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Regulation of myosin expression during myotome formation

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SUMMARY

The first skeletal muscle fibers to form in vertebrate embryos appear in the somitic myotome. PCR analysis and in situ hybridization with isoform-specific probes reveal differences in the temporal appearance and spatial distribution of *fast* and *slow myosin heavy chain* mRNA transcripts within myotomal fibers. *Embryonic fast myosin heavy chain* was the first isoform expressed, followed rapidly by *slow myosin heavy chains 1* and *3*, with *slow myosin heavy chain 2* appearing several hours later. *Neonatal fast myosin heavy chain* is not expressed in myotomal fibers. Although transcripts of *embryonic fast myosin heavy chain* were always distributed throughout the length of myotomal fibers, the mRNA for each slow myosin heavy chain isoform was initially restricted to the centrally located myotomal fiber nuclei. As development proceeded, *slow myosin heavy chain* transcripts spread throughout the length of myotomal fibers in order of their appearance. Explants of segments from embryos containing neural tube, notochord and somites 7-10, when incubated overnight, become innervated by motor neurons from the neural tube and express all four *myosin heavy chain* genes. Removal of the neural tube and/or notochord from explants prior to incubation or addition of d-tubocurare to intact explants prevented expression of *slow myosin chain 2* but expression of genes encoding the other myosin heavy chain isoforms was unaffected. Thus, expression of *slow myosin heavy chain 2* is dependent on functional innervation, whereas expression of *embryonic fast* and *slow myosin heavy chain 1* and *3* are innervation independent. Implantation of sonic-hedgehog-soaked beads in vivo increased the accumulation of both *fast* and *slow myosin heavy chain* transcripts, as well as overall myotome size and individual fiber size, but had no effect on myotomal fiber phenotype. Transcripts encoding embryonic fast myosin heavy chain first appear ventrolaterally in the myotome, whereas *slow myosin heavy chain* transcripts first appear in fibers positioned midway between the ventrolateral and dorsomedial lips of the myotome. Therefore, models of epaxial myotome formation must account for the positioning of the oldest fibers in the more ventral-lateral region of the myotome and the youngest fibers in the dorso-medial region.

Key words: Somite, Myotome, Slow myosin, Innervation, Sonic hedgehog, Chick

INTRODUCTION

In vertebrates, the entire musculature below the head derives from somites, regularly iterated blocks of mesodermal cells that form adjacent to, and under the influence of, the axial structures of neural tube and notochord (Stockdale et al., 2000). Somites initially form as epithelial spheres, a result of the segmentation of unsegmented paraxial mesoderm. The ventral region of each somite undergoes an epithelium-mesenchyme transition to form the sclerotome, the precursor of the axial skeleton, whereas the dorsal portion remains epithelial and forms the dermomyotome. The dermomyotome subsequently undergoes a mesenchymal change to become the source of the dermis and skeletal muscle. From the ventrolateral edge of the dermomyotome arise migratory myogenic cells that give rise to the muscles of the limbs, body wall and tongue (the hypaxial musculature). The dorsomedial aspect of the dermomyotome gives rise to the myotome, the source of the deep back muscles (the epaxial musculature) (Christ et al., 1983; Eloy-Trinquet and Nicolas, 2002; Huang and Christ, 2000; Ordahl and Le Douarin, 1992). The last-formed epithelial somites are located at the caudal end of the embryo, whereas the most-mature somites are found at the rostral end. Thus at any stage of development a rostral-caudal gradient of somite maturation can be observed within an individual embryo.

The neural tube and notochord are both sources of signals that establish myogenic cell lineages during avian and mammalian somitic myogenesis (Buffinger and Stockdale, 1994; Buffinger and Stockdale, 1995; Münsterberg and Lassar, 1995; Stern et al., 1995). Sonic hedgehog (Shh), a product of the notochord and floor plate (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993), has a key role in somite compartmentalization. Although initially thought to promote the formation of sclerotome and to antagonize dermomyotome formation (Fan and Tessier-Lavigne, 1994), other studies have demonstrated that Shh signaling is also required for the initiation of myogenesis in somites (Borycki et al., 1998;

Borycki et al., 1999; Concordet et al., 1996; Currie and Ingham, 1996; Hammerschmidt et al., 1996; Johnson et al., 1994; Münsterberg et al., 1995; Weinberg et al., 1996). It has been proposed that Shh, in combination with members of the Wnt family produced in the dorsal neural tube and surface ectoderm (Münsterberg et al., 1995; Spence et al., 1996; Spörle et al., 1996; Stern et al., 1995; Tajbakhsh et al., 1998), might activate myogenic regulatory factor gene expression and initiate myotome formation. In addition, Shh affects cell proliferation and survival (Cann et al., 1999; Teillet et al., 1998) as well as the expression of specific myotomal muscle phenotypes (Blagden et al., 1997; Cann et al., 1999; Du et al., 1997).

Skeletal muscle fibers are formed by the fusion of mononucleated myoblasts and their subsequent differentiation into multinucleated muscle fibers. Two broad classes of muscle fibers have been defined based on physiological and structural criteria: rapidly contracting oxidative fibers and slowly contracting glycolytic fibers. The rate of contraction is particularly dependent on the specific isoform(s) of the myosin heavy chain (MyHC) family produced within a myofiber (Bárány, 1967; Reiser et al., 1988). Within the limb muscles, rapidly contracting muscle fibers express only *MyHCs* of the *fast* class, whereas slowly contracting muscle fibers frequently express a *MyHC* of the *fast* class, in addition to *MyHCs* of the *slow* class, which are designated *slow MyHC 1*, *2* and *3* in birds. No detailed study has examined the expression of the various *MyHC* isoforms during myogenesis in the somites.

The best-understood system for generating myofiber diversity is in the zebrafish, in which *slow-MyHC*-expressing myofibers appear before those that express *fast MyHC*, and the hedgehog family of signaling molecules is required for slow fibers to form (Devoto et al., 1996). Shh initiates slow-fiber formation when overexpressed in paraxial mesoderm of the zebrafish (Blagden et al., 1997; Du et al., 1997) and, along with the tiggywinkle and echidna hedgehog proteins, controls induction of muscle pioneers from the adaxial cell population (Currie and Ingham, 1996; Lewis et al., 1999). By contrast, little is known about how myotomal fiber diversity develops in embryos of birds and mammals, or of the relationship between the first myotomal fibers and subsequent muscle formation in the vertebrate epaxial musculature. Although Shh has been shown to influence cell survival and proliferation in the avian myotome (Cann et al., 1999; Teillet et al., 1998), it is unclear whether the Shh signaling pathway is instructive for myotomal muscle fiber type in birds or mammals in vivo.

Here, we have investigated the appearance of the three avian isoforms of *slow MyHC* during formation and maturation of the myotome in chick embryos. As shown by whole-mount in situ hybridization and RT-PCR analyses using isoform-specific probes, the *embryonic fast MyHC* (*efast MyHC*) gene and all three *slow MyHC* genes are expressed in myotomal fibers. From the onset of expression, mRNA transcripts from the *efast MyHC* gene are distributed throughout the cytoplasm of myotomal fibers, whereas the mRNA transcripts for all three *slow MyHC* family members are restricted to the central, nuclear domain. To investigate the mechanism regulating the appearance of the various *MyHC* isoforms, we used surgical and pharmacological methods to interfere with innervation of the myotome. The expression of *efast MyHC* and *slow MyHCs 1* and *3* in the myotome occurs independently of innervation or signals from the neural tube or notochord. By contrast, the expression of the *slow MyHC 2* gene requires functional innervation of the myotome.

MATERIALS AND METHODS

Implantation of Shh-releasing beads

Fertile White Leghorn (*Gallus gallus*) eggs were incubated at 37°C to the desired stages of development. To test the effect of Shh on myotomal development in vivo, Affigel beads (BioRad Laboratories) were incubated in 8.5 mg ml^{-1} mouse N-Shh protein, isolated as described by Marti et al. (Marti et al., 1995), or 8.5 mg ml^{-1} bovine serum albumin (BSA) before being implanted into embryos between Hamburger and Hamilton (HH) stages 10 and 15 (Hamburger and Hamilton, 1951). Beads were inserted between the neural tube and somites in the cervical region, and development was allowed to proceed in ovo for ~24 hours. Afterwards, embryos were removed and processed for in situ hybridization with probes specific to *slow MyHC 3* or *efast MyHC* mRNA, and then cryostat sections were made through embryos at the level of the implanted beads.

Whole-mount in situ hybridization

Embryos were harvested and processed for whole-mount in situ hybridization according to the protocol of Nieto and colleagues (Nieto et al., 1996). After fixation in 4% paraformaldehyde, embryos were dehydrated overnight in absolute methanol, rehydrated the following morning in a graded series of methanol-PBT (PBS + 0.1% Tween-20) washes and treated with 10 μg ml^{-1} proteinase K (Boehringer Mannheim) at room temperature for 5-30 minutes. After proteinase-K treatment, the embryos were rinsed in a small volume of PBS and refixed for 20 minutes in a solution of 4% paraformaldehyde, PBS, 2 mM EGTA, 0.1% Tween-20 and 0.1% glutaraldehyde.

Embryos were hybridized overnight at 70°C with digoxigenin-labeled RNA probes. Unbound probe was removed by multiple washes with TBST (Tris-buffered saline, 0.1% Tween-20) and, following a blocking step, embryos were incubated overnight with alkaline-phosphatase-conjugated anti-digoxigenin Fab fragments (Roche) diluted 1:1000. Unbound antibody was removed by extensive washes with TBST containing 2 mM levamisole prior to visualization with 0.225 mg ml^{-1} Nitro Blue Tetrazolium (Sigma) and 0.1167 mg ml^{-1} BCIP (Sigma) dissolved in NTMT (0.1 M Tris, pH 9.5, 50 mM $MgCl_2$, 0.1 M NaCl, 0.1% Tween-20).

Digital images of in situ hybridized myotomes were quantified using Adobe Photoshop 6 software. Multiple points from the central (nuclear domain) and peripheral regions of myotomes cut in sagittal section were measured and the level of blue staining resulting from the alkaline-phosphatase reaction was determined as a proportion of the total (RGB) color.

Immunohistochemistry

To examine the distribution of fast and slow MyHC proteins during myotome development, cryostat sections were made through HH stage 20-21 embryos that had been ethanol fixed and embedded in OCT (Tissue-Tek). Frozen sections 10 μm thick were mounted on poly-L-lysine-treated slides and stained with monoclonal antibodies F59 (specific for fast MyHC isoforms) and S58 (recognizing slow MyHC 2 and 3 isoforms) using previously described methods (Crow and Stockdale, 1986; Miller et al., 1985). F59 was visualized with a Texas-Red-conjugated anti-mouse IgG (Vector Laboratories), S58 with a FITC-conjugated anti-mouse IgA (Southern Biotechnologies) and nuclei with a DAPI counterstain.

The medial and lateral margins of the myotome were determined by whole-mount immunostaining with a rabbit polyclonal anti-desmin antisera (Sigma). Embryos that had been previously processed for in situ hybridization with either the *efast MyHC* or *slow MyHC 3* probes were washed to remove fixative and stained as previously described

(Kahane et al., 2002). Outgrowth of neurons from the spinal cord to the developing myotome was analyzed with monoclonal antibody 16.5H2 (Developmental Studies Hybridoma Bank) specific for motor neurons or monoclonal anti-neurofilament antibody NN18 (Sigma). Both primary antibodies were visualized with Texas-Red-conjugated anti-mouse IgG. To examine the spatial relationship between neurons and the developing myotome, somite explants were double stained with both the rabbit anti-desmin antisera and the mouse anti-neurofilament antibody NN18.

RT-PCR primers and in situ hybridization probes

Isoform-specific primers were made for *embryonic and neonatal fast MyHC*, *slow MyHCs 1*, *2*, and *3*, and *cNkx 2.5* using sequence from the 3′ regions of each gene (Table 1). Each primer pair was used in RT-PCR assays to examine the temporal appearance of each isoform in the myotome. Total RNA was extracted from the three rostral-most somites of 10- to 30-somite embryos and from ED6 wing buds (Qiagen). cDNA was synthesized from 5 μl of each sample and amplified using the isoform-specific primers in the presence of [32P]dCTP. Using an annealing temperature of 65°C, amplification was monitored at 20, 24, 28, 30, 32, 34, 36, 40, 42, 44 and 48 cycles for each developmental stage. At 36 cycles, each primer pair produced product in the linear phase of amplification, and all subsequent analyses were conducted using these conditions. Each primer pair produces a fragment of unique and diagnostic size when analyzed by PAGE and visualized by an overnight exposure to X-ray film. Amplification products were specific to input RNA because, in the absence of reverse transcriptase, no signal was detected.

An RT-PCR reaction was also performed with each primer pair, using mRNA isolated from HH stage 18 embryos as a template, to generate in situ hybridization probes. Each fragment was subcloned into the pUC19 vector (Gibco) and transformed into DH5-α competent cells. T3 or T7 RNA polymerase was used to synthesize antisense RNA probes from linearized plasmid templates in the presence of digoxigenin-labeled UTP (Roche). Labeled probes were passed over successive Sephadex columns to remove unincorporated nucleotides and were then dissolved in 10 ml of hybridization solution per synthesis reaction.

Somite explant cultures

Explants were made from HH stage 14 embryos. Segments containing three or four pairs of somites, the neural tube, notochord and lateral plate were removed from the embryo at the cervical level and transferred to a collagen-coated dish in a single drop of medium. In some instances, tungsten needles were used to separate the neural tube and notochord from somites on one side of the explant. Both halves, one containing somites alone and the other neural tube, notochord and somites were incubated overnight at 37°C in DME containing 5% embryo extract, 10% horse serum, 1% glutamine and 1% penicillin and streptomycin.

To prevent transmission at the neuromuscular junction in explants containing paired somites, neural tube, and notochord, d-tubocurare (d-tubocurarine chloride) was added to the culture medium at a concentration of 16 μM. Following overnight incubation, explants were fixed in 70% ethanol for immunostaining with antibodies directed against desmin and/or motor neurons, or in 4% paraformaldehyde for in situ hybridization with probes for *efast MyHC* and all three *slow MyHCs*.

RESULTS

Appearance of *MyHC* gene transcripts

There is a developmentally controlled sequence to the initiation of expression of genes for *slow* and *fast MyHCs* in the myotome. RT-PCR was used to determine the timing of expression of five chicken *MyHC* gene transcripts using isoform-specific primers to *slow MyHC 1* (*sMyHC1*), *slow MyHC 2* (*sMyHC2*), *slow MyHC 3* (*sMyHC3*), *efast MyHC* and *neonatal fast MyHC* (*nfastMyHC*) (Fig. 1). RNA isolated from the three rostral-most somites of 10- to 38-somite embryos (HH 10 to 19) was analyzed. At HH 10, the earliest time point examined, only *efast MyHC* was expressed. Expression of *sMyHC3* was first detected 6 hours later, at HH 12, and, by HH 15, *sMyHC1* was also detected. Lastly, *sMyHC2* was detected by HH 17. All somites that expressed *sMyHC2* mRNA always expressed *sMyHC1*, *sMyHC3* and *efast MyHC* as well (Fig. 1). At none of these stages of development was *nfast MyHC* detected in the myotome, but it was readily detected in RNA isolated from an ED6 wing bud.

Because the embryonic heart at these stages also expresses *fast* and *slow MyHCs*, and is closely apposed to the somites, each somite sample was amplified with primers specific for *cNkx 2.5*. This gene is expressed throughout the embryonic heart as early as HH 8, but is not expressed in somites. The absence of any detectable *cNkx2.5* demonstrates that each somite sample is free of contaminating heart tissue (data not shown).

The temporal appearance of each *MyHC* gene transcript was also determined by in situ hybridization analysis of HH 13-25 embryos. Using isoform-specific probes, the number of somites expressing each isoform was determined and plotted against the total number of somites that had formed within each embryo (Fig. 2). This confirmed the order of appearance of each *MyHC* isoform and provided information on the rate at which muscle fibers within the maturing somites began to express each *MyHC* isoform. The first muscle fibers to express detectable levels of either *fast* or *slow MyHC* transcripts were found in myotomes of the rostral-most somites (somites 1-4) around HH stage 14 (22 somites) and, with time, there was a

Table 1. Sequence of isoform-specific myosin heavy chain primers

Gene	Forward primer	Reverse primer
Slow MyHC 1 (U85022)	5′-TGCGGCTGGACGAGGCAGAG-3′	5′-ATCGCCACTGCTTTCACTCCTCGT-3′
Slow MyHC 2 (U85023)	5′-CCTGGACGAAGCAGAGCAGATTG-3′	5′-CCAAGGTACAGCAGGTGGCAGGACAGGCAG-3′
Slow MyHC 3 (S78540)	5′-TACCAGACAGAGGAAGACCG-3′	5′-TTGCTGAAAGCAGAGAGATCC-3′
efast MyHC (J00892)	5′-CGAATTCAAGGGGAGGTTGATGCTGAG-3′	5′-CGAATTCTCCATGGGAAAATCTCTAC-3′
nfast MyHC (AB021180)	5′-GAAGGGAGGCAAGAAGCAAA-3′	5′-CATTTTATGCATGCCTCAGGTC-3′
cNkx 2.5 (X91838)	5′-CCTTCCCCGGCCCCTACTAC-3′	5′-CTGCTGCTTGAACCTTCTCT-3′

The upstream and downstream primer pairs used in RT-PCR assays and to generate isoform-specific fragments of each *MyHC* isoform. The GenBank Accession Numbers for the gene sequences used to generate each primer pair are shown in parentheses. Primer sequences are derived from the following references: *slow MyHC 1* and *2* (Chen et al., 1997), *slow MyHC 3* (Yutzey et al., 1994), *efast MyHC* (Kavinsky et al., 1983; Umeda et al., 1983; Umeda et al., 1981), *nfast MyHC* (Machida et al., 2000) and *cNkx 2.5* (Schultheiss et al., 1995).

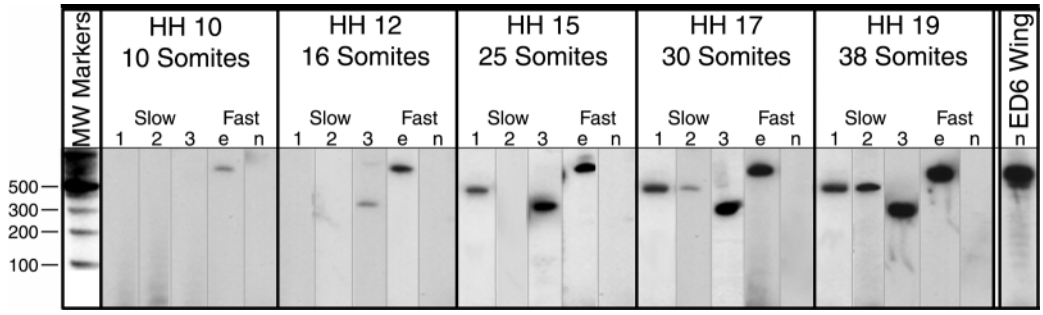

Fig. 1. RT-PCR analysis of somites from HH 10, 12, 15, 17 and 19 embryos, as well as ED6 wing bud, using primers specific for *sMyHC1*, *2*, *3*, *efast MyHC* and *nfast MyHC*. Total RNA was isolated from the three rostral-most somites of embryos at each stage and subjected to 36 cycles of RT-PCR. The first transcripts to appear are *efast MyHC* and *sMyHC3*, followed by *sMyHC1* and finally *sMyHC2*. Transcripts of *nfast MyHC* were not detected in the somites at these stages, but were easily detected with 36 cycles in the wing bud at ED6.

progressive rostral-to-caudal emergence of expression at each developmental stage. As shown by the slope of their lines, the rate of appearance of mRNA transcripts for each gene was approximately the same.

In situ hybridization analysis confirmed that expression of *fast* and *slow MyHC* isoforms began at different times of development (Fig. 2). The first myotomes to express *MyHC* gene transcripts expressed those for *efast MyHC*, whereas transcripts for *slow MyHCs* began to appear a few hours later. *sMyHC1* and *sMyHC3* were detected simultaneously in muscle fibers of rostral somites in 24 somite embryos (HH 15), whereas there was a delay of several hours before *sMyHC2* was first detected, in 27-somite embryos (HH16). This sequence of expression, *efast MyHC*, *sMyHC1* and *sMyHC3*, and finally *sMyHC2* follows the same temporal pattern demonstrated by RT-PCR. The difference in the onset of expression of *sMyHC1* and *sMyHC3*, suggested by the more sensitive RT-PCR assay, was not apparent by in situ hybridization. Both RT-PCR and in situ hybridization revealed a significant delay in the expression of *sMyHC2* relative to the other *MyHC* genes.

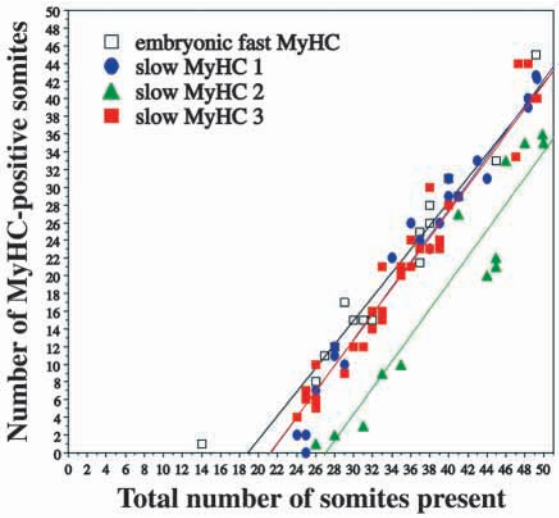

Fig. 2. Developmental expression of *MyHC* transcripts in the myotome detected by *in situ* hybridization. Staged embryos were hybridized with probes specific for *sMyHC1*, *2*, *3* or *efast MyHC*. The number of somites expressing *MyHC* was plotted against the total number of somites that had formed in that embryo. *efast MyHC* (black, open square) was the first myosin expressed, followed a few hours later by nearly simultaneous expression of *sMyHC1* (blue circle) and *sMyHC3* (red square). There was a significant delay before the expression of *sMyHC2* (green triangle).

Intracellular localization of *fast* and *slow MyHC* mRNA initially differs within the myotome

The intracellular location of the *MyHC* gene transcripts is markedly different for the *fast* and *slow* isoforms. From the onset of its expression in the myotome, *efast MyHC* was detected throughout the width of the myotome, extending from its most rostral to its most caudal edge (Fig. 3A,C). By contrast, all three *slow MyHC* gene transcripts were initially located exclusively in a stripe, equidistance from the caudal and rostral edges of each myotome, corresponding to the position of myotomal fiber nuclei (Fig. 3B). Sagittal sections through developmentally immature somites demonstrate the restriction of the *slow* transcripts to the central nuclear domain within myotomal fibers (Fig. 3E). The pattern of nucleus-restricted expression of all *slow MyHC* transcripts was maintained for a substantial time during somite/myotome maturation. By HH stage 18-19, *slow MyHC* mRNA showed a biregional distribution. The strongest signal was still restricted to the nuclear domain, with a weaker signal spanning the width of the fibers (Fig. 3D). By HH 25, the distribution of *slow MyHC* mRNA transcripts became nearly identical to that of *fast MyHC* transcripts (Fig. 3F). A relative measurement of mRNA distribution along the rostral-caudal axis of the myotome was made by sampling the intensity of staining at points from the center to the periphery of sagittally sectioned immature (Fig. 3E) and mature (Fig. 3F) somites (see Materials and Methods). As judged by staining intensity, *slow MyHC* mRNA localized predominately to the center of the myotome in immature somites (78.1±18.8 units), compared to the peripheral regions (33.6±2.2 units). However, as the somite matures, this distinction becomes less apparent, with the central region of the myotome (37.3±4.9 units) staining nearly the same as the remainder of the myotome (35.0±2.8 units). Each of the *slow MyHC* genes showed the same initial pattern of spatial mRNA location and each underwent the same developmental change in location of the transcripts as the somites matured (data not shown).

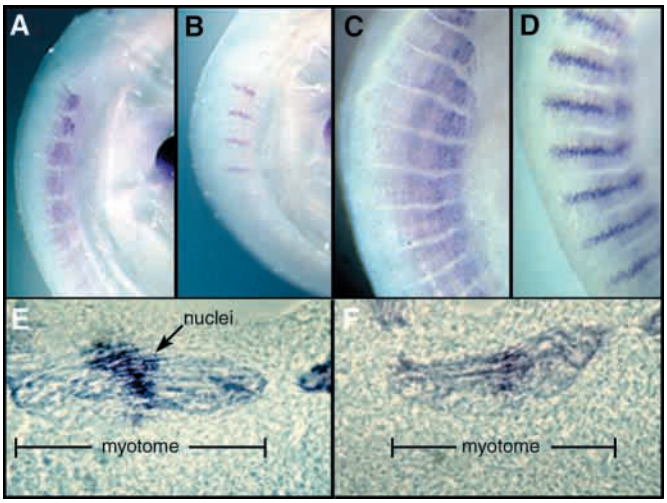

Fig. 3. Intracellular expression patterns of *slow* and *fast MyHC* mRNA within myotomal fibers. HH 15 (A,B) and HH 19 (C,D) embryos were hybridized with probes for *efast MyHC* and *sMyHC3*. (A) At HH 15, *efast MyHC* mRNA is found throughout the cytoplasm of myotomal fibers. (B) *sMyHC3* mRNA is localized to the myotomal nuclear domain. Notice also that fewer somites express *slow MyHC* then *efast MyHC* at this stage. (C) At HH 19, *efast MyHC* expression has expanded along the dorsoventral axis of each somite, but retains its cytoplasmic localization. (D) *sMyHC3* expression has expanded in the dorsoventral direction and some signal is now apparent in the cytoplasm. (E) In sagittal sections through immature somites, in situ hybridization indicates a restriction of the *sMyHC3* mRNA transcripts to the central domain of myotomal fibers where the nuclei are located. (F) In more mature somites, sagittal sections show *sMyHC3* mRNA transcripts distributed beyond the nuclear domain.

Localization of fast and slow MyHC protein and differences in fiber type in the early myotome

There is no distinctive localization of fibers expressing exclusively fast or slow myosin heavy chain protein in the avian myotome. Embryos were fixed and analyzed for fast and slow MyHC protein production using monoclonal antibodies S58 (slow) or F59 (fast). Whole-mount immunohistochemistry first detected fast MyHC isoforms at HH 14-15 (not shown). To determine whether both fast and slow MyHC isoforms accumulate in each myotomal fiber or whether there are fibers that express only fast or slow MyHCs, cross-sections of embryos at the level of the rostral-most somites were double stained with F59 and S58 (Fig. 4A,B). Examination of these cross-sections revealed that most, if not all, myotomal fibers produced both fast and slow MyHC proteins in HH 20 embryos. Sagittal sections through the myotome also revealed that at this stage nearly all myotomal fibers produced both fast and slow MyHCs without there being specific domains for each (Fig. 4C-E). In less-mature somites, occasional fibers located primarily along the dorsal and ventral lips of the myotome were found that produced only fast MyHC protein.

Effects of Shh on MyHC production and myotome size

Experiments were performed to determine the effect of Shh on myosin expression in vivo within the developing somite. The addition of exogenous Shh to cultured somite explants has been shown to produce a dramatic increase in the numbers of *fast*- and *slow-MyHC*-expressing fibers (Cann et al., 1999). Affigel beads soaked in purified Shh protein were implanted between the neural tube and rostral somites of HH stage 10-15 embryos and, following an overnight incubation, an increase in both *slow* and *fast MyHC* expression was detected by in situ hybridization (Fig. 5). The myotomes adjacent to the implanted bead are thickened and larger in both the ventrolateral and dorsomedial aspect compared with myotomes on the opposite side of the embryo (Fig. 5B,D). This distinct thickening of the myotome in response to Shh was particularly evident in cross-sections through affected somites (Fig. 5E). This effect was not seen when control beads soaked in bovine serum albumin (BSA) were implanted (Fig. 5A,C) or in the contralateral somites in embryos implanted with Shh-soaked beads.

The increase in both *efast MyHC* and *sMyHC3* gene expression could be due to an increase in fiber number (as observed in somite explants), fiber hypertrophy or both. Although the resolution of in-situ-hybridized tissue makes it difficult to measure the number of fibers accurately, it is clear that Shh increased the cross-sectional area of myotomal fibers (Fig. 5G). Individual myotomal fibers in somites adjacent to Shh-releasing beads were clearly larger in cross sectional area (23.07±4.34 μm) than those formed in somites on the contralateral side of the same embryo in the absence of exogenously applied Shh (13.60±2.99 μm) (Fig. 5F). These

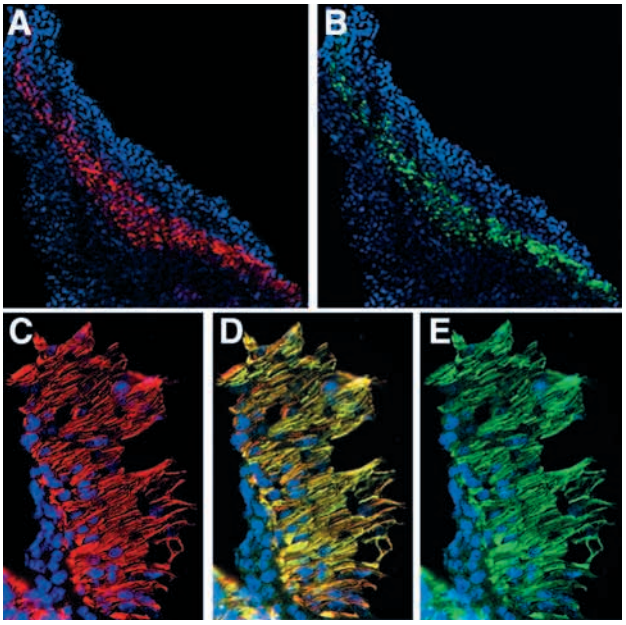

Fig. 4. Myotomal fibers express both fast and slow MyHC proteins. A cross section (A,B) through a rostral somite of a HH 20 embryo and a longitudinal section (C-E) through a rostral myotome of a HH 24 embryo, double-stained with F59 for fast and S58 for slow MyHC protein. (A,C) Fast MyHC (red) is present in fibers of the myotome. (B,E) Slow MyHC (green) appeared in essentially all the fibers as well. (D) Nearly total overlap of the two fluorescent markers (yellow) demonstrates colocalization of fast and slow MyHC proteins in all fibers. The outline of the embryo was visualized by counterstaining nuclei with DAPI (blue). A and B, and C-E depict the same field viewed in different fluorescent channels.

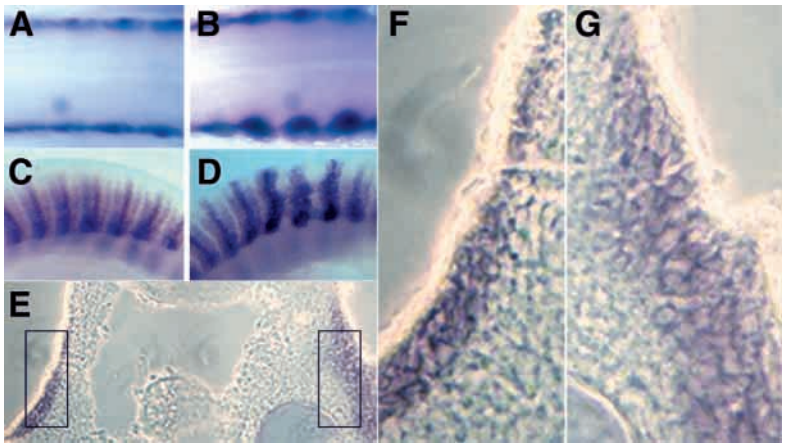

Fig. 5. Implantation of sonic-hedgehog-soaked beads results in an increase in *slow* and *fast MyHC* expression, and a thickening of the myotome. Embryos were implanted with beads soaked in either BSA (A,C) or N-Shh (B,D,E,G) and assayed by whole-mount in situ hybridization with probes specific for either *sMyHC3* (A-D) or *efast MyHC* (E-G). The effect on both myosin isoforms was the same. (A,C) Implantation of a BSA-soaked bead does not cause an alteration to *sMyHC3* expression when viewed dorsally (A) or laterally (C). (B,D) Beads soaked in Shh protein result in a marked increase in *MyHC* expression and thickening of the myotome. (E) When viewed in cross section, it is apparent that Shh-soaked beads cause a thickening of the adjacent myotome, whereas the contralateral myotome is unaffected. (F,G) At higher magnification of the areas shown in E, it is apparent that myotomal fibers adjacent to the Shh-soaked bead are hypertrophied.

results suggest that Shh might have a role in determining the size of muscle fibers in the developing myotome.

Effects of innervation on *MyHC* gene expression in the myotome

The expression of *sMyHC2* is significantly delayed compared with the expression of *sMyHC1* and *sMyHC3* as determined by in situ hybridization assays. This delay prompted an investigation into the developmental events known to occur immediately prior to the stages of development at which *sMyHC2* expression was first seen (~HH 16). Previously published data suggest that, at HH 14-16, nerve fibers first extend from the neural tube toward the myotome (Auda-Boucher et al., 1997; Bo et al., 2000; Hollyday, 1995; Kil and Bronner-Fraser, 1996; King and Munger, 1990; Meiniel and Bourgeois, 1982). It has been demonstrated that myotubes formed in vitro from myoblasts from *slow-MyHC*-expressing muscle will express *slow MyHC 2* only if innervated (DiMario and Stockdale, 1997). Therefore, it was postulated that innervation played a role in the differentiation or developmentally controlled expression of myosin within maturating myotomal fibers. To test this hypothesis, we used both surgical and pharmacological approaches to inhibit innervation.

Explants were made from thick cross-sections through young embryos (~HH 15) at the cervical level. Explants of three or four paired somites were incubated in vitro for 24 hours with or without adjacent neural tube/notochord. In explants including the neural tube, immunostaining with an antibody to neurofilament protein after 4 hours of incubation demonstrated that axons had not grown out from the neural tube, whereas, after an overnight incubation, axons formed and extended into the myotome (Fig. 6A,B). Explants double stained with antibodies to desmin and motor neurons demonstrated that these axons grew from the neural tube and branched to come into physical contact with the myotome (Fig. 6C,D).

To test whether innervation promoted expression of *sMyHC2*, explants of somitic tissue with and without neural tube were cultured overnight and then probed by in situ hybridization with *efast*- or *sMyHC*-specific probes. Somites incubated in contact with the neural tube expressed all three of the *slow MyHC* genes as well as *efast MyHC* (Figs 7, 8). Somites incubated in the absence of the neural tube did not express *sMyHC2* (Fig. 7A) but did express the other *slow MyHC* genes, such as *sMyHC3* (Fig. 7C), or *efast MyHC* (not shown). These results suggested that innervation is required for initiation of expression of *sMyHC2*. Because of the overnight incubation, the *slow MyHC* transcripts appeared throughout the cytoplasm of the fibers, as is typical of myotomes in vivo of comparable developmental stage.

To demonstrate that innervation is required to initiate *sMyHC2*, functional innervation was blocked in explants by exposure to d-tubocurare (Fig. 8). Explants of paired somites, neural tube and notochord grown for 24 hours in the presence

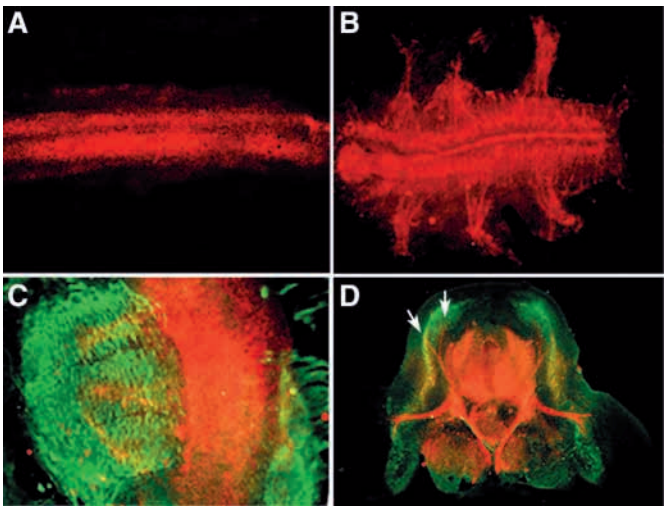

Fig. 6. Nerve fiber outgrowth and innervation of the myotome. Explants were taken from HH 14 (A-C) and HH 23 (D) embryos, and stained with antibodies that identify neurons (red) and desmin (C,D) (green). (A) After 4 hours of incubation, no nerve fiber outgrowth was detected in explants of a HH 14 embryo. (B) Nerve fibers can be seen to extend laterally from the neural tube of explants of HH 14 embryos after 18 hours of incubation. (C) When explants were stained for both desmin and neurofilament protein, it became apparent that nerve fibers were extending to innervate the developing myotomal regions of the somite. (D) In an explant taken of the wing-level somites of a HH 23 embryo and incubated for 18 hours, it can be seen that mature nerve fibers enter the myotomal regions of the somite at two discrete sites (arrows).

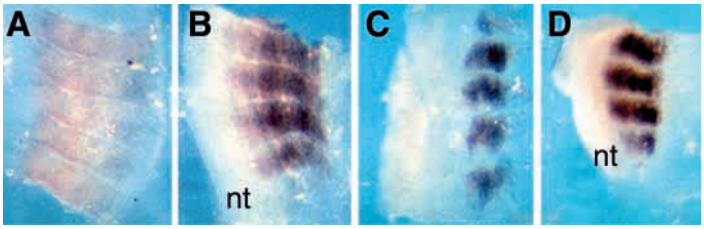

Fig. 7. Surgical separation of somites from the neural tube prevents initiation of *sMyHC2* but not *sMyHC3* expression. Explants from HH 14 embryos were cultured for 24 hours either with or without axial structures (neural tube/notochord) and were then assayed by in situ hybridization. (A) In the absence of neural tube, *sMyHC2* was not expressed. (B) When the contralateral somites of (A) were incubated with the associated neural tube (nt), *sMyHC2* is expressed. (C) Separation of somites from the neural tube/notochord does not inhibit *sMyHC3* expression. (D) Somites contralateral to (C) and incubated in the presence of neural tube/notochord also express *sMyHC3*.

of 16 μM d-tubocurare failed to initiate expression of *sMyHC2* (Fig. 8D), but the expression of the other *slow MyHC* genes and of *efast MyHC* was unaffected (Fig. 8A-C). Control experiments were carried out demonstrating that the outgrowth of nerves from neural tube into the adjacent myotome occurred normally in explants exposed to d-tubocurare (data not shown). With pharmacological blockade by d-tubocurare, diffusible signals from the neural tube/notochord would still be expected to reach myotomal fibers. Thus, the two approaches, surgical and pharmacological prevention of neuromuscular interaction demonstrate that the initiation of *sMyHC2* depends on functional innervation of myotome fibers.

Myotomal fiber differentiation

These studies of the temporal and spatial expression of myosin within myotomal fibers reveal a pattern of differentiation of the first fibers to form in the myotome. It is apparent from whole-mount in situ hybridization that *slow* and *fast MyHC* mRNAs first accumulate in different regions of the myotome (Fig. 9A,C). Although *efast MyHC* transcripts are the first to appear in fibers and virtually all fibers eventually express *slow MyHC* transcripts as well, their respective sites of initiation within the myotome reveal information about the dynamics of myotome formation. To determine the sequence of differentiation within the forming myotome, embryos were assayed by in situ hybridization with probes for *efast MyHC* or *sMyHC3* and were subsequently immunostained with monoclonal antibodies to desmin. Desmin is expressed in all myotomal fibers at the outset of myogenic differentiation (Denetclaw et al., 1997; Lin et al., 1994; Venters et al., 1999), and our results show that *MyHC* genes are expressed shortly after the myotome forms. Thus, desmin expression in the absence of *MyHC* expression provides a marker for the most recently formed myotomal fibers. The first desmin-positive, *efast-MyHC*-expressing fibers appear in the ventrolateral regions of the myotome with desmin-positive, *MyHC*-negative fibers extending to the dorsomedial lip (Fig. 9A). At slightly later stages, as *efast MyHC* expression progressively appears in more medial fibers, *slow MyHC* co-expression first begins within those fibers located near the middle of the myotome. The fibers that first express *slow MyHCs* are flanked both medially and laterally by fibers expressing desmin but no *slow MyHC* (Fig. 9C). As the myotomes matured, transcripts of both types of myosin were detected in all the myotomal fibers. After an initial expansion from the middle of the myotome to the ventrolateral margin, *slow MyHC* expression expanded in the dorsal direction (Fig. 9D), like *efast MyHC* expression (Fig. 9B). Finally, by HH 25, both *fast* and *slow MyHC* expression were seen throughout all fibers of the myotome – medially, centrally and laterally.

DISCUSSION

Differentiation and maturation of myotomal fibers

In vertebrates, the first skeletal muscle to form is the myotome. Initially, a single layer of muscle fibers located beneath the epithelial dermomyotome (the myotome) expands to form the epaxial musculature, primarily muscles of the back. Although there are distinctive differences between hypaxial and epaxial muscle, we and others have shown that the process of myogenesis in the myotome is similar to the more extensively studied process of myogenesis in the limbs (Crow and Stockdale, 1986). As in the limb, the first fibers are mononucleated and all express at least one *fast MyHC* isoform. As muscle fibers in the myotome mature, most, if not all, go on also to express one or more of the *slow MyHC* genes. We used in situ hybridization to examine the temporal order of appearance of individual *MyHC* isoforms expressed in the myotome and found that *efast MyHC* is detected first and *slow MyHC 3* and *slow MyHC 1* appear in rapid succession. These were followed several hours later by the expression of *slow MyHC 2*.

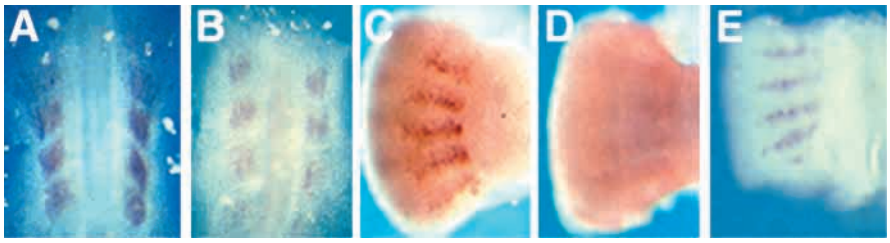

Fig. 8. Exposure of explant cultures to d-tubocurare prevents expression of *sMyHC2* but not of other *MyHC* genes. Explants of somites with the neural tube taken from HH 14 embryos were cultured for 24 hours in the presence (A-D) or absence (E) of 16 μM d-tubocurare and assayed by in situ hybridization with probes specific for *sMyHC1*, *2* and *3*, and *efast MyHC*. (A) *efast MyHC*, (B) *sMyHC1* and (C) *sMyHC3* are expressed in the presence of d-tubocurare. (D) *sMyHC2* was not expressed in explants cultured in the presence of d-tubocurare but (E) was expressed when cultured in its absence.

The progression in the expression of slow myosin heavy chains observed within the myotome is also found in the expression of myosin heavy chains in the hypaxial muscles (Crow and Stockdale, 1986; Hoh, 1979; Kennedy et al., 1986). However, although *slow MyHC* isoforms appear in myotomal fibers in rapid succession over a period of a few hours, changes in fibers of hypaxial muscles occur over a period of days. The

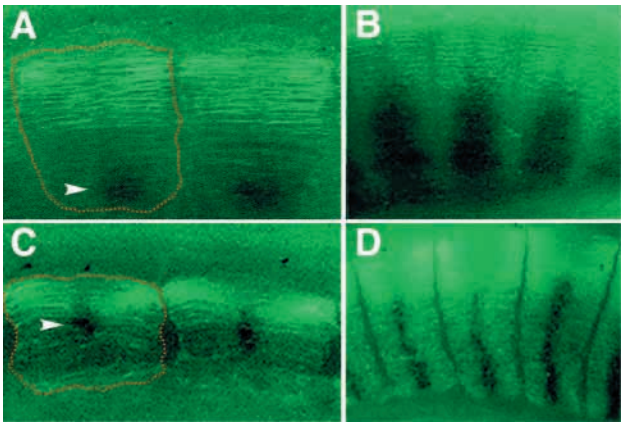

Fig. 9. Localization of *MyHC* message within the developing myotome. Embryos hybridized with isoform-specific *MyHC* probes were subsequently stained with a monoclonal anti-desmin antibody (green). (A) In somites 26 and 27 of an HH19 embryo, the first *efast MyHC* expression is detected by in situ hybridization in the ventrolateral regions of the myotome (arrowhead), and desmin-positive, *MyHC*-negative fibers are positioned primarily in the dorsal region. (B) In more-mature (rostral) somites of the same embryo, the expression of *efast MyHC* extends more dorsomedially. (C) In somites 31 and 32 of an HH21 embryo, the first fibers to express *sMyHC3* are detected near the middle of the myotome (arrowhead), and desmin-positive, *slow-MyHC*-negative fibers are present both dorsal and ventral to this region. (D) After an initial extension to the ventrolateral margin of the myotome, *sMyHC3* expression expands in the dorsomedial direction in more-mature (rostral) somites. The broken line outlines the extent of the desmin-positive fibers in the myotome. Not all fibers are within the focal plane of the photo.

significance of the pattern of myosin heavy chain isoform transitions is not known. These transitions could reflect different roles for the various isoforms in the assembly of sarcomeres, or they could be the result of an evolutionary holdover.

Isoform transitions in birds require the up- and downregulation of individual *slow MyHC* genes in precise sequence (Crow and Stockdale, 1986; Cerny and Bandman, 1987). Although work has elucidated some of the factors regulating *MyHC* gene expression, including thyroid hormone (Gustafson et al., 1986; Izumo et al., 1986), innervation (Pette, 2001) and activity (Cerny and Bandman, 1986; Kennedy et al., 1986), exactly how these and other mechanisms interact to regulate a complex series of isoform changes is not known. Thyroid hormone is perhaps the best documented agent that regulates individual *fast MyHC* isoforms both positively and negatively, depending on the cellular context (Izumo et al., 1986; Morkin et al., 1989). However, it is unlikely that thyroid hormone is a factor in the expression of myosin in the myotome because the changes in *slow MyHC* occur before the hormone is produced by the embryo.

Intracellular localization of slow *MyHC* transcripts

The myotome is initially formed as a single layer of mononucleated muscle fibers subjacent to the dermomyotome. Within each myotomal fiber, the nucleus takes up a central location such that, in the primary myotome, the nuclei form a column extending centrally along the medial-lateral axis of the somite (Kahane et al., 1998). For ~20 hours from the time myosin mRNAs are first detectable in the myotome (HH 14-16), transcripts for each of the three avian *slow MyHC* genes are restricted to the domain where the nuclei are located (Fig. 3). By contrast, *efast MyHC* mRNA does not show this initial intracellular restriction at any time during fiber formation. By HH 18-19 *slow MyHC* transcripts begin to be observed more broadly throughout ventrally located myotomal fibers in the most-rostral somites. That this phenomenon occurs for all three *slow MyHC* genes suggests that it is a true developmentally regulated process specific for *slow MyHC* members of the *MyHC* gene family. This initial localization of *slow MyHC* mRNA was not observed in the mouse myotome for the mammalian homologue, *α-cardiac/slow MyHC* (Lyons et al., 1990).

The in situ hybridization assay used in this study does not have sufficient resolution to determine whether *slow MyHC* transcripts are initially intranuclear or perinuclear, or are in both locations. We hypothesized that, if the *slow MyHC* transcripts are intranuclear, this could be a mechanism of translational control to regulate the appearance of slow MyHC protein in the cytoplasm of myotomal fibers. To test this hypothesis, HH 17-21 embryos were split along the midline of the neural tube. One half of the embryo was then assayed for *slow MyHC 3* mRNA by in situ hybridization, while the other half was assayed immunologically for slow MyHC protein. We were not able to support the hypothesis, because we found that slow MyHC protein was detected in myotomal fibers contralateral to those in which *slow MyHC* mRNA transcripts were confined to the nuclear domain (data not shown). It is also possible that *slow MyHC* transcripts are actually dispersed in the cytoplasm in fibers of less mature myotomes and are below the level of detection by whole-mount in situ hybridization. Alternatively, the *slow MyHC* transcripts could be located in the cytoplasm in a perinuclear location and thus be in a position to be translated. This would require a mechanism whereby all three *slow MyHC* mRNA species would contain sequence information restricting the transcripts to a perinuclear location. Precedence for targeting mRNAs to a specific cytoplasmic location is found in the work of Singer and co-workers (Kislauskis et al., 1994), who have identified a sequence in the 3′ UTR of the *α-actin* gene, designated the ‘zipcode’ that is responsible for the intracellular localization of transcripts to the cell periphery. Regardless of the exact location of the *slow MyHC* transcripts, all three isoforms show the same pattern of temporal and spatial distribution within the nuclear domain and appear to be expressed in the same cells over an extended period of time. Coincidently, the three *slow MyHC* genes are linked to a single locus in the chicken genome (Chen et al., 1997), but the timing of their appearance in the myotome suggests that each is independently regulated.

Innervation-dependent regulation of *slow MyHC 2* in the myotome

The maturation of myotomal muscle requires innervation at an early stage in its formation. We show that innervation of the myotome is necessary for the initiation of *slow MyHC 2* expression. The addition of d-tubocurare to explants to block functional innervation prevents *sMyHC2* expression in the myotome. Because pharmacological blockade by d-tubocurare would not necessarily prevent the release of diffusible signals

from the neural tube/notochord, these observations suggest that it is innervation per se that is important. Thus, the surgical and pharmacological prevention of neuromuscular interaction demonstrates that the initiation of *sMyHC2* depends on functional innervation of myotome fibers. By contrast, the expression of *sMyHC1*, *sMyHC3* and *efast MyHC* is an autonomous process that is independent of nerve outgrowth. Innervation is also an important aspect of *sMyHC2* expression in limb muscles, in which muscle cells isolated from chicken limbs and co-cultured with nerves can initiate *sMyHC2* gene expression, whereas muscle cells cultured alone are not (DiMario and Stockdale, 1997; Lefeuvre et al., 1996).

Effects of sonic hedgehog on the myotome

Shh is expressed by cells of the notochord and floor plate of the neural tube (Echelard et al., 1993; Krauss et al., 1993), and has multiple effects on myogenesis in the somite. Acting in concert with members of the Wnt family of proteins as an activator of the myogenic determination genes *MyoD* and *Myf5*, sonic hedgehog is important for avian myotome formation (Borycki et al., 1998; Borycki et al., 1999; Gustafsson et al., 2002; Münsterberg et al., 1995; Stern et al., 1995; Johnson et al., 1994). In the zebrafish, Shh has been assigned an instructive role in the formation of slow muscle fibers. Adaxial cells, located immediately adjacent to the notochord, form slow muscle fibers that migrate to a superficial position in the adult (Devoto et al., 1996). Ectopic expression of Shh leads to an expansion of slow muscle cells at the expense of fast muscle cells (Blagden et al., 1997; Du et al., 1997; Norris et al., 2000). It is not clear whether this signaling molecule plays an instructive role in the formation of avian slow muscle fibers (Cann et al., 1999; Stockdale et al., 2002). In the chicken, ectopic expression of Shh in the somite leads to an increase in the expression of *slow MyHC 3* as well as an increase in *efast MyHC* in the myotome. Surgical removal of axial sources of Shh from chick embryos prevents myotome formation (Pownall et al., 1996), probably caused by the failure of *MyoD* or *Myf5* to be expressed in precursors located in the dermomyotome (Borycki et al., 1998; Borycki et al., 1999; Münsterberg et al., 1995), making moot the question of whether slow muscle fibers can form in the absence of Shh signaling in birds.

Shh has also been shown to have dramatic effects on cell proliferation and survival in the somite (Cann et al., 1999; Marcelle et al., 1999; Teillet et al., 1998). Somites grown in explant cultures separated from Shh-producing axial structures show greatly reduced levels of cell proliferation and greatly increased levels of apoptosis compared with explants of somites associated with axial structures (Cann et al., 1999). Conversely, the addition of sonic hedgehog to the growth medium prevented apoptosis and expanded the number of muscle cells in somites cultured without axial structures, mimicking the effects of the neural tube. In vivo, the implantation of Shh-expressing cells prevented apoptosis in somites of embryos lacking axial structures (Teillet et al., 1998), and Shh-expressing cells increased proliferation in somites separated from the notochord and neural tube (Marcelle et al., 1999).

Previous work by Amthor and coworkers (Amthor et al., 1999) showed that implantation of a Shh-releasing bead into mature somites with well-formed myotomes increased the expression of *MyoD* in the epaxial muscle after 24 hours of incubation. In response to Shh-soaked beads, we also observed locally increased amounts of *MyoD* mRNA in the somite, particularly in the epaxial myotome (data not shown) and in *MyHC* gene expression. The observed expansion of both *fast* and *slow MyHC* expression in the myotome adjacent to a Shh-releasing bead is consistent with the conclusion that Shh signals induce precocious differentiation of muscle fibers in the myotome. The ectopic addition of Shh also leads to hypertrophy of individual myotomal muscle fibers (Fig. 5), suggesting a previously unknown role for this important signaling molecule in the regulation of muscle fiber size [for a review of muscle size, see Patel et al. (Patel et al., 2002).

Implications for myotome formation

In the myotome, as in skeletal muscle in general, one of the first indicators of myoblast differentiation is the expression of the intermediate filament protein desmin (Denetclaw et al., 1997; Lin et al., 1994; Venters et al., 1999), which defines the boundaries of the myotome. Our in situ hybridization data demonstrate that maturation of myotomal fibers, as defined by the expression of *MyHCs*, begins in a subset of desmin-positive fibers, located ventrolaterally in the myotome (Fig. 9A). These fibers first express *efast MyHC* and, as somites mature, the domain of *fast MyHC* expression expands dorsomedially in the myotomes (Fig. 9B). At the last time examined (HH 21), even in the most mature somites, desmin-positive *MyHC*-negative fibers remained in the dorsomedial region of the myotome (Fig. 9B). These are the last fibers to express *MyHCs*. In a similar way, Duxson and co-workers (Venters et al., 1999) demonstrated in the mouse that there exists a gradient of increasingly more mature muscle fibers as one proceeds ventrally from the dorsomedial edge of the myotome.

A few hours after the first appearance of *fast MyHC* transcripts, *slow MyHC* gene expression begins within these same fibers (Fig. 2). Fibers that first express *slow MyHC* transcripts are first seen midway between the medial and lateral borders of the myotome (Fig. 9C), in fibers that are continuing to express *efast MyHC*. As somites mature, the domain of *slow MyHC* expression expands first ventrolaterally and subsequently dorsomedially (Fig. 9D). Denetclaw and colleagues (Denetclaw and Ordahl, 2000) have shown that myoblasts first enter into the myotome from the dorsomedial lip and only later from the ventrolateral lip. It is possible that the muscle fibers located ventrolateral to those expressing *slow MyHC* are younger than those in the mid-myotome, having entered from the ventrolateral lip. Such fibers, formed from cells of the ventrolateral lip, could have initiated expression of *efast* MyHC but, because they are younger, have not initiated *slow MyHC* expression. Alternatively, it is possible that a signal originating from a restricted region of the overlying dermomyotome could induce the underlying myotomal fibers to activate *slow MyHC* genes. Based on gene expression patterns and morphology, Spörle (Spörle, 2001; Spörle et al., 2001) has identified a centrally located region in the myotome, termed the intercalated (dermo)myotome, that is in a similar location to that of the initial *slow MyHC* activation and can be characterized by specific molecular markers (Hadchouel et al., 2000; Teboul et al., 2002). One marker of the intercalated dermomyotome is the homeobox-containing gene *engrailed* (Spörle, 2001). In the zebrafish, *engrailed* is expressed in

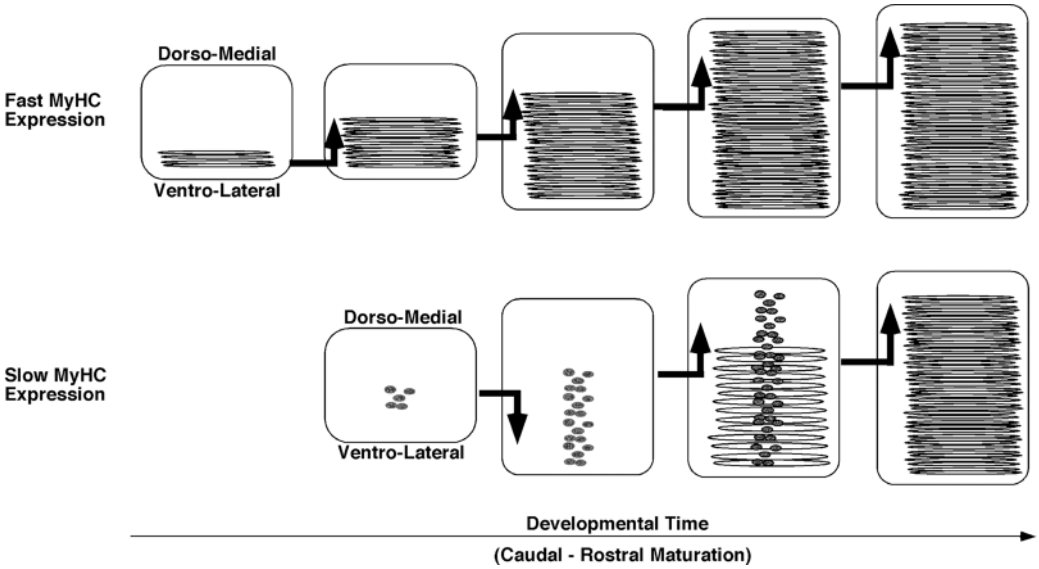

Fig. 10. Model of fiber formation in the myotome. The myotome as defined by desmin staining is represented as closed boxes. *Fast MyHC* transcripts appear first in the myotome and are distributed throughout the width of myotomal fibers, whereas transcripts for *slow MyHCs* appear slightly later and are initially confined to the central nuclear domain. *Fast MyHC* expression begins near the ventrolateral edge of the myotome and expands dorsomedially as development continues. *Slow MyHCs* are initially expressed more centrally in the myotome and expression initially expands ventrolaterally. Subsequently, *slow MyHC* expression expands dorsomedially within the myotome. In the most-mature somites, *slow MyHC* transcripts become distribute more evenly throughout the myotomal fibers, eventually losing their nuclear localization completely to mimic the distribution of *fast MyHC* transcripts.

muscle pioneers, the first muscle cells to differentiate within the myotome and a subset of slow-fiber skeletal muscle precursors (Hatta et al., 1991; Devoto et al., 1996). However, it should be realized that, unlike the zebrafish, the myotome of avian embryos does not show the same distinct separation of fast and slow muscle fibers. Although *slow MyHC* genes appear to be expressed first in a subset of myotomal fibers located centrally within the myotome, during the early stages of development examined here, all (or nearly all) myotomal fibers eventually show a single phenotype in which both *fast* and *slow MyHCs* are expressed (Fig. 4).

These observations have implications for the proposed mechanisms of myotome formation (Fig. 10). The appearance of myosin gene expression should be a temporal matter with regard to the maturation of fibers within the myotome and thus should reflect the age of the fibers. As measured by the expression of *MyHC*, maturation of the myotome first occurs ventrolaterally, with an increasing number of fibers expressing *MyHC*. This pattern suggests that the youngest fibers are the most medial ones in the early myotome, leading to the suggestion that the origin of most myotomal fibers must be the dorsomedial region of the somite. This conclusion is in agreement with models of early myotome formation, which have demonstrated that the dorsomedial lip of the dermomyotome is the source of epaxial myotomal fibers (Christ et al., 1978; Cinnamon et al., 2001; Denetclaw and Ordahl, 2000; Denetclaw et al., 1997; Denetclaw et al., 2001; Kahane et al., 1998; Kahane et al., 2002; Venters and Ordahl, 2002; Venters et al., 1999).

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