with the dorso-ventral position of the superficial muscle cell into which it differentiated (data not shown).

Although lateral presomitic cells also become muscle cells, their behavior and embryonic cell fate is quite different from those of the adaxial cells. Injected lateral presomitic cells (Fig. 3A) remained in approximately the same medio-lateral position; none of them migrated extensively during the same period that all of the adaxial cells moved radially away from the notochord. The lateral presomitic cells developed into deep muscle cells (Fig. 3B,C).

### Migration of the adaxial cell population

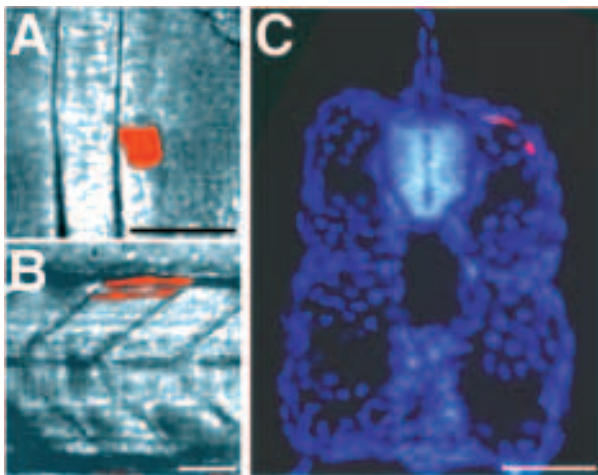
These experiments demonstrate that injected adaxial cells



**Fig. 1.** Adaxial and lateral presomitic cells can be distinguished in the segmental plate. (A) Dorsal view of the segmental plate of an approximately 13 h (8 somites have formed) live embryo. The cuboidal adaxial cells (ad) on either side of the notochord (N) can be distinguished from the irregularly shaped lateral presomitic cells (lps). (B) Transverse semithin section of the segmental plate of a 12 h (6 somites) embryo. The adaxial cells (ad) are large cells adjacent to the notochord (N). Lateral presomitic cells (lps) are smaller, more irregularly shaped and not contacting the notochord. nk, neural keel. (C) Dorsal view of a 13 h (8 somites) embryo labeled by whole-mount RNA in situ hybridization for *myoD*. In the segmental plate (sp), only adaxial cells abundantly express *myoD*; as somites (S) form, other somitic cells also express *myoD*. Horizontal lines mark somite borders. (D) Transverse section through the most rostral portion of the segmental plate of an approximately 15 h (12 somites) embryo labeled by whole-mount in situ hybridization for *myoD*. Adaxial cells express very abundant levels of *myoD*. Some of the lateral presomitic cells are beginning to express low levels of *myoD*. (E) Schematic drawing of the segmental plate. The adaxial cells (ad) are arranged as a sheet between the notochord (N) and the lateral presomitic cells (lps); the neural keel (nk) is also shown. Approximately 20 adaxial cells contribute to each somite. In dorsal views, anterior is to the top of the figure; in transverse sections, dorsal is up. Scale bars: A,B, 25  $\mu$ m; C, D, 50  $\mu$ m.

migrate away from the notochord to become superficial muscle cells. Several possible routes exist by which the adaxial cells could migrate laterally. They might each move radially away from the notochord, migrating individually between the other somitic cells. Alternatively, they could all follow the same path, either moving around the outside edge of the somite or along one path through the middle of the somite, for example at the level of the future horizontal myoseptum. With single cell injections, it would be difficult to determine the paths taken by the entire population of adaxial cells and to characterize the time course of their migration. Thus, we sought an antibody that would recognize all of the adaxial cells during their migration.

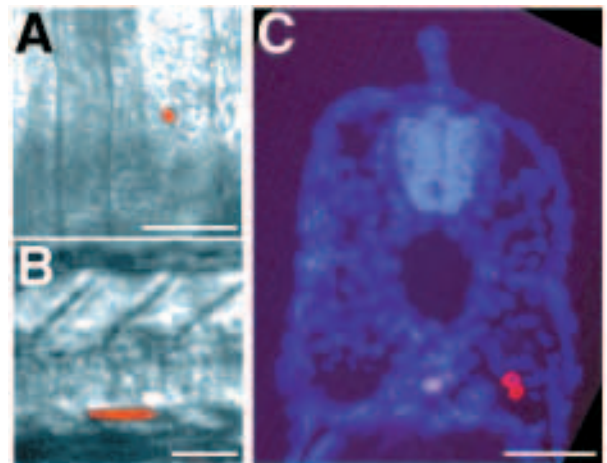
One such antibody is the F59 monoclonal antibody, which recognizes myosin heavy chain in fish (Miller et al., 1989). To determine that F59 labels adaxial cells, we immunostained sections containing injected adaxial and lateral presomitic cells. Injected adaxial cells differentiated into one of the super-



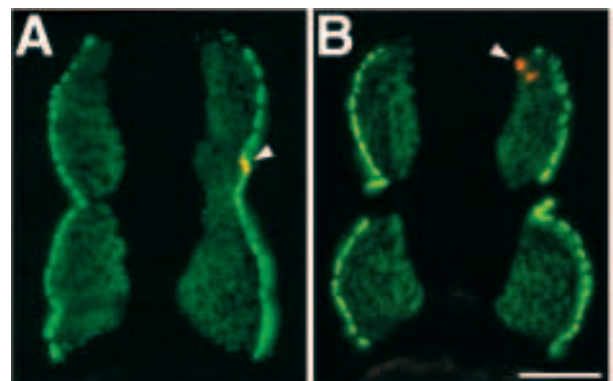
**Fig. 2.** Adaxial cells become the most superficial muscle cells in the somite. (A) Dorsal view of the segmental plate of an approximately 15 h (12 somites) live embryo, after injection of two adaxial cells with lysinated rhodamine dextran. (B) Side view of the same embryo at about 40 h. Both of the injected cells developed into dorsal muscle cells in somite 15. (C) Transverse section of the same embryo, counter stained with Hoechst 33258 to show cell nuclei (blue). The two injected adaxial cells (red) are located superficially. In a series of similar experiments, 72 out of 73 injected adaxial cells became superficial muscle fibers (the single exception was a dorsal cell that may have been adjacent to the neural keel instead of the notochord, and hence misidentified as an adaxial cell at the time of injection). In side views, anterior is to the left; in transverse sections, dorsal is up. Scale bars, 50  $\mu$ m.

ficial monolayer of muscle fibers strongly labeled by F59 (Fig. 4A). In contrast, injected lateral presomitic cells always differentiated into the deeper muscle fibers only weakly labeled by F59 (Fig. 4B). Approximately 20 muscle fibers comprise the F59-labeled superficial muscle layer in each somite, which corresponds closely with the number of adaxial cells that contribute to each somite, suggesting that F59 recognizes all of the adaxial cells. Moreover, F59 also recognizes injected adaxial cells during their migration (data not shown).

We therefore used F59 as a specific label for adaxial cells during the time that they differentiate and migrate (Fig. 5). F59 first labels adaxial cells while they are still cuboidal shaped segmental plate cells, just prior to somite formation (Fig. 5A). 1–2 hours after somite formation, as adaxial cells elongate, F59 labeling becomes more localized into a pattern that may reflect the formation of myofilaments (Fig. 5B, data not shown). During the next 2 hours, many of the F59-positive cells move dorsally and ventrally (Fig. 5B,C). At this time, what was previously a four by five array of cuboidal adaxial cells in each somite, all adjacent to the notochord, has become a one by twenty array of elongated muscle fibers, each spanning the entire length of the somite, with the array distributed along the medial surface of the somite. As individual adaxial cells elongate along the notochord, their intercalation may drive this dorso-ventral movement. Between 3 and 6 hours after somite formation, the F59-positive cells become located progressively farther lateral (Fig. 5D,E), until they are the most superficial cells in the myotome (Fig. 5F). Thus, over the course of approximately 7 hours after somite formation, adaxial cells elongate,

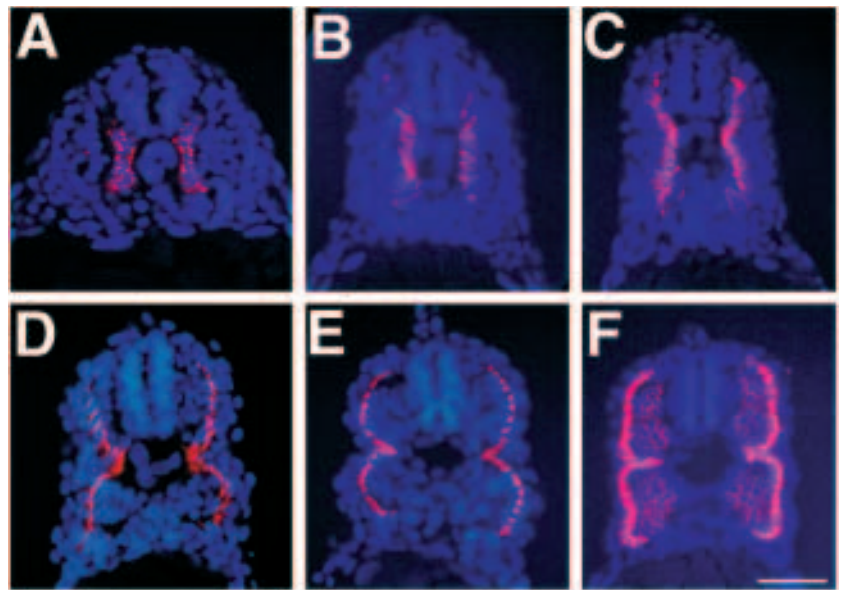


**Fig. 3.** Lateral presomitic cells become deep muscle cells. (A) Dorsal view of the segmental plate of an approximately 15 h embryo (12 somites), after injection of a lateral presomitic cell with lysinated rhodamine dextran. (B) Side view of the same embryo at about 40 h. The lateral presomitic cell developed into two ventral muscle cells located in somite 16. (C) Transverse section of the same embryo, counter stained with Hoechst 33258 to show cell nuclei. Both cells are deep muscle fibers. In a series of similar experiments, 25 out of 25 injected lateral presomitic cells became deep muscle fibers. In side views, anterior is to the left; in dorsal views, anterior is up; in transverse sections, dorsal is up. Scale bars, 50  $\mu$ m.



**Fig. 4.** F59 intensely labels a monolayer of superficial muscle cells derived from adaxial cells and weakly labels deep muscle cells derived from lateral presomitic cells. Adaxial (A) or lateral presomitic (B) cells were injected at about 14 h with vital dye. At 40 h, embryos were fixed and sections stained with F59. (A) The injected adaxial cell (arrowhead, yellow due to double fluorescence of red and green) became a superficial muscle cell strongly labeled by F59 (green). (B) The injected lateral presomitic cells (arrowhead, red) became deep muscle cells only weakly labeled by F59 (green). In a series of similar experiments, 6 out of 6 injected adaxial cells became part of the superficial layer of strongly F59 reactive muscle fibers; 12 out of 12 injected lateral presomitic cells differentiated into the deeper, weakly F59 reactive muscle fibers. We have also used the monoclonal antibody zn-5, which labels the same population of superficial muscle cells that are strongly labeled by F59 at this time (data not shown). In a series of similar experiments, 18 out of 19 injected adaxial cells differentiated into one of the superficial monolayer of muscle fibers positive for zn-5 (the single exception was the same cell mentioned in the legend to Fig. 2, that was dorsal and may have been adjacent to the neural keel instead of the notochord). In these transverse sections, dorsal is up. Scale bar, 50  $\mu$ m.





**Fig. 5.** Adaxial cells migrate radially away from the notochord, between other somitic cells. In the caudal trunk, migration occurs over approximately 5 hours. Transverse sections through the caudal trunk (somites 14-17) were immunolabeled with F59 to mark adaxial cells and counter stained with Hoechst to reveal the nuclei (A-F). (A) 17 h (16 somites) embryo. F59 immunoreactivity is present only in adaxial cells, the labeling is perinuclear. (B) 18.5 h (19 somites) embryo. The adaxial cells have begun to move dorsally and ventrally; this is approximately the time that these cells are elongating in the anterior posterior dimension. (C) 20.5 h (23 somites) embryo. The adaxial cell population now extends almost the full dorso ventral extent of the myotome. (D) 21.5 h (25 somites) embryo. Radial migration of the adaxial cells has begun. (E) 23 h (28 somites) embryo. Most of the adaxial cells have reached the lateral surface. (F) 24 h embryo. The adaxial cells are now lateral. A subset of adaxial cells remains apposed to the notochord, generating an hourglass shape to the layer of superficial muscle cells. The deep cells of the myotome are now beginning to express the F59 epitope weakly. In these transverse sections, dorsal is up. Scale bar, 50  $\mu$ m.

move dorsally and ventrally, then migrate radially through the somite to become a layer of superficial muscle cells.

### Muscle pioneers

Muscle pioneers are a previously identified subpopulation of muscle cells in zebrafish (Felsenfeld et al., 1991). Two to six muscle pioneers develop in each somite; they are distinguished by their morphology, their position in the region of the future horizontal myoseptum and their high levels of *Engrailed* expression (Hatta et al., 1991a; Ekker et al., 1992). During the course of the above experiments, we noticed that a subset of the adaxial cells do not migrate completely to the lateral surface of the myotome, and instead extend from the notochord to the lateral surface of the somite, at the level of the future horizontal myoseptum (c.f. Fig. 5E,F). The behavior and position of these cells are similar to those described for muscle pioneers (Hatta et al., 1991a).

We used cell fate analysis and immunostaining to examine whether muscle pioneers are derived from adaxial cells. We found that a subset of injected adaxial cells differentiated into muscle pioneers, as determined by *Engrailed* expression (Fig. 6A,B). We confirmed this with double immunolabeling for the superficial muscle cells and for the muscle pioneers. In zebrafish, the S58 myosin antibody (Crow and Stockdale, 1986) labels all of the F59-positive superficial muscle cells (data not shown). The muscle pioneers, which are strongly labeled by the 4D9 *Engrailed* antibody, are also labeled by S58 (Fig. 6C,D), confirming that muscle pioneers are a subset of adaxial cells. Although they are part of the layer of superficial muscle cells, they remain apposed to the notochord on their medial surface. Other muscle cells surrounding the muscle pioneers also express *Engrailed* at lower levels (Hatta et al., 1991a); these cells are not derived from adaxial cells.

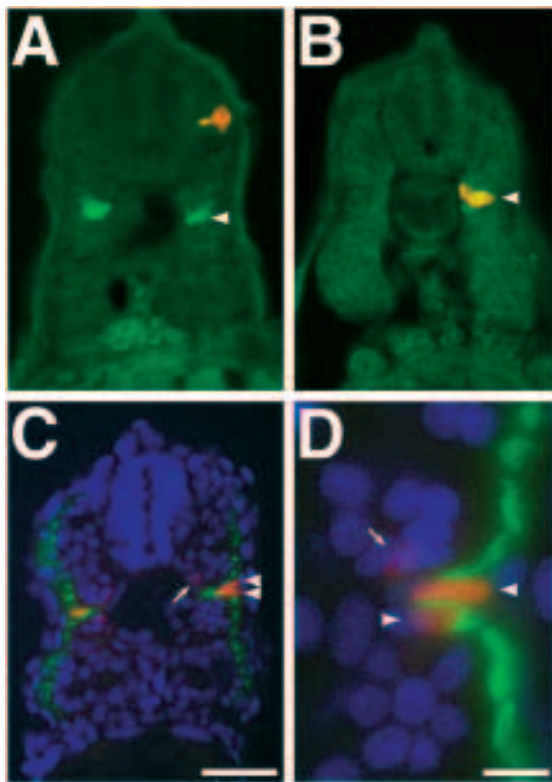
### Muscle fiber type fates in vivo

The presence of distinct populations of segmental plate cells, adaxial and lateral presomitic cells, and their differentiation

into distinct embryonic muscle cells, superficial muscle cells and deep muscle cells, suggested that these populations may be the precursors of distinct muscle fiber types in the adult animal. In adult fish, the fiber type of a specific muscle cell can be inferred from its position and morphology (Bone, 1978). All of the muscle fibers in the wedge-shaped region at the lateral end of the horizontal myoseptum are slow fibers, whereas the muscle fibers in the deeper region are fast. Moreover, we have identified two monoclonal antibodies that reliably distinguish fiber types in larval and adult zebrafish (Fig. 7). The S58 antibody (Crow and Stockdale, 1986) labels slow muscle but does not recognize intermediate or fast muscle (Fig. 7A). The 12/101 antibody (Griffin et al., 1987) labels fast and intermediate muscle fibers, but does not recognize slow muscle (Fig. 7B). Reactivity with these two antibodies is mutually exclusive, we have never seen muscle fibers co-express both the S58 and 12/101 epitopes (Fig. 7C).

The adult muscle pattern appears between two and three weeks of development, when the wedge-shaped region of slow muscle begins to appear. There is little difference in the pattern of slow and fast muscle between a three week larva and a three month adult, other than a considerable increase in the amount of both slow and fast muscle (Waterman, 1969). In addition, the S58 and 12/101 staining patterns are very similar in larvae and adults (data not shown). Thus, we can learn the adult fates of adaxial and lateral presomitic cells by determining the fiber types they form in late larval fish.

We injected adaxial and lateral presomitic cells with vital dye (Figs 8A, 9A, respectively), recorded their positions within the embryo (Figs 8B, 9B), and then raised the injected embryos to the late larval stage, keeping each embryo separate. We then sectioned and labeled the larvae for the presence of the injected dye, and for 12/101 and S58 immunoreactivity. We used position, morphology and antibody labeling to identify the fiber type that each injected cell became. All injected adaxial cells differentiated into slow muscle fibers, on the basis of their position and morphology, S58 immunoreactivity and lack of



**Fig. 6.** Muscle pioneer cells are a subset of adaxial cells. (A,B) Transverse sections of ~22 h embryos in each of which an adaxial cell had been injected as in Fig. 2. Sections were immunostained with the 4D9 Engrailed antibody, which labels the muscle pioneers (green). One out of 7 injected adaxial cells became Engrailed-labeled muscle pioneers. (A) An adaxial cell (red) that became a superficial muscle cell that is not a muscle pioneer (green, arrowhead). (B) An adaxial cell (arrowhead, yellow due to double labeling in green and red fluorescence) that differentiated into an Engrailed-expressing muscle pioneer. (C) Transverse section through a 24 h embryo immunolabeled with the S58 myosin antibody (green) to mark the superficial muscle cells derived from adaxial cells and the 4D9 engrailed antibody (red) to label the muscle pioneers. The Engrailed-labeled muscle pioneers (arrowheads, bright red nuclei) are a subset of the superficial muscle cells. Other somite cells near the muscle pioneers also weakly express Engrailed (arrow, Hatta et al., 1991a), these cells are unlabeled by the S58 antibody and are not part of the superficial muscle cell population derived from adaxial cells. (D) Higher magnification view of the region of the myotome containing muscle pioneers. The two cells that express Engrailed at high levels (arrowheads, bright red nuclei), are also S58 positive (green). Another somite cell that weakly expresses Engrailed, not a muscle pioneer, does not express S58 and is not part of the adaxial-derived superficial muscle cells (arrow, nucleus light red). In these transverse sections, dorsal is up. Scale bars: A-C, 50  $\mu\text{m}$ , D, 10  $\mu\text{m}$ .

12/101 immunoreactivity (Fig. 8C,D). In contrast, injected lateral presomitic cells invariably developed into fast muscle fibers (Fig. 9C,D).

## DISCUSSION

We have found that, during axial muscle development, slow and fast muscle fibers arise from cells which can be recognized *in vivo* before somites form (Fig. 10). In the zebrafish

segmental plate, adaxial cells are large, cuboidal cells adjacent to the notochord; they migrate radially to the lateral surface of the myotome where they differentiate into slow muscle fibers. Lateral presomitic cells, in contrast, are smaller, irregularly shaped cells that do not contact the notochord; they contribute to fast muscle. Thus, slow and fast muscle precursors develop in quite different cellular environments and exhibit distinct cellular behaviors.

### Fiber type development in fish

Our results resolve a controversy over the temporal sequence of muscle fiber type generation in fish. Some studies had suggested that deep myoblasts differentiate into fast fibers and superficial myoblasts later form slow fibers (Waterman, 1969; Nag and Nursall, 1972; Proctor et al., 1980), while others had suggested that myoblasts initially express slow properties and a subset subsequently switches to express fast properties (van Raamsdonk et al., 1978, 1982a). We demonstrated that adaxial cells begin to express slow muscle fiber properties while they are still deep within the myotome. Later, they migrate to a superficial position, as other cells begin to differentiate into fast muscle. Thus, although slow fiber differentiation begins deep within the myotome where fast fibers are later found, this is not a result of fiber type switching; rather, these early differentiating slow muscle precursors migrate from a deep to a superficial location.

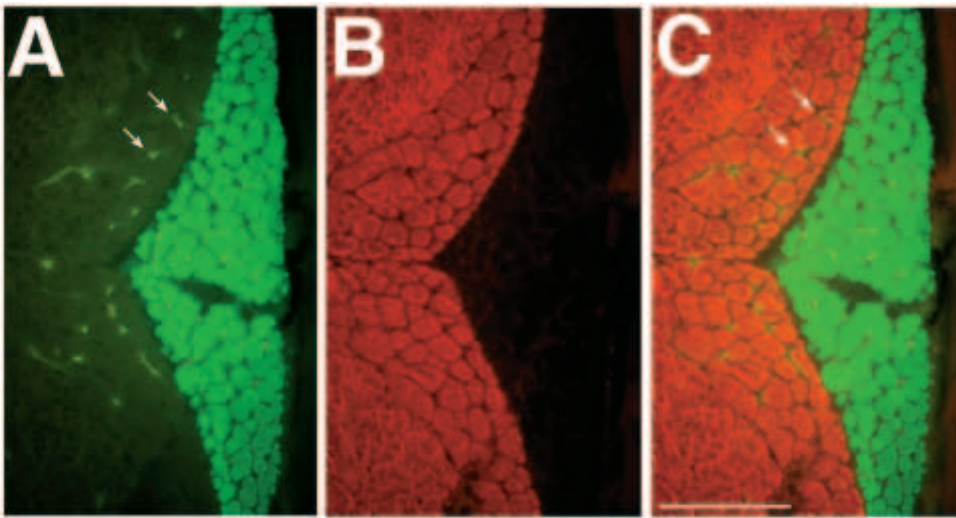
### Development and commitment of fiber type fate

Our results are consistent with previous studies in amniotes, which suggested that the earliest embryonic myoblasts are already specified to give rise to particular muscle fiber types (Stockdale, 1992). In contrast, fiber type identity of later-developing muscle cells, can be labile. Postnatal myoblasts can contribute by cell fusion to several different fiber types (Hughes and Blau, 1992), suggesting that the muscle fiber to which they fuse determines their fiber type identity. Neural activity also influences the fiber type properties of later-developing muscle fibers; even in adults, cross-innervation of a predominately slow muscle by the motor nerve of a fast muscle changes the originally slow muscle into a fast muscle (Buller et al., 1960; for review see Pette and Vrbová, 1985). These experiments have demonstrated that later-developing myoblasts are generally uncommitted to a specific fiber type identity (but see also Hoh and Hughes, 1988) In zebrafish, there is a large increase in the number and size of both slow and fast muscle fibers during larval growth (Koumans and Akster, 1995), suggesting that there may be later-developing myoblasts in zebrafish. We do not know whether these myoblasts have an intrinsic fiber type identity, or whether neural activity or the earlier-developing adaxial and lateral presomitic cells influence their identity.

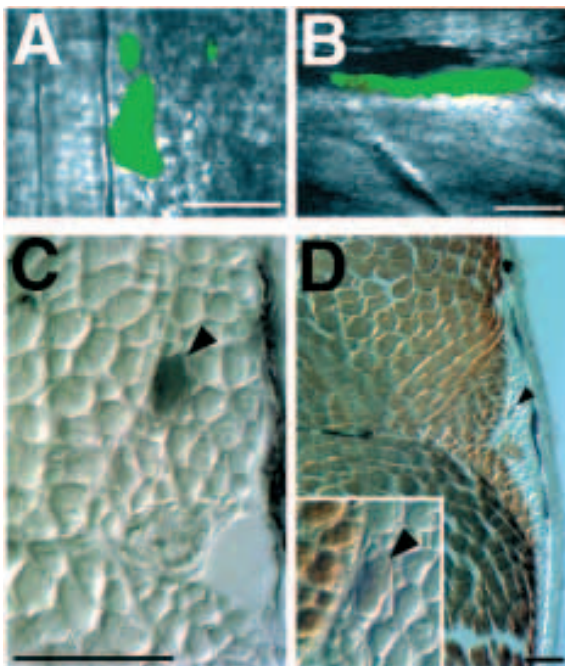
### Muscle pioneers

Muscle pioneers were previously identified by their early differentiation, compared to other myotomal cells, and by their high levels of expression of the *engrailed1* and *engrailed2* genes (Waterman, 1969; van Raamsdonk et al., 1974; Felsenfeld et al., 1991; Hatta et al., 1991a; Ekker et al., 1992). Our present results further show that muscle pioneers develop from a subset of adaxial cells and are part of the superficial muscle

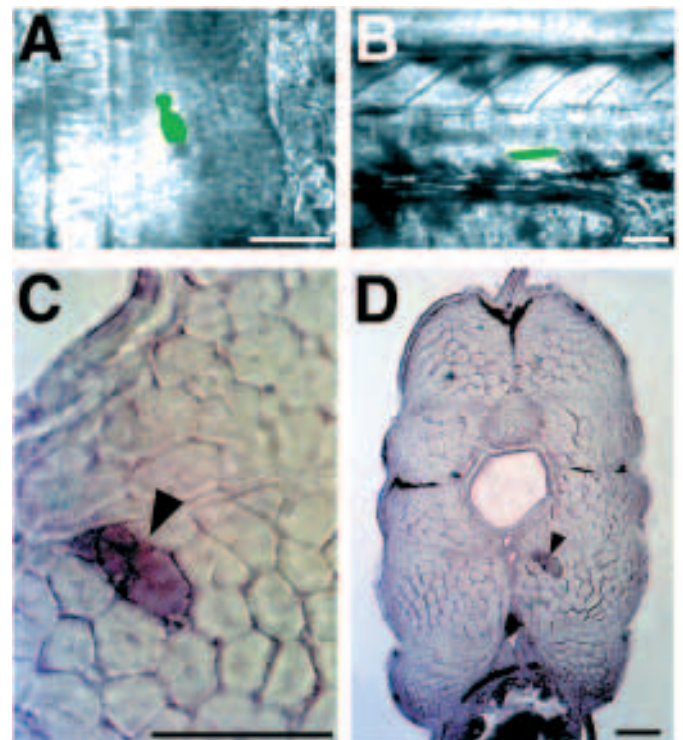




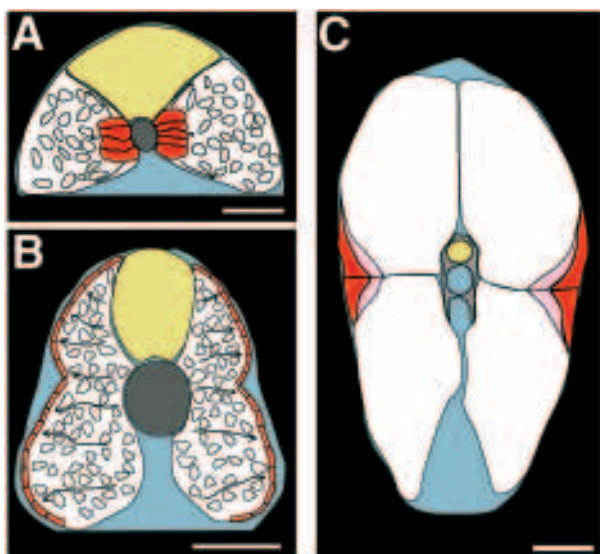
**Fig. 7.** Slow and fast muscle fibers can be unambiguously distinguished by immunolabeling. A transverse section of a 32 d zebrafish was doubly immunolabeled with the S58 antibody and the 12/101 antibody (A-C). (A) S58 (green) recognizes only the slow muscle fibers. Some non-specific fluorescence is present within blood vessels in all muscle regions (e.g. arrows). (B) 12/101 (red) recognizes fast and intermediate, but not slow muscle fibers. (C) 12/101 (red) and S58 (green) label mutually exclusive muscle fibers. In these transverse sections, dorsal is up. Scale bar, 100  $\mu\text{m}$ .



**Fig. 8.** Adaxial cells become slow muscle fibers. (A) Dorsal view of the segmental plate of an approximately 12 h embryo (6 somites), after injection of adaxial cells with lysinated fluorescein dextran. Two or three adaxial cells were injected in this embryo, acellular fluorescent debris is present lateral to the injected adaxial cells. (B) Side view of the same embryo at 8 days. Only one of the injected adaxial cells survived. It developed into a muscle cell located in somite 11. The hexagonal background pattern in this image is due to the video camera. (C) Transverse section of the same embryo at 20 d. The section has been labeled with an antibody to fluorescein, thus the injected cell is black (arrowhead). (D) The same section after labeling with the 12/101 antibody. The adaxial cell developed into a small diameter muscle fiber located in the slow muscle region, not labeled by 12/101 (arrowhead). **Inset:** higher magnification (as in C) view of the injected cell (arrowhead). In a series of similar experiments, 4 out of 4 injected adaxial cells became slow muscle fibers, as determined by antibody staining, position and morphology. In dorsal views, anterior is up; in side views, anterior is to the left; in transverse sections, dorsal is up. Scale bars, 50  $\mu\text{m}$ .



**Fig. 9.** Lateral presomitic cells become fast muscle fibers. (A) Dorsal view of the segmental plate of an approximately 15.5 h embryo (13 somites), after injection of lateral presomitic cells with lysinated fluorescein dextran. (B) Side view of the same embryo at about 38 h of development. One of the lateral presomitic cells developed into a ventral muscle fiber located in somite 15. (C) Transverse section of the same embryo at 15 d. The injected lateral presomitic cell developed into a large diameter muscle fiber (arrowhead) located deep in the muscle. (D) Lower magnification view of the injected cell (arrowhead). The muscle fiber is within the fast muscle region. In a series of similar experiments, 3 out of 3 injected lateral presomitic cells became fast muscle fibers. In dorsal views, anterior is up; in side views, anterior is to the left; in transverse sections, dorsal is up. Scale bars, 50  $\mu\text{m}$ .



**Fig. 10.** Summary of fiber type development in zebrafish axial muscle. (A) Schematic transverse section of the caudal trunk (e.g. somite 16) is shown as it appears in the segmental plate prior to somite formation (15 h). Adaxial cells (red) in the segmental plate, elongate shortly after somite formation and at this time begin to move dorsally and ventrally (arrows), still on the medial surface of the somite. The lateral presomitic cells are shown in white. (B) Schematic drawing of the same somite after the adaxial cells have finished migration (36 h). Adaxial cells (red) have migrated radially away from the notochord (arrows), to become a layer of superficial muscle cells. The lateral presomitic cells do not migrate, but differentiate into muscle cells remaining deep in the myotome. (C) Schematic drawing of the same myotome in the adult zebrafish. Adaxial cells become slow muscle fibers, while lateral presomitic cells differentiate into fast muscle fibers. A layer of intermediate fibers (pink), is present between the slow (red) and fast (white) muscle. In these schematic transverse sections, dorsal is up. Scale bars: A,B, 50  $\mu$ m, C, 250  $\mu$ m.

cell population. Following elongation, the medial surface of each muscle pioneer remains adjacent to the notochord as the rest of the cell extends laterally, generating a flattened, elongated cell that extends from the notochord to the lateral surface of the myotome, at the level of the future horizontal myoseptum (Waterman, 1969). In contrast to other adaxial cells, which lose their contact with the notochord shortly after beginning to migrate, muscle pioneers do not lose their contact with the notochord until after 48 h (Waterman, 1969). Further experiments will be needed to learn whether muscle pioneers later develop into a population of muscle fibers that is distinct from the slow muscle fibers formed by the remainder of the adaxial cells.

#### Fiber type differentiation in vivo

It is unclear whether slow and fast muscle precursors are similarly distinct and localized within the segmental plate in other vertebrates. In both zebrafish and amniotes, the first cells to express muscle differentiation markers are the most medial cells of the future myotome. In zebrafish, these cells are adjacent to the notochord (Fig. 1B; Weinberg et al., 1996), whereas in amniotes they are adjacent to the dorsal half of the

spinal cord (Ott et al., 1991; Pownall and Emerson, 1992). It is unknown whether the earliest differentiating muscle precursor cells in amniotes migrate laterally or whether they are precursors of slow muscle fibers. The early in vivo development of muscle fiber type in amniotes has been mainly examined in limb muscles (for reviews see Stockdale, 1992; Donoghue and Sanes, 1994; see also Van Swearingen and Lance-Jones, 1995). Therefore, direct comparisons with our results, which are from axial muscles, are difficult. Whereas different muscle fiber types are intermingled in axial muscle of amniotes, the distribution of slow and fast muscle fibers in axial muscle of amphibian larvae is strikingly similar to that in the late zebrafish embryo; a superficial layer of slow muscle surrounds the deeper fast muscle fibers (Muntz et al., 1989; Radice et al., 1989). If amphibian slow muscle fibers derive, as in zebrafish, from segmental plate cells adjacent to the notochord, then the timing and localization of slow and fast muscle precursors may be a feature of other vertebrates as well.

At the levels of the limbs, the medial half of the avian somite contributes to axial musculature, whereas the lateral half contributes to limb musculature (Ordahl and Le Douarin, 1992; see also Tosney et al., 1994). Dermamyotome cells of the lateral half somite lose their epithelial character and migrate laterally to enter the limb bud, where they form limb muscle fibers. It is tempting to compare the lateral migration of zebrafish adaxial cells with the migration of these avian myogenic precursor cells into the limb bud. However, several differences caution against this analogy. First, as noted above, limb muscle precursors derive from the lateral half of the somite, whereas adaxial cells are initially the most medial cells in the somite. Second, adaxial cells are slow muscle precursors, whereas the limb myogenic cells generate both slow and fast muscle fibers. Finally, limb myogenic precursors express muscle differentiation markers only after they have migrated into the limb bud (de la Brousse and Emerson, 1990; Williams and Ordahl, 1994), whereas adaxial cells express *myoD*, *myogenin* and *tropomyosin* prior to their migration (Thisse et al., 1993; Weinberg et al., 1996). Thus, it seems unlikely that the adaxial cells are equivalent to the limb myogenic precursors of amniotes. Whether there are other slow muscle precursors in amniotes that are equivalent to adaxial cells is currently unknown.

#### Possible mechanisms regulating early development of fiber type precursors

Our results suggest that the cellular environment in which the earliest myogenic precursor cells develop might influence their fiber type fate. Several embryonic tissues and growth factors have been suggested to pattern the somite and play a role in myogenesis. The notochord is capable of inducing and patterning several cell types in amniotes, amphibians and teleosts, including floor plate cells and motoneurons in the spinal cord, and sclerotome and muscle cells in the somite (Kitchin, 1949; Watterson et al., 1954; van Straaten et al., 1988; Placzek et al., 1991; Brand Saberi et al., 1993; Halpern et al., 1993; Pourquié et al., 1993). The signaling molecule Sonic Hedgehog underlies many of these properties of the notochord (for review see Smith, 1994, see also Hammerschmidt et al., 1996). The surface ectoderm, dorsal neural tube and lateral plate are all potential regulators of myogenesis in chick and mouse



(Pourquié et al., 1995). Members of the Wnt family of secreted proteins may underlie neural tube signaling (Munsterberg et al., 1995), while BMP4 may underlie lateral plate signaling (Pourquié et al., 1996).

The cellular environment of slow and fast muscle precursors suggests that some of these same tissues or growth factors may play a role in the development of muscle fiber type identity. Before their radial migration, adaxial cells are immediately adjacent to the notochord, while the lateral presomitic cells are closer to the surface ectoderm. Interactions between notochord and paraxial mesoderm, or between the surface ectoderm and the paraxial mesoderm, might be involved in the development of muscle fiber type identity. Several zebrafish mutations that disrupt notochord development or signaling have recently been isolated (Hatta et al., 1991b; Halpern et al., 1993; Talbot et al., 1995). By examining the development of adaxial and lateral presomitic cells in these mutant embryos, it will be possible to test whether notochord signaling is required for the development of slow or fast muscle precursors.

We wish to thank Michele McDowell and Ruth BreMiller for invaluable help with histology, Truus te Kronnie for helpful discussions during the course of these experiments, and Sharon Amacher, Christine Beattie, Andreas Fritz, Stephen Hauschka, Charles Kimmel, Frank Stockdale, Truus te Kronnie, Katherine Tosney and Kate Whitlock for critical comments on the manuscript. David Fashena, Bernard Thisse, and Christine Thisse kindly contributed to Fig. 1. The F59 and S58 antibodies were generously provided by Frank Stockdale; the myoD probe was a gift of Eric Weinberg. The 12/101 monoclonal antibody developed by C. R. Kintner and J. P. Brookes was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD 21205 and the Department of Biological Sciences, University of Iowa, Iowa City, IA 52242, under contract N01-HD-2-3144 from the NICHD. This work was supported by fellowships to S. H. D. from the Muscular Dystrophy Association and the Oregon Affiliate of the American Heart Association, and NIH grants NS21132, NS23915 and HD22486.

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