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RED AND WHITE MUSCLE DEVELOPMENT IN THE TROUT (*ONCORHYNCHUS MYKISS*) AS SHOWN BY *IN SITU* HYBRIDISATION OF FAST AND SLOW MYOSIN HEAVY CHAIN TRANSCRIPTS

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Summary

The axial muscle of most teleost species consists of a deep bulk of fast-contracting white fibres and a superficial strip of slow-contracting red fibres. To investigate the embryological development of fast and slow muscle in trout embryos, we carried out single and double *in situ* hybridisation with fast and slow myosin heavy chain (MyHC)-isoform-specific riboprobes. This showed that the slow-MyHC-positive cells originate in a region of the somite close to the notochord. As the somite matures in a rostrocaudal progression, the slow-MyHC-positive cells appear to migrate radially away from the notochord to the lateral surface of the myotome, where they form the superficial strip of slow muscle. Surprisingly, the expression pattern of the fast MyHC showed that the differentiation of fast muscle commences in the medial domain of the somite before the differentiation and migration of the slow muscle precursors. Later, as the differentiation of fast muscle progressively spreads from the inside to the outside of the myotome, slow-MyHC-expressing cells become visible medially. Our observations that the initial differentiation of fast muscle takes place in proximity to axial structures and occurs before the differentiation and migration of slow muscle progenitors are not in accord with the pattern of muscle formation in teleosts previously described in the zebrafish *Danio rerio*, which is often used as the model organism in fishes.

Key words: myosin heavy chain, somitogenesis, muscle, differentiation, teleost, trout, *Oncorhynchus mykiss*.

Introduction

In contrast to the muscle of terrestrial vertebrates, in which the different muscle fibre types form a mosaic within the same anatomical muscle, slow and fast muscle fibres of fishes are grouped into physically distinct areas. The slow muscle occurs as a thin continuous strip that lies above the fast muscle, which constitutes the major part of the myotomal musculature (Van Raamsdonk et al., 1982). Some studies in trout *Oncorhynchus mykiss* (Nag and Nursall, 1972; Proctor et al., 1980) and zebrafish *Danio rerio* (Waterman, 1969) had suggested that deep myoblasts differentiate into fast fibres and that superficial myoblasts later form slow fibres. Using labelling with vital dyes, Devoto et al. (Devoto et al., 1996) obtained data that indicated, to the contrary, that the slow muscle fibres of the zebrafish originate from adaxial cells next to the notochord that migrate radially to reach the lateral surface of the myotome. These authors and others suggested that, as the slow muscle cells migrate radially, others myogenic cells begin to differentiate into fast muscle (Devoto et al., 1996; Bladgen et al., 1997). It was also proposed that Hedgehogs derived from the notochord induce the commitment of adaxial cells to a slow lineage (Bladgen et al., 1997; Du et al., 1997). Accordingly, a mutation in the gene encoding sonic hedgehog or Gli2, a zinc finger transcription factor implicated in mediating Hedgehog signalling, was found to result in an alteration of adaxial cell differentiation in the zebrafish mutants *sonic you* (sonic hedgehog) and *you-too* (Gli2) (Schauerte et al., 1998; Karlstrom et al., 1999; Lewis et al., 1999).

In the present study, to investigate the developmental origins of the slow and fast muscle fibres, *in situ* hybridisations were carried out using species-specific cRNA probes for fast and slow myosin heavy chain (MyHC) transcripts. We observed an apparent lateral migration of slow-MyHC-expressing cells from the medial to the lateral domain of the somite, where they ultimately form the superficial slow muscle. However, in contrast to the general conception of teleost muscle differentiation inferred from data obtained in zebrafish, our work showed that the fast muscle also differentiates initially in a medial domain of the somite adjacent to the notochord, well before the lateral migration of slow muscle progenitors.

Materials and methods

Generation of a slow-MyHC-encoding cDNA by rapid amplification of cDNA ends/polymerase chain reaction (Race–PCR)

First-strand cDNA was synthesised from trout (*Oncorhynchus mykiss*) slow muscle RNA using the RoRidT$_{(17)}$ (5′-ATCGATGGTCGACGCATGCGGATCCAA-AGCTTGAATTCGAGCTCT$_{(17)}$) primer. For the polymerase chain reaction (PCR), we used, in addition to the RoRi primer, the oligonucleotide 5′-GGAGCTGAAGAAGGAG/ACAGG-ACACC-3′, which is highly conserved in human myosin sequences. The PCR conditions were 25 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 80 s. The PCR product was cloned into pCRII vector (InVitrogen) and sequenced using an automatic sequencing system (ABI PRISM 310, PE Biosystems).

Whole-mount in situ *hybridisation*

After appropriate linearisation of the plasmid that contained the slow MyHC cDNA or a 2 kb fast MyHC cDNA (Gauvry and Fauconneau, 1996), sense and anti-sense RNA probes were generated using bacteriophage T3, T7 or SP6 RNA polymerases in the presence of digoxigenin-11UTP* or fluorescein-12UTP* (Boehringer Mannheim). The chorion was removed from embryos using fine forceps. Embryos were then fixed overnight at 4 °C in paraformaldehyde in phosphate-buffered saline (PBS). Specimens were dehydrated and stored in methanol at −20 °C. Following rehydration in a graded series of methanol/PBS, embryos were processed according to established procedures (Joly et al., 1993) with minor modifications. Double, whole-mount *in situ* hybridisations were carried out essentially according to the method of Jowett and Yan (Jowett and Yan, 1996). Briefly, the slow MyHC antisense riboprobe was labelled with digoxigenin-11UTP* and the fast MyHC antisense riboprobe was labelled with fluorescein-12UTP* (Boehringer Mannheim). Hybridisation was performed simultaneously with the two riboprobes. After reaction with anti-digoxigenin alkaline phosphatase Fab fragments, the first colour staining was performed with NBT-BCIP (Boehringer Mannheim). Alkaline phosphatase was inactivated by incubation with 0.1 mol l^{-1} glycine-HCl (pH 2.2) twice for 10 min. After extensive washing with PBS, the embryos were incubated with anti-fluorescein alkaline phosphatase Fab fragments. The second colour staining was performed with Fast Red substrate (Boehringer Mannheim).

Histological methods

For histological examinations, embryos were dehydrated and mounted in paraffin, and 10 μm sections were cut. Sections were counterstained with nuclear Fast Red, mounted in Eukitt (Prolabo) and observed using a Zeiss 47.50.57 stereo microscope. The embryos used for double *in situ* hybridisation experiments were embedded in 30 % ovalbumin, 0.5 % gelatine and 1 % glutaraldehyde in PBS. Blocks were sectioned at 40 μm on a Leica vibratome. Nomarski optics were used for the examination of dual-colour sections.

Results

Derivation of a slow MyHC clone by reverse transcriptase–polymerase chain reaction

We have previously identified a MyHC selectively expressed in fast muscle of trout (Gauvry and Fauconneau, 1996). Its cDNA was used in this study as a marker for the fast myogenic lineage. Given that no slow MyHC has been isolated so far from trout, the first aim of our work was to clone such a MyHC cDNA for use in specifically labelling the slow muscle precursor cells. A cDNA of approximately 0.67 kb that includes an open reading frame of 169 amino acid residues was isolated (Fig. 1) using the 3′RACE–PCR method with cDNA

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ELKKEQDTSSHLERMKKNMEQTIKDLQHRLDEAEQIAMKG    Trout slow MyHC
ELKKEQDTSAHLERMKKNMEQTIKDLQHRLDEAEQIALKG    Human alpha-MyHC
ELKKEQDTSSHLERMKKNLEQSVKDLQHRLDEAEQLAMKG    Frog MyHC
ELKKEQDTSSHLERMKKNMEQTIKDLQMRLDEAEQIAMKG    Chicken slow MyHC

GKKQIQKLESRVRELESEVEMEQRRSSDAVKGVRKYERRI    Trout slow MyHC
GKKQLQKLEARVRELEGELEAEQKRNAESVKGMRKSERRI    Human alpha-MyHC
GKKQLQKLEVRVRELESELDNEQKRGVESIKGVRKYERRV    Frog MyHC
GKKQIQKLEARVRELEGELDMEQKKMAEAQKGIRKYERRI    Chicken slow MyHC

KELTYQTEEDRKNLSRLQDLVDKLQLKVKSYKRTSEEAEE    Trout slow MyHC
KELTYQTEEDKKNLLRLQDLVNKLQLKVKAYKGQAEEAEE    Human alpha-MyHC
KELTYQTEEDRKNLLRLQDLVDKLQLKVKAYKRQAEESEE    Frog MyHC
KELSYQTEGDRKNLTRMQDLIDKLQSKVKSYKRQFEGAEQ    Chicken slow MyHC

QANSSLGKFRKIQHELDEAEERADIAESQVNKMRAKSRDS    Trout slow MyHC
RANTNLSKFRKVQHELDEAEERADIAESQVNKLRAKSRDS    Human alpha-MyHC
QANAHLGRFRKVQHELEEAEERADIAESQVNKLKAKSRDS    Frog MyHC
QANSNLVKYRKVQHELDDAEERADIAETQVNKLRARTNEV    Chicken slow MyHC

GSKKGKEEE                                   Trout slow MyHC
GAKKMHDEE                                   Human alpha-MyHC
GGKK-EIEE                                   Frog MyHC
ITFK---HE                                   Chicken slow MyHC
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Fig. 1. Comparison of the amino acid sequences of the carboxy-terminal part of the slow trout myosin heavy chain (MyHC) with MyHCs from other species. The accession numbers for the sequences are as follows: trout slow MyHC (AF211172), human alpha-MyHC (M21664), frog MyHC (AF097907), chicken slow MyHC (U85023). Identical residues are shaded.

generated from slow muscle mRNA. After alignment, the predicted sequence of our clone was found to show a high degree of similarity with MyHC from various species. Northern blotting confirmed that the transcript is selectively expressed in slow muscle of trout and is not detectable in fast muscle (data not shown). This cDNA therefore served as a convenient marker for slow muscle.

Lateral migration of slow-MyHC-positive cells

In situ hybridisation on whole embryos using a digoxigenin-labelled riboprobe indicated that the slow MyHC was first detected when approximately 30 somites had been formed (stage 13 of Ballard, 1973). At this stage, the labelling was within the most rostral somites but not in the newly formed posterior somites. Thus, the onset of slow MyHC expression is considerably later than that of the myogenic bHLH genes (Delalande and Rescan, 1999). As somitogenesis proceeded along an anteroposterior axis, labelling appeared progressively in more caudal somites (Fig. 2A). Transverse sections through the developing somites showed that labelling for slow MyHC was at first restricted to medial cells close to the notochord (Fig. 2B). As the somites increase in height dorsoventrally, the number of slow-MyHC-positive cells increases to span the whole depth of the developing myotome (Fig. 2C). These presumptive slow cells then appear progressively more laterally (Fig. 2D) until they form the outermost regions of the myotomes (Fig. 2E). This apparent lateral migration of slow-MyHC-positive cells towards the superficial domain of the myotome was not completed rostrally before segmentation was complete.

Differentiation of fast muscle

The fast MyHC transcript was first detected in 25-somite embryos, and its presence was limited to the most rostral region of the trunk (Fig. 2F). Expression of fast MyHC progressed caudally as somites formed in a rostral-to-caudal wave (Fig. 2G). During somitogenesis, a dorsal view of the whole embryo showed that the fast MyHC labelling was restricted posteriorly to a thin medial domain of the somite close to the notochord whereas, more anteriorly, the labelling was found to fill the medial bulk of the somite (Fig. 2H). This medial-to-lateral spread of differentiation of fast muscle was confirmed

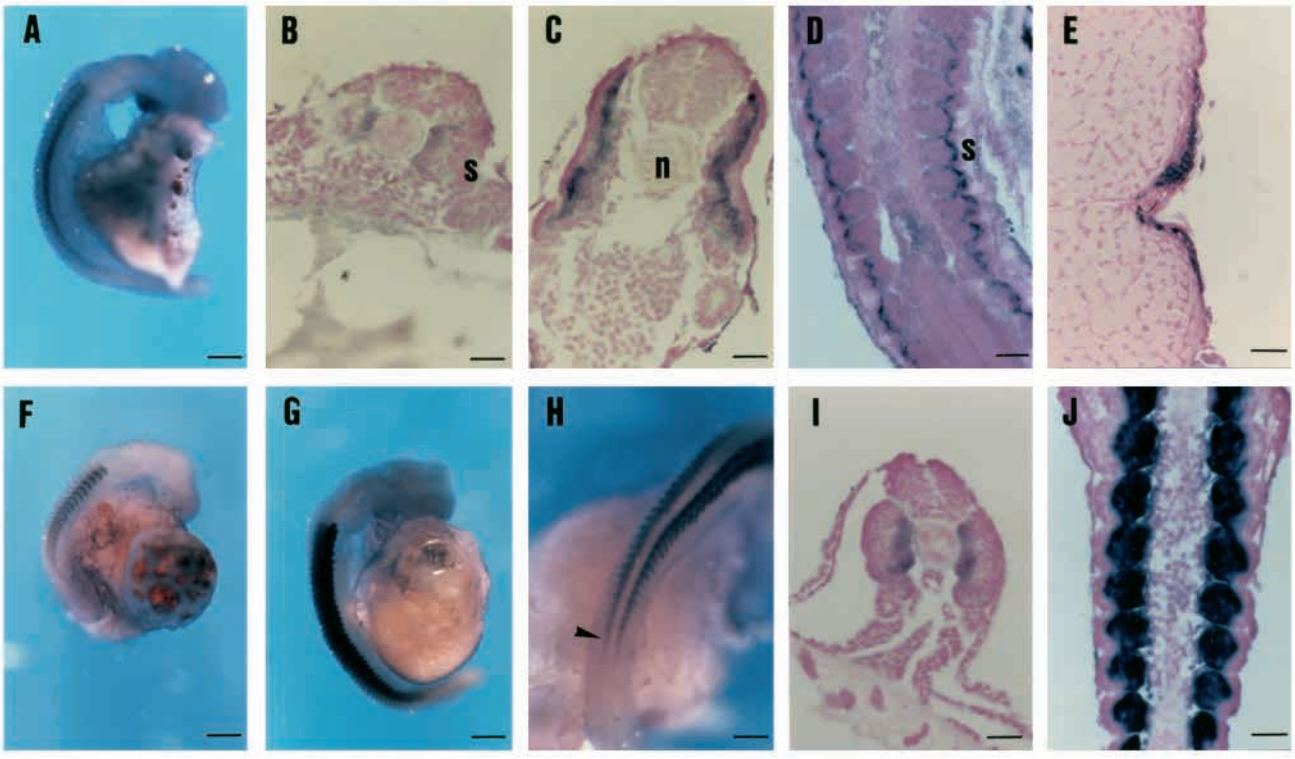

Fig. 2. Expression of slow (A–E) and fast (F–J) myosin heavy chain (MyHC) in trout embryo. (A) Stage 16 embryo (approximately 45 somites), lateral view. (B) Stage 15 embryo (35 somites), transverse section within a rostral somite pair. The label is restricted to the medial domain of the somite. (C) Stage 16 embryo, transverse section within a rostral somite pair. The slow-MyHC-positive cells have begun to move dorsally and ventrally. (D) Stage 17 embryo (50 somites), horizontal section. The slow-MyHC-positive cells progressively reach the lateral surface of the myotome. (E) Stage 22 embryo, transverse section. The slow-MyHC-positive cells are present in the outermost domains of the myotome. (F) Stage 14 embryo (30 somites), lateral view. The fast MyHC label is observed in the most rostral somites. (G) Stage 17 embryo, lateral view. The label progresses caudally as somites form. (H) Stage 16 embryo, dorsal view. The label is initially confined to medial cells in caudal somites (arrowhead) and spreads laterally as the somite matures rostrally. (I) Stage 15 embryo, transverse section within a somite of the trunk. The label is restricted to the medial domain of the somite. (J) Stage 17 embryo, horizontal section. The label fills most of the medial bulk of the somite. s, somite; n, notochord. Scale bars: A, 300 μm; B,C,D,J, 25 μm; E, 14 μm; F, 250 μm; G, 350 μm; H, 200 μm; I, 30 μm.

by an examination of sections through the trunk of embryos at different stage (Fig. 2I,J). The medial fast muscle cells differentiated before the lateral muscle cells within each individual somite.

Differentiation of fast muscle commences before the differentiation and lateral migration of slow muscle cells

To compare the timing and location of the differentiation of slow and fast muscle throughout somite development in trout, we performed double *in situ* hybridisation in embryos using a mixture of digoxigenin- and fluorescein-labelled probes. In all these embryos, it was clear that the expression of fast MyHC occurred medially before slow-MyHC-expressing cells were detected (Fig. 3A–C). Later, as the differentiation of fast muscle spread laterally, slow-MyHC-expressing cells became evident in the medial domain of the somite (Fig. 3D). These slow muscle cells progressively migrated lateral to the fast muscle cells (Fig. 3E). They eventually formed a distinct superficial monolayer of muscle fibres at the lateral surface of the myotome (Fig. 3F).

Discussion

In this study, we have examined the development of slow and fast muscle in the trout myotome using *in situ* hybridisation of mRNAs specific to the slow and fast muscle lineages. Our results show that the slow-MyHC-positive cells are initially present in the medial domain of the somite next to the axial structures. As the somite matures, an apparent migration of the slow-MyHC-positive cells occurs from the medial domain to the lateral surface of the somite, where they form the subcutaneous slow-twitch (red) muscle. We cannot exclude the possibility that this apparent migration reflects a wave of expression of slow MyHC. However, in the light of the work of Devoto et al. (Devoto et al., 1996) and Stoiber et al. (Stoiber et al., 1998), it is likely that the expression pattern we describe here for slow MyHC mRNA in the trout corresponds to a true migration of the slow muscle precursors within the developing somite. Comparing the trout with the zebrafish, it is apparent that there are differences in the timing of slow myoblast migration with respect to the somite stage. In the zebrafish, the slow muscle cells of the rostral somites migrate laterally before the end of somitogenesis; in the trout, the radial migration of the slow progenitors of the most rostral somites is not completed before the end of segmentation. Interestingly, the timing of differentiation of slow muscle described here contrasts with previous ultrastructural and histochemical analyses that led to the proposal that the differentiation of slow-twitch muscle occurs in salmonids only at around the time of hatching, when the transition from nutritional dependence on the yolk sac to exogenous feeding takes place (Nag and Nursall, 1972; Proctor et al., 1980).

Somewhat surprisingly, double *in situ* hybridisation with specific riboprobes for the slow and fast muscle lineages indicated that the expression of fast MyHC in the trout also commences immediately adjacent to axial structures and occurs before the differentiation and lateral migration of slow muscle cells. Hence, this challenges the concept, based on the zebrafish work, that the somitic medial cells give rise only to slow muscle progenitors, which differentiate before fast muscle progenitors (Devoto et al., 1996; Bladgen et al., 1997; Du et al., 1997). Although there are unquestionably common

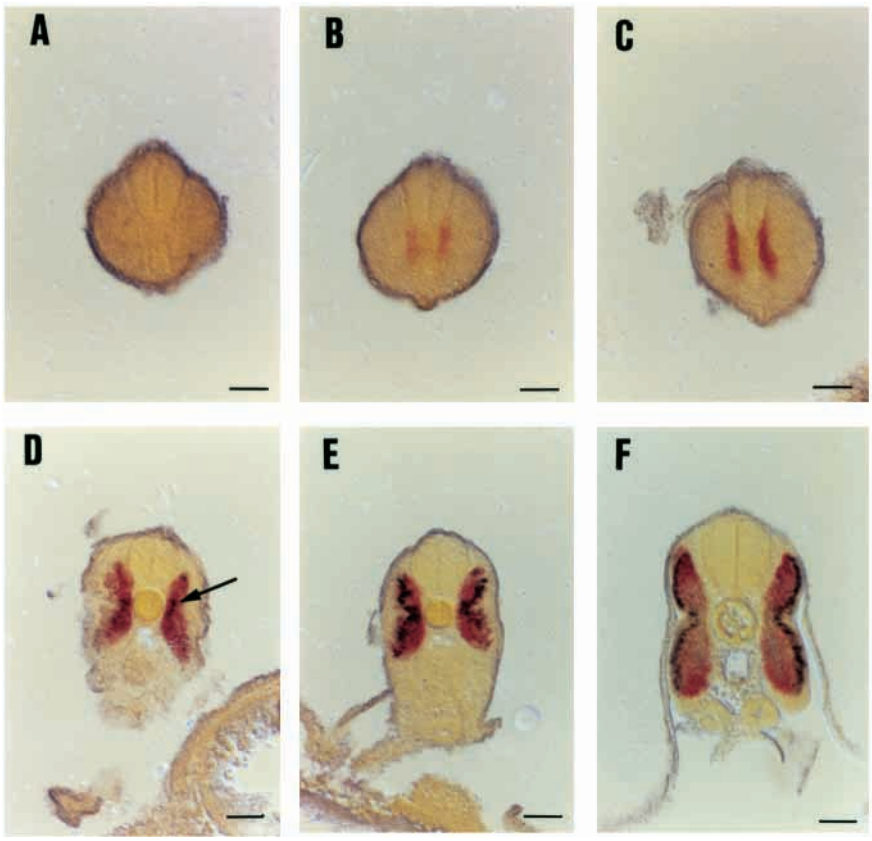

Fig. 3. Dual-colour *in situ* hybridisation for slow (dark blue) and fast (red) myosin heavy chain (MyHC). (A–E) Serial transverse sections of a stage 16 embryo (approximately 45 somites). (A) Posterior tail; fast and slow MyHC are still undetectable. (B) Middle tail; fast MyHC expression is evident in the medial domain of the somite. (C) Anterior tail; fast muscle cells now extend almost the full dorsoventral extent of the somite. (D) Posterior trunk (immediately anterior to the anal vent); the fast muscle cells fill the medial bulk of the somite. Slow muscle precursors are now evident and are mostly present in the medial domain of the somite (arrow). (E) Anterior trunk; the migrating slow muscle cells are now lateral to the differentiated fast muscle cells. (F) Transverse section through the middle trunk of an eyed-stage embryo (stage 20); the slow muscle cells have reached the outermost regions of the myotomes; other myogenic cells have differentiated into fast muscle. Scale bars, 45 μm.

features of muscle development among fishes, it would be a mistake to define a stereotypic myogenic pattern in teleosts by referring only to data published for the zebrafish.

In the trout, the initial differentiation of both fast and slow muscle takes place in a domain of the somite that is topologically under the influence of signals originating from the notochord. Thus, muscle-cell-type specification in this fish cannot simply result from differential exposure of the medial and lateral somitic cells to axial Hedgehogs, as demonstrated in zebrafish (Devoto et al., 1996; Bladgen et al., 1997; Du et al., 1997), but must involve a more complicated signal-induction network. If axial Hedgehog signalling plays a role in the induction of slow muscle differentiation in the trout, we must suppose that the trout fast muscle cell precursors, which are in the vicinity of the notochord, are insensitive to Hedgehog signals and that the slow muscle cell precursors are able to respond to these signals. In this regard, it would be interesting to analyse whether all the medial cells in the medial domain of the somite express the genes encoding patched (*ptc*), a transmembrane receptor involved in mediating Hedgehog signalling.

Bladgen et al. (Bladgen et al., 1997) used a monoclonal antibody raised against avian MyHC to detect fast myosin expression in their zebrafish studies. Fish myosins (Gerlach et al., 1990) are known to differ considerably from avian MyHCs. In the chicken, there are at least 36 different MyHC genes (Robbins et al., 1986). In fish, as in mammals and birds, there are both developmental and adult-type myosins. Therefore, the specificity and sensitivity of the avian antibody in detecting early expression of fast myosin in zebrafish somites should be questioned. This is one of several reasons why no conclusions could be drawn from previous work regarding the origin of the white myotomal muscle in fish. It is possible that the early expression of fast MyHC in medial somitic cells is actually a transient expression in slow muscle precursors that then later turn on slow MyHC expression. However, even accepting this hypothesis, our observations showing that the differentiation of fast muscle occurs within the developing myotome well before the lateral migration of the slow muscle progenitors are not in accord with the temporal sequences of muscle differentiation and myotome formation described in zebrafish embryos (Bladgen et al., 1997).

It is interesting to note that the medial portion of the somite, in which both fast- and slow-MyHC-positive cells are initially detected, corresponds to the part of the somite that is the first to express the bHLH myogenic regulators MyoD and myogenin (Delalande and Rescan, 1999) and where differentiated myotubes are first formed (Killeen et al., 1999). However, the main finding reported here is that, in trout, differentiation of both slow and fast muscle begins medially next to the notochord before extending progressively to more lateral regions of the developing somite.

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