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 dorsomedial 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 (Bárany, 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 MgCl₂, 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 antiserum (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...
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 antiserum and the mouse anti-neurofilament antibody NN18.

RT-PCR primers and in situ hybridization probes
Isomorph-specific primers were made for embryonic and neonatal fast MyHC, slow MyHCs 1, 2, and 3, and cNkx2.5 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 isomorph-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 a product 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-tubocurarine (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 isomorph-specific primers to slow MyHC 1 (sMyHC1), slow MyHC 2 (sMyHC2), slow MyHC 3 (sMyHC3), efast MyHC and neonatal fast MyHC ( nefastMyHC) (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 nefast 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 cNkx2.5. 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 isomorph-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>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>Slow MyHC 1 (U85022)</td>
<td>5′-TGCCTGCTGAGGAAGAGAGGAG-3′</td>
<td>5′-ATCGCCACTGTTTCTACCTTCGTC-3′</td>
</tr>
<tr>
<td>Slow MyHC 2 (U85023)</td>
<td>5′-CCTGGCAACACAGAGCAAGATTG-3′</td>
<td>5′-CCAAGGTACAGCAGGTGGGAGG-3′</td>
</tr>
<tr>
<td>Slow MyHC 3 (U85024)</td>
<td>5′-CTGCTGAAAGCAGAGAATCC-3′</td>
<td>5′-TGTCGAGGCAAGAGAGAGAG-3′</td>
</tr>
<tr>
<td>efast MyHC (J00892)</td>
<td>5′-AAATTTAAAGGGGAGTATTGTCCTAG-3′</td>
<td>5′-GAAATTTAAAGGGGAGTATTGTCCTAG-3′</td>
</tr>
<tr>
<td>nefast MyHC (AB021180)</td>
<td>5′-GAAAAAAGGAGCAGACAGCAA-3′</td>
<td>5′-CATTTGATAGCCTACACTG-3′</td>
</tr>
<tr>
<td>cNkx2.5 (X91838)</td>
<td>5′-CTTCCCTCGCCCGCTTACAT-3′</td>
<td>5′-CTTCCCTCGCCCGCTTACAT-3′</td>
</tr>
</tbody>
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The upstream and downstream primer pairs used in RT-PCR assays and to generate isomorph-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 (Yutzy et al., 1994), efast MyHC (Kawinsky et al., 1983; Umeda et al., 1983; Umeda et al., 1981), nefast MyHC (Machida et al., 2000) and cNkx2.5 (Schultheiss et al., 1995).
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.

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 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).
Myosin expression in the myotome

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 fast MyHC and slow MyHC3 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).
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 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 of d-tubocurare did not express sMyHC2, whereas unblock innervation (Fig. 8A) led to expression of sMyHC2 in the presence of d-tubocurare (Fig. 8B). These results suggested that innervation is required for initiation of expression of sMyHC2, and that the slow MyHC transcripts appeared throughout the cytoplasm of the fibers, as is typical of myotomes in vivo of comparable developmental stage.

**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.

**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 (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).
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; Venter 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 to also 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.

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

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**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.

**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 dorsomedial direction in more-mature (rostral) somites. The slow MyHC3 dorsal and ventral to this region. (D) After an initial extension to the fast-negative fibers are present both somites 31 and 32 of an HH21 embryo, the first fibers to express expression of MyHC extends more dorsomedially. (C) In more-mature (rostral) somites of the same embryo, the expression of efast MyHC (green). (A) In somites 26 and 27 of an HH19 embryo, the first fibers to express sMyHC3 are detected near the middle of the myotome (arrowhead), and desmin-positive, MyHC-negative fibers are present both dorsal and ventral to this region. (B) 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.

**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.

The 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. Coincidentally, 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; Lefevre 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; Venter 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 (Venter 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 Or Dahl, 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 (Hadhoeul 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.

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 dermatomyotome 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 et al., 1999).

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