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Temperature and the expression of seven muscle-specific protein genes during embryogenesis in the Atlantic cod *Gadus morhua* L.

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Summary

Seven cDNA clones coding for different muscle-specific proteins (MSPs) were isolated from the fast muscle tissue of Atlantic cod *Gadus morhua* L. *In situ* hybridization using cRNA probes was used to characterize the temporal and spatial patterns of gene expression with respect to somite stage in embryos incubated at 4°C, 7°C and 10°C. *MyoD* transcripts were first observed in the presomitic mesoderm prior to somite formation, and in the lateral compartment of the forming somites. *MyoD* expression was not observed in the adaxial cells that give rise to the slow muscle layer, and expression was undetectable by *in situ* hybridization in the lateral somitic mesoderm after the 35-somite stage, during development of the final ~15 somites. RT-PCR analysis, however, confirmed the presence of low levels of the transcript during these later stages. A phylogenetic comparison of the deduced amino-acid sequences of the full-length *MyoD* cDNA clone and those from other teleosts, and inference from the *in situ* expression pattern suggested homology with a second paralogue (*MyoD2*) recently isolated from the gilthead seabream *Sparus aurata*. Following *MyoD* expression, *α-actin* was the first structural gene to be switched on at the 16-somite stage, followed by *myosin heavy chain, troponin T, troponin I* and *muscle creatine kinase.* The final mRNA in the series to be expressed was *troponin C.* All genes were switched on prior to myofibril assembly. The *troponin C* sequence was unusual in that it showed the greatest sequence identity with the rainbow trout *Oncorhynchus mykiss* cardiac/slow form, but was expressed in the fast myotomal muscle and not in the heart. In addition, the third TnC calcium binding site showed a lower level of sequence conservation than the rest of the sequence. No differences were seen in the timing of appearance or rate of posterior progression (relative to somite stage) of any MSP transcripts between embryos raised at the different temperatures. It was concluded that myofibrillar genes are activated asynchronously in a distinct temporal order prior to myofibrillar assembly and that this process was highly canalized over the temperature range studied.

Key words: *Gadus morhua*, temperature, development, muscle, *in situ* hybridization, cod, myofibril, MyoD, myosin, troponin, creatine kinase.

Introduction

Muscle is an unusual and highly specialized tissue in that it consists predominantly of post-mitotic syncytial cells. Many proteins are expressed uniquely in muscle cells (termed muscle-specific proteins or MSPs; Xu et al., 2000). These include structural and contractile proteins (e.g. α-actins, myosins, troponins) as well as soluble muscle proteins and enzymes (e.g. muscle creatine kinase and parvalbumin). During differentiation, non-muscle isoforms of many proteins are downregulated, while muscle-specific isoforms begin to be expressed (Rubenstein and Spudich, 1977; Goncharova et al., 1992; Phillips et al., 1995). Much is known about the biochemistry of muscle contraction, but comparatively less about the process of myofibrillogenesis, and there are relatively few models. Most studies have relied on cell culture systems (Lin et al., 1994; Yoshimi et al., 1995; van der Ven and Furst, 1998), which may not reflect the *in vivo* situation (Costa et al., 2002). At the current time, whole embryo studies are limited to the mouse *Mus musculus* (Furst et al., 1989), zebrafish *Danio rerio* (Xu et al., 2000) and *Xenopus laevis* (Martin and Harland, 2001), and the relative timing of the onset of MSP expression appears to differ considerably between these species (see Costa et al., 2002). Teleost muscle is unique in many aspects of its structure compared to other vertebrates (Luther et al., 1995), and the extent to which myofibril assembly shows phylogenetic variation is unknown.

The first aim of the present study was therefore to characterize and investigate the expression of MSP genes required for myofibril assembly in the Atlantic cod *Gadus*

morhua L., including a full-length cDNA of *MyoD*. Myogenic regulatory factors (MRFs) of the *MyoD* gene family play a key role in lineage determination (*MyoD*, *Myf-5*) and in initiating and stabilizing the differentiation programme (*myogenin*, *MRF4*), in cooperation with other basic helix–loop–helix and MADS box transcription factors (MEF-2 proteins) (for reviews, see Sabourin and Rudnicki, 2000; Pownall et al., 2002; Johnston et al., 2002). The promoter regions of most muscle-specific genes, including *MyHC*, contain *MyoD* and *MEF-2* recognition sites (Giger et al., 2000; Wheeler et al., 1999). It has been shown that *MyoD* mRNA expression precedes the *de novo* expression of *MyHC IIB* mRNA in rat fast muscle following hind limb suspension (Wheeler et al., 1999). Other MSP cDNA clones characterized included *α-actin*, which forms the backbone of the thin filament in the myofibril (Gordon et al., 2000); *myosin heavy chain* (*MyHC*), which is the major component of the thick filament and the most abundant protein of the sarcomere (Lu et al., 1999); *muscle creatine kinase* (*CK-M*), which plays a central role in the catalysis of ADP to form high energy ATP (Wallimann et al., 1992), and the three troponin (Tn) subunit genes *troponin C* (*TnC*), *troponin I*, (*TnI*) and *troponin T* (*TnT*), which are involved in calcium binding and signal transduction (Filatov et al., 1999).

Temperature is known to have major effects on early muscle development in teleosts, altering the timing of myofibril assembly with respect to somite stage (Atlantic herring, Johnston et al., 1995), as well as the number and size of embryonic muscle fibres in numerous species (Stickland et al., 1988; Vieira and Johnston, 1992; Brooks and Johnston, 1993; Gibson and Johnston, 1995; Hanel et al., 1996; Matschak et al., 1998), including Atlantic cod, *Gadus morhua* (Galloway et al., 1998; Hall and Johnston, 2003). During ontogeny, embryonic isoforms of the myofibrillar proteins are gradually replaced by larval and adult isoforms, reflecting increases in body sizes and associated changes in swimming behaviour (Martinez et al., 1991; Chanoine et al., 1992; Mascarello et al., 1995). The relative timing of expression of developmental-stage specific isoforms varies for different myofibrillar components and is altered by rearing temperature (Johnston et al., 1997, 1998). For example, in Atlantic herring *Clupea harengus* the appearance of adult isoforms of myosin light chain 2, troponin T and troponin I occurred at longer body lengths in larvae reared at 5°C compared to 12°C (Johnston et al., 1997).

In a recent study in rainbow trout *Oncorhynchus mykiss* it was shown that MRF expression was delayed and prolonged at low compared to high egg incubation temperatures, and it was suggested that this resulted in a higher number of muscle fibres in hatched embryos, due to a longer period for proliferation of the myogenic precursor cells prior to terminal differentiation (Xie et al., 2001). In contrast, the onset of *MyoD* and *myogenin* expression relative to developmental stage was found to be similar at a range of temperatures in the Atlantic herring (Temple et al., 2001).

The second aim of the present study was therefore to test the hypothesis that differences in muscle cellularity with temperature in cod embryos (Hall and Johnston, 2003) are correlated with changes in the relative expression of MSP genes required for myofibril assembly. Somite stage was used as a normalized index of development at the different temperatures studied (Hall and Johnston, 2003; Hall et al., 2003).

Materials and methods

Larval rearing

Six female and six male cod *Gadus morhua* L. broodstock, approximately 6 years old, were caught by gillnet from Ardtoe Bay, Scotland, and kept in spawning tank systems of the type described by Huse and Jensen (1983). Three discrete clutches of newly fertilized eggs from different females were collected during March 2001. Clutches taken on consecutive days from the same tank were known to be spawned from different individuals, since the release of eggs is subject to an ovarian cycle lasting 36 h or more (Kjesbu et al., 1996). A third clutch was taken from a separate closed system. Within clutches, eggs were almost certainly of multiple paternity due to the spawning behaviour of this species (Hutchings et al., 1999; Bekkevold et al., 2002). Clutches were divided into three groups and incubated in 10 litre containers under three temperature conditions: 4°C, 7°C and 10°C (range ±0.2°C). The incubators were supplied with 0.1 μm filtered, UV-sterilized seawater (34‰) with a flow rate of 10 ml min^{-1}. Gentle aeration was constant and photoperiodicity was 8 h:14 h light (1.4 lux):total darkness. Prior to use, embryos were killed with an overdose of anaesthetic (MS-222).

Isolation of cDNA clones

Total RNA was extracted from embryos of mixed developmental stages using Tri-reagent (Sigma, Poole, UK). mRNA was purified from the total RNA using a poly-T^+ spin column (Amersham Pharmacia Biotech, Little Chalfont, UK). A first-strand reaction was carried out with 1 μg poly$(A)^+$ RNA using Superscript II reverse-transcriptase (Gibco BRL, Paisley, UK) and either an oligo-DT, 3′ RACE cDNA synthesis primer (Gibco BRL) or an oligo-DT, 5′ RACE cDNA synthesis primer (Clontech, Basingstoke, UK) in conjunction with a SMART II oligonucleotide (Chenchik et al., 1998). Primers were designed to conserved regions of multiple nucleotide sequence alignments of genes from related species (Tables 1, 2), prepared using the Clustal algorithm in Lasergene (DNAstar Inc, Madison, USA). Polymerase chain reaction (PCR) conditions were complex and often involved multiple rounds and touchdown cycles. Final PCR products were purified by agarose gel electrophoresis followed by gel extraction on a spin column (Qiagen, Crawley, UK). cDNAs were ligated into the PCR-4-TOPO vector (Invitrogen, Paisley, UK) and transformed into TOP-10-F competent cells (Invitrogen) according to the manufacturer's instructions. All clones were sequenced twice in either direction, and the nucleotide and deduced amino acid sequences submitted to the NCBI database (Table 1).

Table 1. *Details of muscle-specific protein clones and the most homologous cDNAs, determined by the DDJP blast engine*

Gene name	Abbreviation	Most homologous cDNA (species, GenBank accession no.)	Amino acid sequence identity (%)	Whole insert length (nt)	Coding region (nt) (F, full length)	GenBank accession no.
Myogenic determination factor	*MyoD*	Zebrafish, Z36945	64	1568	804 F	AF329903
Skeletal α-actin	*α-actin*	Alaskan pollack AB073380	100	1598	1131 F	AF500273
Myosin heavy chain	*MyHC*	Chum salmon AB076182	88	897	782	AY093703
Troponin T	*TnT*	Atlantic salmon AF072687	86	767	690 F	AF500272
Troponin I	*TnI*	Atlantic salmon U84394	62	817	516 F	AF498091
Muscle creatine kinase	*CK-M*	Mozambique tilapia AY034098	89	1187	777	AF329904
Troponin C	*TnC*	African clawed frog AB003080	81	589	367	AF500274

DDJP, DNA data bank of Japan.

In addition to the MSP genes, the 60S ribosomal subunit gene L15 was cloned for use as an internal standard during RT-PCR analysis. None of the clones have been previously isolated or sequenced elsewhere, all are novel sequences reported for the first time. Sequence manipulation was carried out using DNAman (Lynnon Biosoft, Vaudreuil, Canada) and Lasergene (DNAstar Inc.). The phylogenetic tree was constructed from a Clustal alignment followed by neighbour joining in PHYLIP (Felsenstein, 1995). Initial homology searches were carried out on the DNA data bank of Japan (DDBJ) protein-blast engine.

Preparation of DIG-labelled cRNA probes

Plasmids were linearized using *Spe*I or *Not*I restriction endonucleases and purified on an enzymatic cleanup spin column (Qiagen). 50 pg μl^{-1} of linear plasmid was used to transcribe the probes in a reaction containing $1/10^{th}$ volume DIG-RNA labeling mix (Roche, Lewes, UK), transcription buffer and 2 U μl^{-1} of the appropriate RNA polymerase (T3 for *Not*I digests, T7 for *Spe*I digests). Following incubation at 37°C for 2 h, the labelled probe was purified by lithium chloride/ethanol precipitation and dissolved in diethyl-pyrocarbonate (DEPC)-treated water, before storage at –80°C. All probes were made using the longest plasmid insert possible, with the exception of α-actin, the 3′ UTR of which was used to avoid cross-hybridization with β-actin, which is ubiquitously expressed (Xu et al., 2000).

In situ *hybridization*

20 embryos of mixed somite stages from each temperature group per cRNA probe were used for *in situ* hybridization. Sense probes were also used in each case as negative controls. *In situ* hybridization was carried out using a procedure incorporating aspects of those described by Wilkinson (1992) and Ennion et al. (1999), which permitted a high throughput with small embryos and gave an excellent signal. The

Table 2. *Primer sequences used for first-strand cDNA synthesis, and 3′ and 5′ RACE*

cDNA	3′ RACE forward primer	Product size (bp)	5′ RACE reverse primer	Product size (bp)
MyoD	AGATGCACGTCCACCAACCCGAACC	1143*	AAGGATCCCCACTTTGGGCAGCCTCTGG	512
α-actin	ACCTTCCAGCAGATGTGGATCAGCA	494*	AAGCACTTCCTGTGGACGATGGAGG	1194
MyHC	CAAGGAGCAGGCTGCTATGGTTGAGC	934*		
TnT			CCAAGCAGCAGAAGGGCCCGTT	447*
TnI	GATGAGGAGCGGTACGATGCTGCGG	516*	GCAGACATGCGCACCTTCTTCAGGG	447
CK-M	GGTGGCGATGACCTGGACCCC	1205*		
TnC	TGCATCAGTACCAAGGA	606*		

cDNA	RT-PCR analysis primers: Forward	RT-PCR analysis primers: Reverse	Product size (bp)
MyoD	GAGCCTTGGTCGAACATCACCGAC	GGAACTTTGATGAGCTTGCTTCTTATCAG	1262
L15	GCTCCCAGACCCACCAGACCC	ATGGCCTTGTGGAAGGTGTCG	311

MyoD, Myogenic determination factor; *α-actin*, skeletal α-actin; *MyHC*, myosin heavy chain; *TnT*, troponin T; *TnI*, troponin I; *CK-M*, muscle creatine kinase; *TnC*, troponin C.

RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase-polymerase chain reaction. For details, see Materials and methods.

Final product sizes calculated include the primer sequences.

*Clones used for *in situ* hybridization.

triethanolamine wash, antibody pre-absorption step and RNAse digestion step were found to be unnecessary, and use of CHAPS rather than SDS allowed briefer stringency washes carried out at a single temperature. Importantly, the length of hybridization was lengthened considerably and appeared to result in a stronger final signal.

Details of the *in situ* hybridization are as follows. Embryos were dechorionated on ice under a dissecting microscope using no. 10 watchmaker's forceps, then rehydrated at room temperature through 75% methanol:25% 0.1% Tween-20 in phosphate-buffered saline (PBST), 50%:50% methanol:PBST, 25%:75% methanol:PBST, followed by two washes in 100% PBST. Permeabilization was achieved by digestion in 20 μg ml^{-1} proteinase K in PBST for 10 min, followed by 2× 5 min washes in PBST. Embryos were refixed in 4% (w/v) paraformaldeyde, 0.1% gluteraldehyde in PBST, followed by 3× 5min washes in PBST. A pre-hybridization step was carried out in hybridization buffer [50% formamide, 2% (w/v) blocking reagent (Roche), 0.1% Triton X-100, 0.1% (w/v) CHAPS, 20 μg ml^{-1} yeast tRNA, 50 μg ml^{-1} heparin in 5 mmol l^{-1} EDTA] for 20 min at 70°C before addition of 0.5 μg ml^{-1} digoxygenin (DIG)-labelled cRNA probe. The hybridization step lasted for 3 days at 70°C. Subsequent to hybridization, the embryos were washed with decreasing stringency to remove unbound probe. The post-hybridization washes were carried out at 70°C and consisted of 2× 10 min in 2× SSC, 3× 20 min in 2× SSC, 0.1% (w/v) CHAPS, and a further 3× 20 min washes in 0.2× SSC, 0.1% (w/v) CHAPS. Embryos were then rinsed 2× 10 min in 'Heaven Seven' (HS) solution (150 mmol l^{-1} NaCl, 1% Tween-20 in 100 mmol l^{-1} Tris, pH 7.5) at room temperature followed by a 20 min blocking step in 20% sheep serum in HS. Bound probe was conjugated to an alkaline-phosphatase labelled anti-DIG antibody (Roche), which was used at a dilution of 1:4000, overnight at 4°C. Free antibody was removed by 4× 1 h washes in 1 mmol l^{-1} levamisole in HS. The final colour reaction was carried out in 'Divine Nine' (DN) solution (100 mmol l^{-1} NaCl, 1% Tween-20, 1 mmol l^{-1} levamisole, 100 mmol l^{-1} Tris, pH 9.5) containing 1 mg ml^{-1} of nitroblue tetrazolium (NBT) and 0.5 mg ml^{-1} of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). After 24 h of development in the dark at 4°C, the reaction was stopped with 4% paraformaldehyde in PBS. Photographs were taken on a binocular microscope

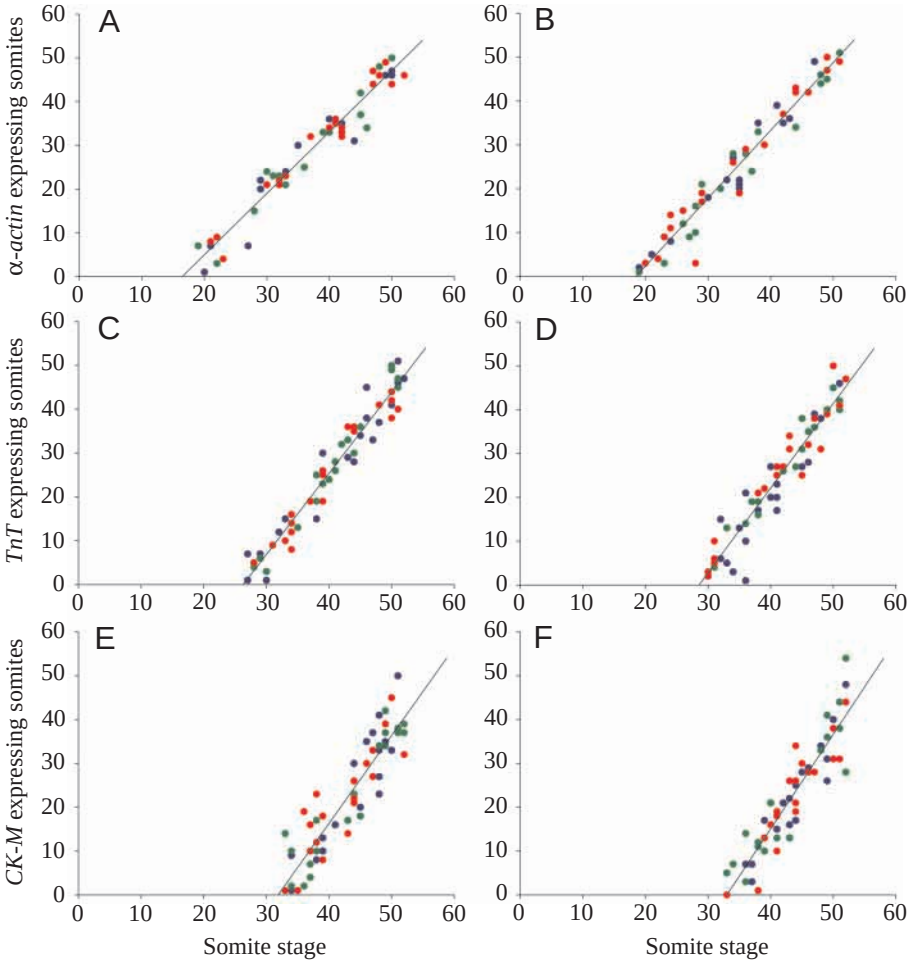

Fig. 1. Expression of myofibrillar muscle-specific protein mRNAs at different temperatures according to somite stage. (A) *α-actin*, (B) *myosin heavy chain* (*MyHC*), (C) *troponin T* (*TnT*), (D) *troponin I* (*TnI*), (E) *muscle creatine kinase* (*CK-M*) (F) *troponin C* (*TnC*). Blue, 4°C; green, 7°C; red, 10°C.

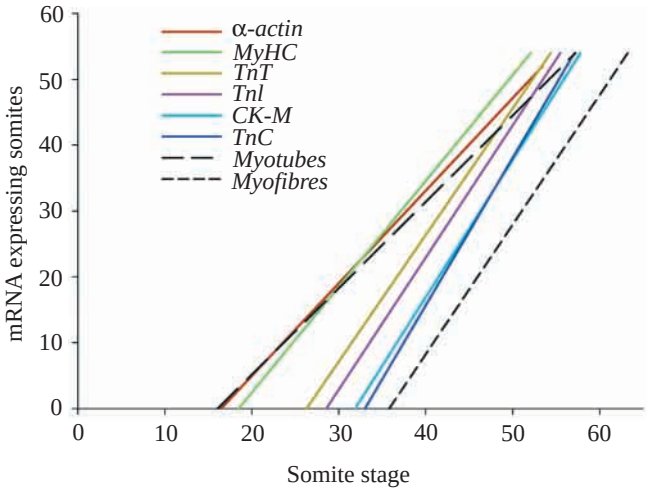

Fig. 2. Comparison of the regression lines of muscle-specific protein expression with somite stage, together with the regression lines of myotube and myofibril synthesis (from Hall and Johnston, 2003) against somite stage.

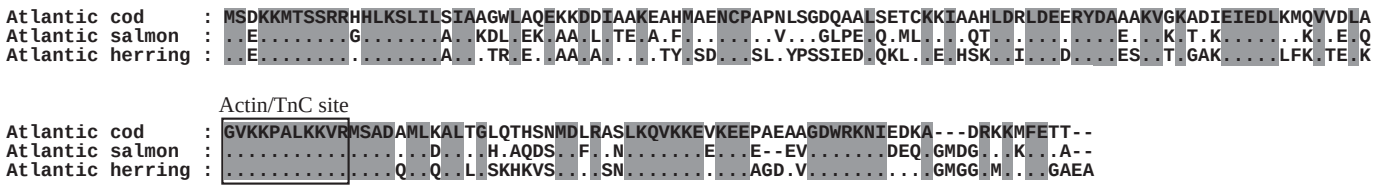

Fig. 3. Multiple sequence alignment of the deduced amino acid sequence of Atlantic cod *Gadus morhua* troponin I (TnI), with those of Atlantic salmon *Salmo salar* (sequence identity 62%) and Atlantic herring *Clupea harengus* (sequence identity 55%). The actin\Troponin C (TnC) binding site is boxed. Identical regions are shaded.

Fig. 4. Multiple sequence alignment of the deduced amino acid sequence of Atlantic cod *Gadus morhua* troponin T (TnT), with those of Atlantic salmon *Salmo salar* (sequence identity 86%) and zebrafish *Danio rerio* (sequence identity 81%). A region of low homology near to the N terminus is boxed. Identical regions are shaded.

(Lieca MZ7.5, Milton Keynes, UK) using darkfield illumination and Zeiss (Welwyn Garden City, UK) Axiocam imaging system.

Analysis of expression patterns.

Expression patterns were determined for the structural/contractile mRNAs by scoring the most posterior somite expressing a particular mRNA against the somite stage. For *MyoD*, however, this was not considered appropriate since expression preceded somite formation and was confined transiently to a small number of somites. Instead, expression was compared visually and RT-PCR analysis was carried out on RNA→cDNA extracted from different stages of development, as in Wyzykowski et al. (2002) and Lin-Jones and Hauschka (1997). Primers are shown in Table 2. Statistics, one-way analysis of variance (ANOVA) and multiple analysis of covariance (MANCOVA) were performed according to Zar (1999).

Results

The sequence of development of somites, myotubes and myofibril assembly was identical to that described by Hall and Johnston (2003). Somite development proceeded in a rostral-caudal wave, beginning at 122 h in the 4°C group, 75 h in the 7°C group and 53 h in the 10°C group. Further somites were added at intervals of 4.5 h, 3.0 h and 2.3 h, respectively. The first myotubes appeared in the anteriormost somites at approximately the 12-somite stage and the first myofibrils could be discerned in the most rostral somites at approximately the 37-somite stage. There was no difference in the rate of timing of myotube or myofibril appearance, or rate of their posterior progression, between temperature groups (Figs 1, 2; Hall and Johnston, 2003).

Sequence analysis

Of the amplified cDNAs, deduced amino acid sequence conservation was generally high. α-actin was the most highly

Fig. 5. Multiple sequence alignment of the deduced amino acid sequence of Atlantic cod *Gadus morhua* troponin C (TnC), with those of rainbow trout *Oncorhynchus mykiss* and Atlantic salmon *Salmo salar* (sequence identity 83% in both cases). Ca^{2+} binding sites II, III and IV are boxed. Identical regions are shaded.

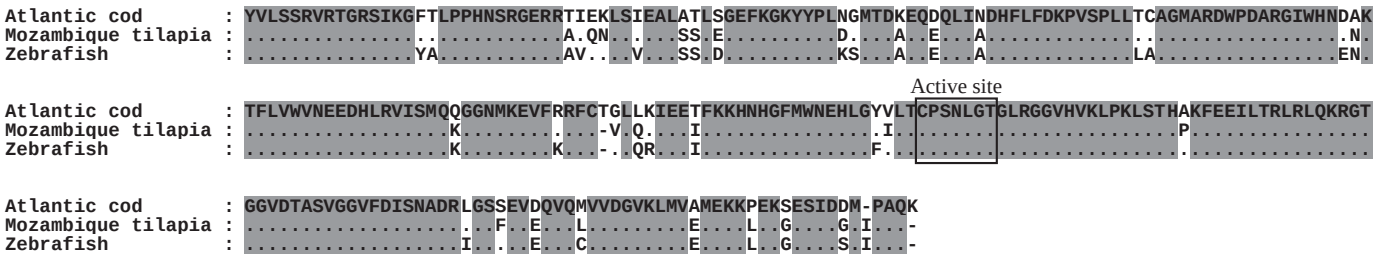

Fig. 6. Multiple sequence alignment of the deduced amino acid sequence of Atlantic cod *Gadus morhua* creatine kinase (CK-M), with those of Mozambique tilapia *Oreochromis mossabicus* (sequence identity 89%) and zebrafish *Danio rerio* (sequence identity 87%). The active site motif is boxed. Identical regions are shaded.

conserved, with an identical deduced amino acid sequence to those of Alaskan pollack *Theragra chalcogramma* and rat-tail fish *Coryphaenoides cinereus* (Table 1).

The MyHC cDNA was a partial sequence (the full coding cDNA is >5000 bp in carp; Hirayama and Watabe, 1997). The isolated clone came from the 3′ end and spanned 779 bp of coding region and 89 bp of untranslated region (UTR). The deduced amino acid sequence most closely matched that of chum salmon *Oncorhynchus keta* adult fast skeletal muscle MyHC, exhibiting 88% sequence identity (Table 1). No specific functional domains were defined in this region.

Troponin I (TnI) exhibited the lowest identity with published sequences, 62% when compared with Atlantic salmon *Salmo salar*, and just 55% when compared to Atlantic herring (Table 1, Fig. 3). The most highly conserved region within the TnI molecule was the actin/troponin C (TnC) binding site located towards the center of the sequence. This binding site exhibited the motif characteristic of invertebrates (*K*PXL*K*) rather than that usually seen in vertebrates (*R*PXL*R*).

Troponin T (TnT) showed 86% sequence identity with the Atlantic salmon isoform 3 (Table 1). A short region of low conservancy at the N terminus (Fig. 4) was present in a position that spans several known splice sites in avian and mammalian TnTs (Smillie et al., 1988).

Surprisingly, troponin C most closely matched the trout cardiac/slow isoform (Table 1), despite being expressed exclusively in the skeletal muscle and not in the heart (see Fig. 8, below). Sequence identity was 83% with both the trout and salmon cardiac/slow sequences (Fig. 5) and 81% with the *Xenopus* cardiac/slow sequence (not shown). The portion of the TnC sequence isolated incorporated the second, third and fourth Ca^{2+} binding sites (Fig. 5). Sites II and IV were highly conserved, exhibiting only five differences in 65 residues (92% identity) with the trout sequence. Site III showed less conservation, sharing 64% sequence identity with that of the trout.

The fast muscle creatine kinase (CK) clone was most closely related to that from Mozambique tilapia *Oreochromis mossambicus* showing 89% identity in deduced amino acid sequence (Table 1), and 87% with that of the zebrafish. It was possible to recognise the active site motif CPSNLGT (Fig. 6), which is absolutely conserved between all known CK isoforms (Taylor et al., 1990; Fritz-Wolf et al., 1996).

When blast searched on the DDBJ protein-blast engine, the full-length cod MyoD amino acid sequence showed greatest identity with zebrafish MyoD (64%; Table 1). The most conserved regions were the basic and the helix–loop–helix domains, with the extent of sequence identity declining towards the C terminus (Fig. 7).

In situ *hybridization*

mRNA signals for all of the MSP genes began in the most differentiated somites at the anterior of the embryo and progressed rostro–caudally, mirroring the pattern of somite formation (Fig. 8). The onset of expression of different genes was sequential, *MyoD* being the first to be expressed in a single band in the presomitic mesoderm. Upon formation of somites,

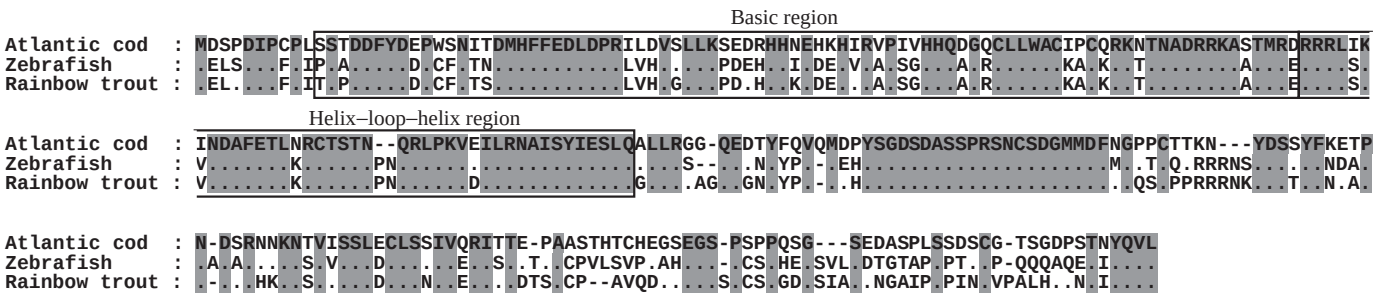

Fig. 7. Multiple sequence alignment of the deduced amino acid sequence of Atlantic cod *Gadus morhua* MyoD, with those of zebrafish *Danio rerio* and rainbow trout *Oncorhynchus mykiss* (sequence identity 64% in both cases). Basic and helix–loop–helix domains are boxed. Identical regions are shaded.

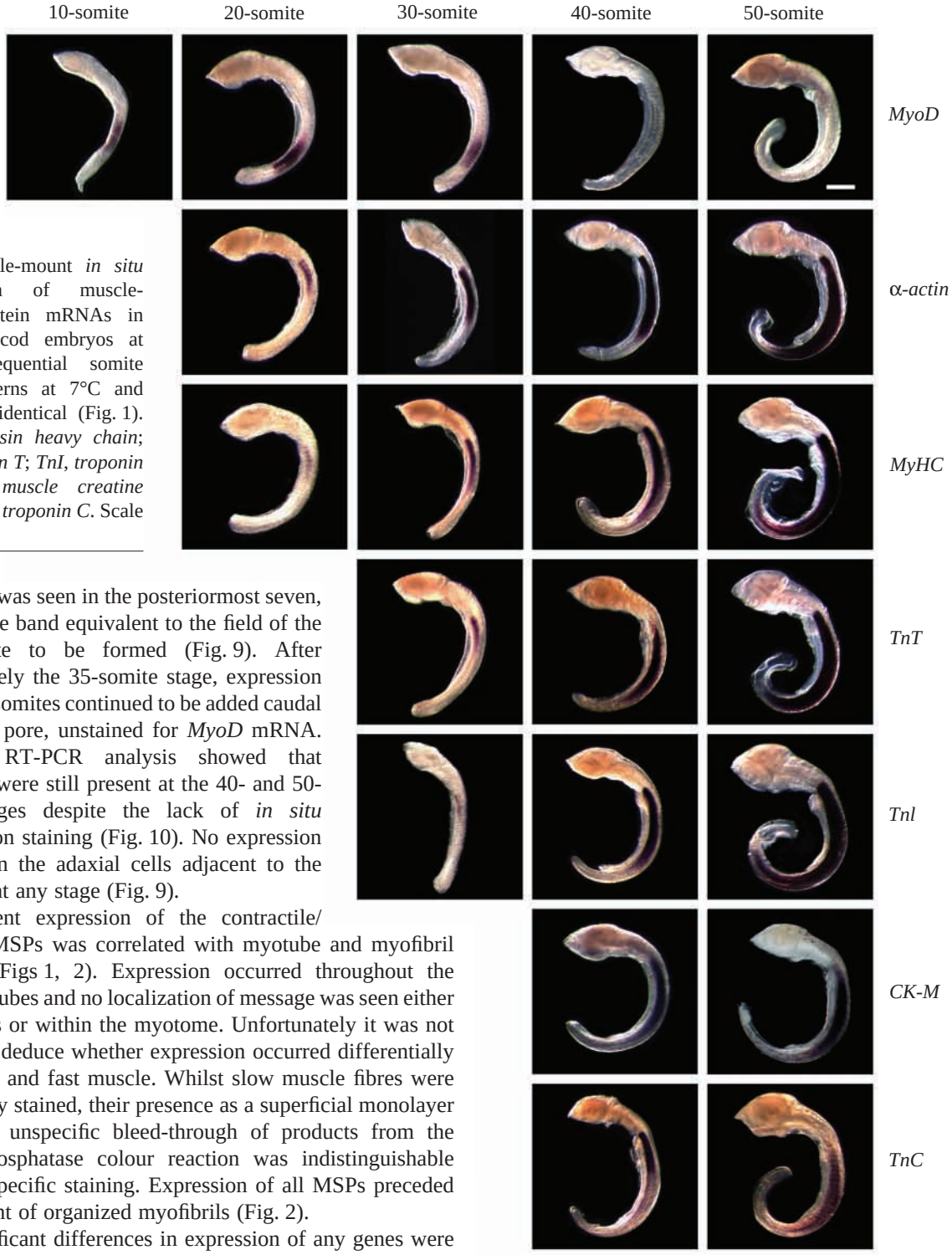

Fig. 8. Whole-mount *in situ* hybridization of muscle-specific protein mRNAs in developing cod embryos at 4°C at sequential somite stages. Patterns at 7°C and 10°C were identical (Fig. 1). *MyHC*, *myosin heavy chain*; *TnT*, *troponin T*; *TnI*, *troponin I*; *CK-M*, *muscle creatine kinase*; *TnC*, *troponin C*. Scale bar, 200 μm.

expression was seen in the posteriormost seven, plus a single band equivalent to the field of the next somite to be formed (Fig. 9). After approximately the 35-somite stage, expression faded, and somites continued to be added caudal to the anal pore, unstained for *MyoD* mRNA. However, RT-PCR analysis showed that transcripts were still present at the 40- and 50-somite stages despite the lack of *in situ* hybridization staining (Fig. 10). No expression was seen in the adaxial cells adjacent to the notochord at any stage (Fig. 9).

Subsequent expression of the contractile/structural MSPs was correlated with myotube and myofibril synthesis (Figs 1, 2). Expression occurred throughout the fibres/myotubes and no localization of message was seen either within cells or within the myotome. Unfortunately it was not possible to deduce whether expression occurred differentially in the slow and fast muscle. Whilst slow muscle fibres were undoubtedly stained, their presence as a superficial monolayer meant that unspecific bleed-through of products from the alkaline-phosphatase colour reaction was indistinguishable from any specific staining. Expression of all MSPs preceded development of organized myofibrils (Fig. 2).

No significant differences in expression of any genes were seen between embryos raised at different temperatures (Table 3, Fig. 1). Expression of *MyoD* was not scored against somite stage, because it was expressed before the first somite formed, but the expression pattern remained the same at different temperatures nonetheless. In all cases the characteristic pattern of expression of a single stained band in the presomitic mesoderm followed by seven stained somites was observed. At the initiation of myogenesis, however, the body axis of embryos incubated at 10°C was frequently slightly shorter than those incubated at lower temperatures, as a result of asynchrony between the extent of epiboly and segmentation in these fish (Fig. 11; Hall and Johnston, 2003). RT-PCR analysis showed that no transcript was detectable at the embryonic shield stage, shortly prior to the initiation of myogenesis, but as segmentation began, expression of *MyoD* was switched on (Fig. 10). Furthermore, low-level expression persisted even after completion of formation of somites at the

~50-somite stage, when transcripts are no longer visible by *in situ* hybridization (Fig. 8).

When the expression patterns of different MSP transcripts were compared with each other by MANCOVA, all were highly significantly different in terms of slope or intercept, with the exception of *MyHC*/*α-actin* and *TnC*/*CK-M* (Tables 4, 5, Fig. 2).

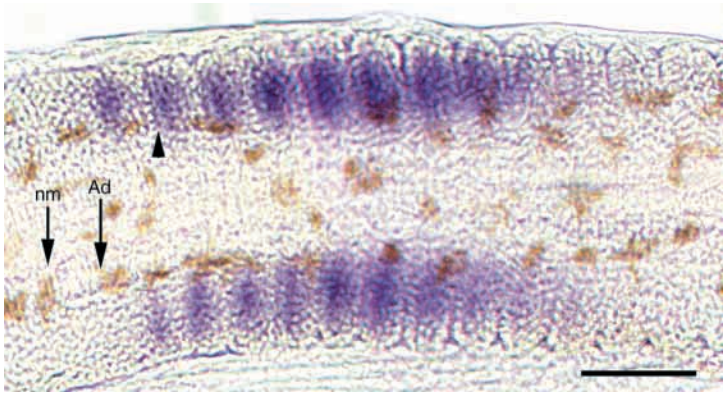

Fig. 9. *MyoD* expression in the developing somites of a 15-somite embryo. Expression can be seen the posteriormost seven somites, and in a single band in the presomitic mesoderm at the site of imminent somite formation. The anteriormost somites are by this time unstained for *MyoD*. Arrowhead, most recent somite furrow. nm, notochord/mesoderm boundary; Ad, unstained adaxial cells. Scale bar, 100 μm.

Discussion

Despite the differences in fibre number shown previously (Hall and Johnston, 2003), there was no difference in the timing of expression of the seven MSPs studied between temperature groups. Although there are many additional MSPs involved in myofibrillar assembly, these data do not support the hypothesis that differences in fibre number are related to differences in the timing of expression of myofibrillar genes. However, the discrete, sequential patterns seen between most of the mRNAs reinforces the notion of muscle differentiation as a set of centrally regulated, concomitant but autonomous steps (Costa et al., 2002).

A common feature of many muscle proteins is that they have multiple isoforms, which are generated either from separate genes or by alternate mRNA splicing from the same gene. This situation is complicated in fish by an ancestral whole-genome duplication event (Meyer and Schartl, 1999; Taylor et al., 2001a), which is thought to have occurred after the radiation of the sarcopterygian lineage, which includes all terrestrial vertebrates. A second tetraploidization of the salmonid genome is also thought to have taken place more recently (Allendorf and Thorgaard, 1984; Rescan and Gauvry, 1996). Evidence for genome duplication events is far from conclusive (see Taylor et al., 2001a,b,c; Robinson-Rechavi et al., 2001a,b), but whatever the reason, teleost genomes are characterized by expanded gene families (Robinson-Rechavi et al., 2001c).

There are at least six different actin genes present in mammals, the products of which are expressed in various and overlapping cell types. β- and γ-actins are constituents of the cytoskeleton and are ubiquitously expressed. A further four actins are specific to muscle cells. These include two striated muscle (α-skeletal and α-cardiac) and two smooth muscle (α-aortic and γ-enteric) actins (Vandekerckhove and Weber, 1978, 1979). Polymerized actin chains form the backbone of the thin filament, consisting predominantly of mixed α and β chains (Gordon et al., 2000). Single cDNAs encoding skeletal muscle α-actin have been isolated from channel catfish *Ictalurus punctatus* (Kim et al., 2000), common carp and goldfish (Watabe et al., 1995). However, in a recent study of the Japanese pufferfish *Fugu rubripes*, Venkatesh et al. (1996) isolated nine distinct genomic actin clones. These were classified as two skeletal α-actins, three α-cardiac actins, one testis-type α-actin, two β-actins and one vascular β-cytoplasmic actin. The two skeletal muscle types had identical genomic organization and differed in only five amino acid residues. Such high sequence identity is a common feature of

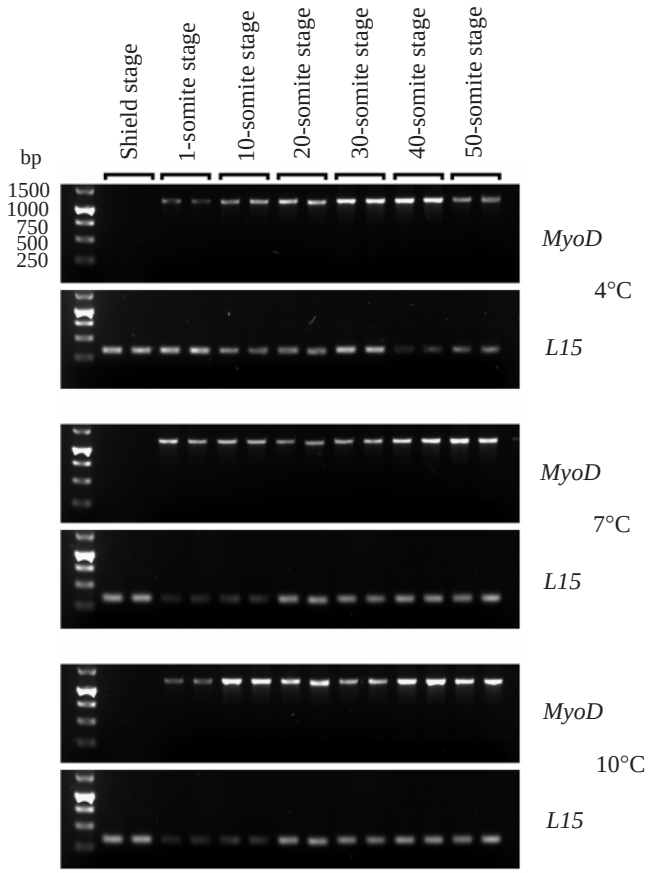

Fig. 10. RT-PCR analysis of *MyoD* expression in different stage embryos developing at different temperatures. At all temperatures, *MyoD* transcripts are switched on as the somites began to form. Note that despite the lack of *in situ* staining for *MyoD* beyond approximately the 35-somite stage (Fig. 10), transcripts are still present. L15 is a 60S ribosomal subunit 'housekeeping' gene, expressed at all temperatures and in all stages, used as an internal standard. The positions of marker nucleotides (bp) are shown at left.

Table 3. *Regression data for the expression of muscle-specific protein genes in relation to somite stage*

Gene expressed	Slope	Intercept	r^2 (adjusted)	*F* (slopes)	*F* (intercepts)	Residual d.f.
α-actin	1.42	–23.55	94.6	0.031	0.326	58
MyHC	1.55	–28.62	94.9	0.079	0.235	58
TnT	1.85	–48.64	94.4	1.064	0.358	58
TnI	1.93	–55.09	90.9	0.051	0.152	58
CK-M	2.01	–63.96	83.1	0.708	0.140	58
TnC	2.16	–71.15	83.8	0.204	0.724	58

α-actin, skeletal α-actin; *MyHC*, myosin heavy chain; *TnT*, troponin T; *TnI*, troponin I; *CK-M*, muscle creatine kinase; *TnC*, troponin C.
$P<0.001$ for all regressions.
F values represent the results of analysis of covariance (ANCOVA) between 4, 7 and 10°C groups; in all cases there was no significant interaction between temperature and expression ($P>0.05$).

actins, even between distantly related species. This was consistent with the finding that cod skeletal *α-actin* exhibited 100% deduced amino acid sequence identity with that of Alaskan pollack and rat-tail fish (Table 1). The expression pattern was also comparable with that of zebrafish skeletal *α-actin* (Xu et al., 2000), being switched on shortly after the onset of somitogenesis and before the expression of *MyHC*.

The MyHCs of vertebrates are encoded by multi-gene families and are expressed in a tissue-specific manner (Konig et al., 2002). It has been reported that there are at least eight skeletal and two cardiac MyHCs in humans (Soussi-Yanicostas et al., 1993). However, in the common carp *Cyprinus carpio* over 29 different genomic sequences have been identified (Kikuchi et al., 1999). Different *MyHC* mRNA isoforms in teleosts have been shown to be expressed under different conditions of temperature (Imai et al., 1997), at different stages of development (Ennion et al., 1999) and in different fibre types (Rescan et al., 2001). In the present study, no difference in *MyHC* expression was seen with different rearing temperature. It is possible that the probe used in this study bound heterologously to multiple *MyHC* mRNA isoforms, since the proportion of 3′ UTR:cds was much greater in this case than with the other clones used (89 bp:779 bp, respectively). It is equally possible that the probe hybridized to a specific isoform, which showed a broad pattern of expression.

Troponin (Tn), is an actin-associated protein complex, consisting of three interacting subunits, each with an identifying letter from the first identified property: troponin C (TnC) binds Ca^{2+}, troponin I (TnI) binds to actin and inhibits the actomyosin ATPase and troponin T (TnT) links the Tn complex to tropomyosin (Gordon et al., 2000). Three *TnT* genes exist in mammals, encoding fast, slow and cardiac forms (Huang et al., 1999). In the mouse, these are alternatively spliced into at least 13 fast isoforms (Wang and Jin, 1997) and three slow isoforms (Jin et al., 1998). Splicing usually occurs close to the N terminus and different splice variants are thought to be involved in ontogenetic changes in phenotype and in different fibre-typing in the adult (Briggs and Schachat, 1996). Five TnT protein isoforms have been isolated from Atlantic salmon, two from slow muscle and three from fast muscle (Waddleton et al., 1999). A single fast muscle cDNA has been isolated from the zebrafish (Xu et al., 2000). The *TnT* isoform isolated from cod showed greatest identity with an Atlantic salmon fast muscle isoform. The most degenerate area of sequence was at the N terminus, in a region spanning several known splice sites in mammals (Smillie et al., 1988).

As with *TnT*, the *TnI* isoform found in this study showed greatest identity with an Atlantic salmon fast muscle isoform. Three genes code for slow, fast and cardiac isoforms of TnI in birds and mammals (Guenet et al., 1996; Mullen and Barton,

Table 4. *MANCOVA comparison of the slopes of the regression lines of expression between different muscle-specific protein genes (all temperatures)*

	MyHC	*TnT*	*TnI*	*CK-M*	*TnC*
α-actin	NS	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.001$
MyHC		$P<0.05$	$P<0.01$	$P<0.001$	$P<0.001$
TnT			NS	NS	NS
TnI				NS	NS
CK-M					NS

MANCOVA, multivariate analysis of covariance.
NS, not significant.
α-actin, skeletal α-actin; *MyHC*, myosin heavy chain; *TnT*, troponin T; *TnI*, troponin I; *CK-M*, muscle creatine kinase; *TnC*, troponin C.

Table 5. *MANCOVA comparison of the intercepts of the regression lines of expression between different muscle-specific protein genes (all temperatures)*

	MyHC	*TnT*	*TnI*	*CK-M*	*TnC*
α-act	NS	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.001$
MyHC		$P<0.001$	$P<0.001$	$P<0.001$	$P<0.001$
TnT			$P<0.01$	$P<0.001$	$P<0.001$
TnI				$P<0.001$	$P<0.001$
CK-M					NS

MANCOVA, multivariate analysis of covariance.
NS, not significant.
α-actin, skeletal α-actin; *MyHC*, myosin heavy chain; *TnT*, troponin T; *TnI*, troponin I; *CK-M*, muscle creatine kinase; *TnC*, troponin C.

2000). Much less is known about TnI in fish. Three cDNAs have been isolated from salmon (Jackman et al., 1998) and one from herring (Hodgson et al., 1996). Surprisingly, however, in each case the amino acid sequence identity was considered too low to assign orthology to either the fast, slow or cardiac varieties seen in other vertebrates. In addition, the actin/TnC binding site contained a motif (*K*PXL*K*) peculiar to invertebrates, rather than that seen in avian and mammalian species (*R*PXL*R*). This same, invertebrate-type motif was also seen in the cod sequence found in this study (Fig. 3). The significance of this finding is unknown, but it appears that TnI is more heterologous in fishes than in other vertebrates. At the protein level too, Crockford et al. (1991) resolved two different isoforms of TnI being co-expressed in the fast fibres of *Oreochromis niloticus* and *O. andersoni*, using two-dimensional gels and affinity chromatography. No genomic, cDNA or proteomic information on TnI has yet been presented for the zebrafish.

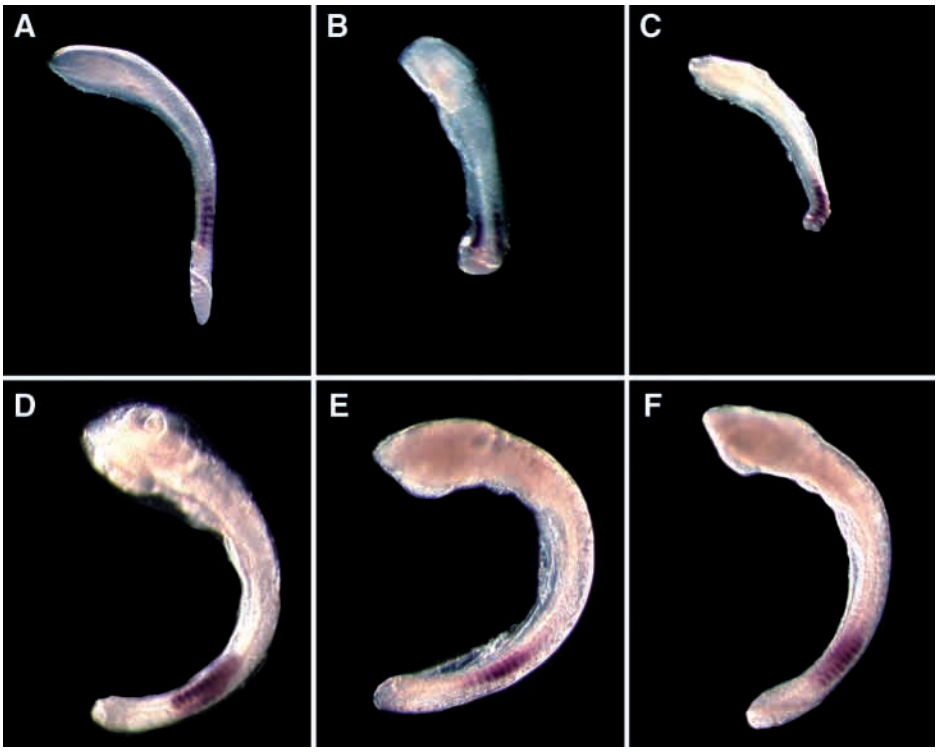

Fig. 11. *MyoD* expression in 5-somite (A–C) and 35-somite (D–F) embryos at 4, 7 and 10°C. Note that the 7 and 10°C embryos are shorter than the 4°C embryos, reflecting differences in the extent of epiboly between temperature groups (Hall and Johnston, 2003). However, the expression of *MyoD* in relation to somite development remains constant. Scale bar, 300 μm.

Troponin C exists as two distinct tissue-specific types rather than the three exhibited by the other Tn subunits. In birds, mammals and amphibians, a fast-fibre type and a type specific to both slow and cardiac fibres have been identified (Reinach and Karlsson, 1988; Gahlmann and Kedes, 1990; Parmacek et al., 1990; Jin et al., 1995; Tiso et al., 1997; Warkman and Atkinson, 2002). Numerous studies have investigated aspects of TnC function in teleosts at the protein level (Demaille et al., 1974; McCubbin et al., 1982; Gerday et al., 1984; Feller and Gerday, 1989; Crockford and Johnston, 1993; Francois et al., 1997), but until now the only published nucleotide sequences were those from zebrafish (showing highest identity to *Xenopus* fast-type; Xu et al., 2000) and trout (showing highest identity to *Xenopus* slow/cardiac-type; Moyes et al., 1996). Because protein sequences with homology to both fast-skeletal and slow/cardiac forms have been isolated from fish, it has been supposed that their nature is equivalent to those in birds and mammals (Yuasa et al., 1998). In this study, however, the cod *TnC* sequence showed highest identity with the trout cardiac form, but was only expressed in the myotomal muscle, and not in the heart. Tissue-specific expression has not been investigated in the trout, but the timing of expression shown in this study was quite different to that of the zebrafish fast-type *TnC* shown by Xu et al. (2000). In zebrafish, fast-type *TnC* was one of the first MSPs to be switched on, whereas in the cod, expression occurred last out of the seven MSPs, towards the end of somitogenesis. It may be, therefore, that TnC expression in teleosts is more complex than previously thought, as has been demonstrated in the case of TnI.

Fast skeletal muscle TnC contains four Ca^{2+} binding sites, which facilitate conformational changes in the protein according to calcium concentration. Sites I and II, in close proximity to the N terminus, have a lower Ca^{2+} affinity than sites III and IV, which are located towards the C terminus. In the cardiac/slow form of TnC, site I is non-functional. The nucleotide sequence isolated from cod did not cover site I, so comparison of this region could not be made with fast and slow isoforms from other species. However, binding sites II and IV were highly conserved, sharing 89% identity with the trout sequence. Site III was less highly conserved, sharing 64% sequence identity with the trout sequence.

In cells and tissues with intermittently high and fluctuating energy requirements, including skeletal muscle, creatine kinase plays a central role in the catalysis of the reversible transfer of a phosphate ion from phosphorylcreatine to ADP to form high energy ATP (Wallimann et al., 1992). Creatine kinase enzymes constitute a family of different isoforms with tissue-specific expression and isoenzyme-specific subcellular localization (Stolz and Wallimann, 1998). Four isoform types are present in all vertebrates: a cytosolic brain-type (CK-B), a cytosolic muscle-type (CK-M) and two mitochondrial types, CK-MiA and CK-MiB (Benfield et al., 1984; Ordahl et al., 1984; Sun et

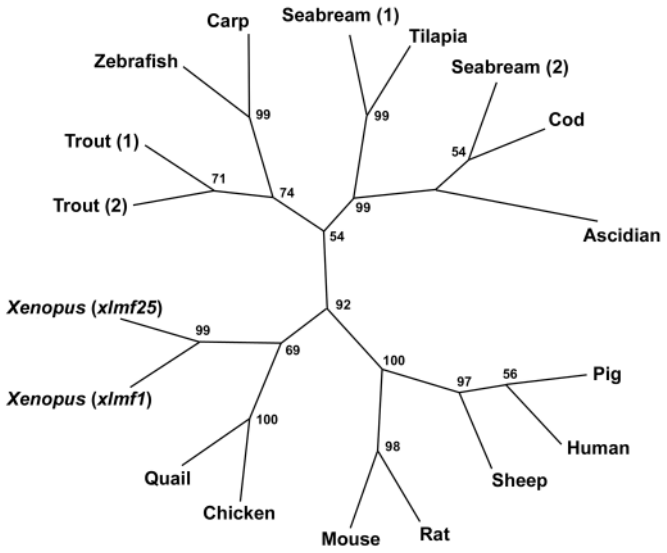

Fig. 12. Phylogenetic relationships of the vertebrate MyoD sequences published to date. Note that the cod sequence clusters with the seabream MyoD2 sequence. Similarities are also evident in the *in situ* expression patterns. The tree was constructed in PHYLIP (Felsenstein 1995) by the neighbour-joining method. Node numbers refer to the percentage of bootstrap trials supporting a clade. Bootstrap confidence is based on 1000 pseudoreplications. The amino acid sequence for the single myogenic factor (AMD1) of the ascidian *Halocynthia roretzi* (accession no. D13507) was used as the outgroup. Other accession numbers are seabream *Sparus aurata* MyoD1, AF478568, MyoD2 AF478569; blue tilapia *Oreochromis aureus*, AF270790; Atlantic cod *Gadus morhua*, AF329903; pig *Sus scrofa*, U12574; human *Homo sapiens*, NM_002478; sheep *Ovis aries*, X62102; rat *Rattus norvegicus*, M84176; mouse *Mus musculus*, XM_124916; chicken *Gallus gallus*, L34006; *Cotornix cotornix*, L16686; *Xenopus laevis xlmf1*, M31116, *xlmf25* M31118; rainbow trout *Oncorhynchus mykiss* MyoD1, X75798, MyoD2, Z46924; zebrafish *Danio rerio*, NM_131262; common carp *Cyprinus carpio*, AB012882.

al., 1998). The genetic organization of the creatine-kinase enzymes has been little studied in any species, although it is known that three sub-isoforms of CK-M are encoded by three different genes in the common carp (Sun et al., 1998) and that at least two *CK-M* genes exist in the zebrafish (Harder and McGowan, 2001) and in the channel catfish (Liu et al., 2001). Only one *CK-M* gene has been identified in any mammalian species and it has been argued that the multiple copies found in teleosts play a role in overcoming the rate-depressing effect of seasonal cooling, and therefore help to retain muscle function over a broad temperature range (Sun et al., 1998). The number of *CK-M* encoding genes in the cod is not known, but the *CK-M* mRNA isoform examined in this study did not show any change in its timing of expression, with respect to somite stage, between different rearing temperatures.

There is an apparent paradox regarding the myofibrillar proteins, in that despite the large number of splice variants found, and the enormous number theoretically possible (Miyadzaki et al., 1999), only a relatively small number of protein isoforms have been isolated (Yao et al., 1992). This could be for several reasons; it might be that the differences in protein structure are so subtle as to not be resolved by currently available techniques, or that many proteins are inefficiently transcribed or not stably incorporated into the myofibrils. Alternatively, the explanation could simply be that isoform diversity is not so important at the level of the protein. Duplication of genes at the genomic level might allow them to be placed under different conditions of transcriptional control, and divergence in mRNA primary structure might be a prerequisite for differential timing and maintenance of translation.

Expression of *MyoD* in the cod was very unusual in that the expression pattern appeared to be more limited than that shown previously in the zebrafish (Weinberg et al., 1996) and in the herring (Temple et al., 2001). *MyoD* was not expressed in the adaxial cells adjacent to the notochord, and was undetectable with ISH after approximately the 35-somite stage (Fig. 8). It is well known that two paralogues of *MyoD* exist in the salmonids, apparently produced from a recent tetraploidization of the salmonid genome (Rescan and Gauvry, 1996), and that these genes have diverged in function (Delalande and Rescan, 1999). However, two non-allelic *MyoD* genes have recently been cloned from the (non-salmonid) gilthead seabream *Sparus aurata* (Tan and Du, 2002). In this case, *MyoD2* transcripts are much more restricted in their expression pattern than *MyoD1*. To test the hypothesis that the cod *MyoD* clone was an orthologue of seabream *MyoD2*, a neighbour-joining phylogenetic tree was constructed in PHYLIP (Felsenstein, 1995), using all available full-length vertebrate *MyoD* sequences. Whilst the two trout paralogues clustered together, the seabream paralogues were more highly divergent. In addition, the seabream *MyoD2* and cod *MyoD* sequences also clustered together (Fig. 12).

In summary, muscle development in the Atlantic cod is canalized over the temperature range studied (4–10°C). Although the number of fibres has been shown to differ between temperature groups (Hall and Johnston, 2003), the relative timing of muscle development and expression patterns of the myofibrillar mRNAs are independent of temperature. Myofibrillar genes are activated asynchronously and follow a distinct temporal order during myogenesis; a potentially exciting prospect is the application of these and related cDNAs to the characterization of teleost embryos from pelagic sampling studies. Surveys of fish egg abundance have been used to estimate spawning biomass in stock assessments, and require the determination of the age distributions and mortality rates of eggs (Armstrong et al., 2001). It is suggested that the timing of MSP gene expression using *in situ* hybridisation could be employed as the basis of a convenient species-specific method of identification and staging.

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References

Allendorf, F. W. and Thorgaard, G. H. (1984). Tetraploidy and evolution of salmonid fishes. In *Evolutionary Genetics of Fishes* (ed. B. J. Turner), pp. 1-53. New York: Plenum.

Armstrong, M. J., Connolly, P., Nash, R. D. M., Pawson, M. G., Alesworth, E., Coulahan, P. J., Dickey-Collas, M., Milligan, S. P., O'Neill, M. F., Witthames, P. R. et al. (2001). An application of the annual egg production method to estimate the spawning biomass of cod (*Gadus morhua* L.), plaice (*Pleuronectes platessa* L.) and sole (*Solea solea* L.) in the Irish Sea. *ICES J. Mar. Sci.* **58**, 183-203.

Bekkevold, D., Hansen, M. M. and Loeschcke, V. (2002). Male reproductive competition in spawning aggregations of cod (*Gadus morhua*, L.). *Mol. Ecol.* **11**, 91-102.

Benfield, P. A., Zivin, R. A., Miller, L. S., Sowder, R., Smythers, G. W., Henderson, L., Oroszlan, S. and Pearson, M. L. (1984). Isolation and sequence analysis of cDNA clones coding for rat skeletal muscle creatine kinase. *J. Biol. Chem.* **259**, 14979-14984.

Briggs, M. M. and Schachat, F. (1996). Physiologically regulated alternative splicing patterns of fast troponin T RNA are conserved in mammals. *Am. J. Physiol. C* **270**, 298-305.

Brooks, S. and Johnston, I. A. (1993). Influence of development and rearing temperature on the distribution, ultrastructure and myosin subunit composition of myotomal muscle fiber types in the plaice *Pleuronectes platessa*. *Mar. Biol.* **117**, 501-513.

Chanoine, C., Guyot-Lenfant, M., el Attari, A., Saadi, A. and Gallien, C. L. (1992). White muscle differentiation in the eel (*Anguilla anguilla* L.): changes in the myosin isoforms pattern and ATPase profile during post-metamorphic development. *Differentiation* **49**, 69-75.

Chenchik, A., Zhu, Y. Y., Diatchenko, L., Li, R., Hill, J. and Siebert, P. (1998). Generation and use of high-quality cDNA from small amounts of total RNA by SMART PCR. In *RT-PCR Methods for Gene Cloning and Analysis* (ed. P. Siebert and J. Larrick), pp. 305-319. Baltimore: BioTechniques Books.

Costa, M. L., Escaleira, R. C., Rodrigues, V. B., Manasfi, M. and Mermelstein, C. S. (2002). Some distinctive features of zebrafish myogenesis based on unexpected distributions of the muscle cytoskeletal proteins actin, myosin, desmin, [alpha]-actinin, troponin and titin. *Mech. Dev.* **116**, 95-104.

Crockford, T., Wommack, K. E., Johnston, I. A., McAndrew, B. J., Mutungi, G. and Johnson, T. P. (1991). Inter- and intra-specific variation in myosin light chain and troponin I composition in fast muscle fibres from two species of fish (genus *Oreochromis*) which have different temperature-dependent contractile properties. *J. Muscle Res. Cell Motil.* **12**, 439-446.

Crockford, T. and Johnston, I. A. (1993). Developmental changes in the composition of myofibrillar proteins in the swimming muscles of Atlantic herring, *Clupea harengus*. *Mar. Biol.* **115**, 15-22.

Delalande, J. M. and Rescan, P. Y. (1999). Differential expression of two nonallelic MyoD genes in developing and adult myotomal musculature of the trout (*Oncorhynchus mykiss*). *Dev. Genes Evol.* **209**, 432-437.

Demaille, J., Dutruge, E., Eisenberg, E., Capony, J. P. and Pechere, J. F. (1974). Troponin C from reptile and fish muscles and their relation to muscular parvalbumins. *FEBS Lett.* **42**, 173-178.

Ennion, S., Wilkes, D., Gauvry, L., Alami-Durante, H. and Goldspink, G. (1999). Identification and expression analysis of two developmentally regulated myosin heavy chain gene transcripts in carp (*Cyprinus carpio*). *J. Exp. Biol.* **202**, 1081-1090.

Feller, G. and Gerday, C. (1989). Troponin C of the Antarctic icefish (*Champsocephalus gunnari*) white muscle. *Comp. Biochem. Physiol.* **94B**, 769-773.

Felsenstein, J. (1995). PHYLIP (Phylogeny Inference Package) Version 3.6a3. Seattle: http:www//evolution.genetics.washington.edu/phylip.html.

Filatov, V. L., Katrukha, A. G., Bulargina, T. V. and Gusev, N. B. (1999). Troponin: structure, properties, and mechanism of functioning. *Biochemistry (Moscow)* **64**, 969-985.

Francois, J.-M., Altintas, A. and Gerday, G. (1997). Characterization of the single tyrosine containing troponin C from lungfish white muscle. Comparison with several fast skeletal muscle troponin C's from fish species. *Comp. Biochem. Physiol.* **118A**, 589-598.

Fritz-Wolf, K., Schnyder, T., Wallimann, T. and Kabsch, W. (1996). Structure of mitochondrial creatine kinase. *Nature* **381**, 341-345.

Furst, D. O., Osborn, M. and Weber, K. (1989). Myogenesis in the mouse embryo: differential onset of expression of myogenic proteins and the involvement of titin in myofibril assembly. *J. Cell Biol.* **109**, 517-527.

Gahlmann, R. and Kedes, L. (1990). Cloning, structural analysis, and expression of the human fast twitch skeletal muscle troponin C gene. *J. Biol. Chem.* **265**, 12520-12528.

Galloway, T., Kjorsvik, E. and Kryvi, H. (1998). Effect of temperature on viability and axial muscle development in embryos and yolk sac larvae of the Northeast Arctic cod (*Gadus morhua*). *Mar. Biol.* **132**, 559-567.

Gerday, C., Francois, J.-M. and Dubois, I. (1984). Troponin C from eel (*Anguilla anguilla*) skeletal muscle: a protein containing one single tryptophan residue. Comparison with carp skeletal muscle troponin C. *Mol. Physiol.* **6**, 43-54.

Gibson, S. and Johnston, I. A. (1995). Temperature and development in larvae of the turbot *Scophthalmus maximus*. *Mar. Biol.* **124**, 17-25.

Giger, J. M., Haddad, F., Qin, A. X. and Baldwin, K. M. (2000). In vivo regulation of the beta-myosin heavy chain gene in soleus muscle of suspended and weight-bearing rats. *Am. J. Physiol. C* **278**, 1153-1161.

Goncharova, E. J., Kam, Z. and Geiger, B. (1992). The involvement of adherens junction components in myofibrillogenesis in cultured cardiac myocytes. *Development* **114**, 173-183.

Gordon, A. M., Homsher, E. and Regnier, M. (2000). Regulation of contraction in striated muscle. *Physiol. Rev.* **80**, 853-924.

Guenet, J. L., Simon-Chazottes, D., Gravel, M., Hastings, K. E. and Schiaffino, S. (1996). Cardiac and skeletal muscle troponin I isoforms are encoded by a dispersed gene family on mouse chromosomes 1 and 7. *Mamm. Genome* **7**, 13-15.

Hall, T. E. and Johnston, I. A. (2003). Temperature and developmental plasiticity during embryogenesis in the Atlantic cod *Gadus morhua* L. *Mar. Biol.* **142**, 833-840.

Hall, T. E., Smith, P. and Johnston, I. A. (2003). Stages of embryonic development in the Atlantic cod *Gadus morhua*. *J. Morphol.* In press.

Hanel, R., Karjalainen, J. and Wieser, W. (1996). Growth of swimming muscles and its metabolic cost in larvae of whitefish at different temperatures. *J. Fish Biol.* **48**, 937-951.

Harder, G. and McGowan, R. (2001). Isolation and characterization of the muscle-specific isoform of creatine kinase from the zebrafish, *Danio rerio*. *Biochem. Cell. Biol.* **79**, 779-782.

Hirayama, Y. and Watabe, S. (1997). Structural differences in the crossbridge head of temperature-associated myosin subfragment-1 isoforms from carp fast skeletal muscle. *Eur. J. Biochem.* **246**, 380-387.

Hodgson, P. A., Leaver, M. J., George, S. G., MacLean, D. W. and Hastings, K. E. (1996). Molecular cloning of troponin I expressed in fast white muscle of a teleost fish, the Atlantic herring (*Clupea harengus* L.). *Biochim. Biophys. Acta* **1306**, 142-146.

Huang, Q.-Q., Chen, A. and Jin, J.-P. (1999). Genomic structure and organization of mouse slow skeletal muscle troponin T gene. *Gene* **229**, 1-10.

Huse, I. and Jensen, P. A. (1983). A simple and inexpensive spawning and egg collection system for fish with pelagic eggs. *Aquacult. Eng.* **2**, 165-171.

Hutchings, J. A., Bishop, T. D. and McGregor-Shaw, C. R. (1999). Spawning behaviour of Atlantic cod, *Gadus morhua*: evidence of mate competition and mate choice in a broadcast spawner. *Can. J. Fish. Aquat. Sci.* **56**, 97-104.

Imai, J., Hirayama, Y., Kikuchi, K., Kakinuma, M. and Watabe, S. (1997). cDNA cloning of myosin heavy chain isoforms from carp fast skeletal muscle and their gene expression associated with temperature acclimation. *J. Exp. Biol.* **200**, 27-34.

Jackman, D. M., Pham, T., Noel, J. J., Waddleton, D. M., Dhoot, G. K. and Heeley, D. H. (1998). Heterogeneity of Atlantic salmon troponin-I. *Biochim. Biophys. Acta* **1387**, 478-484.

Jin, J. P., Zhang, J. and Wang, J. (1995). An embryonic alternative transcription initiation site and the 5′-upstream structure of mouse cardiac troponin T gene. *Biochem. Biophys. Res. Commun.* **214**, 1168-1174.

Jin, J. P., Chen, A. and Huang, Q. Q. (1998). Three alternatively spliced mouse slow skeletal muscle troponin T isoforms: conserved primary structure and regulated expression during postnatal development. *Gene* **214**, 121-129.

Johnston, I. A., Vieira, V. L. A. and Abercromby, M. (1995). Temperature

and myogenesis in embryos of the Atlantic herring *Clupea harengus*. *J. Exp. Biol.* **198**, 1389-1403.

Johnston, I. A., Cole, N. J., Vieira, V. L. V. and Davidson, I. (1997). Temperature and developmental plasticity of muscle phenotype in herring larvae. *J. Exp. Biol.* **200**, 849-868.

Johnston, I. A., Cole, N. J., Abercromby, M. and Vieira, V. L. V. (1998). Embryonic temperature modulates muscle growth characteristics in larval and juvenile herring. *J. Exp. Biol.* **201**, 623-646.

Johnston, I. A., Hall, T. E. and Fernandez, D. A. (2002). Genes regulating the growth of myotomal muscle in teleost fish. In *Aquatic Genomics – Steps Towards a Great Future* (ed. N. Shimizu T. Aoki I. Hirono and F. Takashima), pp. 153-166. Heidelberg: Springer-Verlag.

Kikuchi, K., Muramatsu, M., Hirayama, Y. and Watabe, S. (1999). Characterization of the carp myosin heavy chain multigene family. *Gene* **228**, 189-196.

Kim, S., Karsi, A., Dunham, R. A. and Liu, Z. J. (2000). The skeletal muscle alpha-actin gene of channel catfish (*Ictalurus punctatus*) and its association with piscine specific SINE elements. *Gene* **252**, 173-181.

Kjesbu, O. S., Solemdal, P., Bratland, P. and Fonn, M. (1996). Variation in annual egg production in individual captive Atlantic cod (*Gadus morhua*). *Can. J. Fish. Aquat. Sci.* **53**, 610-620.

Konig, S., Burkman, J., Fitzgerald, J., Mitchell, M., Su, L. and Stedman, H. (2002). Modular organization of phylogenetically conserved domains controlling developmental regulation of the human skeletal myosin heavy chain gene family. *J. Biol. Chem.* **277**, 27593-27605.

Lin, Z., Lu, M. H., Schultheiss, T., Choi, J., Holtzer, S., DiLullo, C., Fischman, D. A. and Holtzer, H. (1994). Sequential appearance of muscle-specific proteins in myoblasts as a function of time after cell division: evidence for a conserved myoblast differentiation program in skeletal muscle. *Cell Motil. Cytoskel.* **29**, 1-19.

Lin-Jones, J. and Hauschka, S. D. (1997). Skeletal and cardiac alpha-actin isoforms exhibit unanticipated temporal and tissue-specific gene expression patterns in developing avian limbs and embryos. *Dev. Biol.* **189**, 322-334.

Liu, Z., Kim, S., Kucuktas, H. and Karsi, A. (2001). Multiple isoforms and an unusual cathodic isoform of creatine kinase from channel catfish (*Ictalurus punctatus*). *Gene* **275**, 207-215.

Lu, B. D., Allen, D. L., Leinwand, L. A. and Lyons, G. E. (1999). Spatial and temporal changes in myosin heavy chain gene expression in skeletal muscle development. *Dev. Biol.* **216**, 312-326.

Luther, P. K., Munro, P. M. G. and Squire, J. M. (1995). Muscle ultrastructure in the teleost fish. *Micron* **26**, 431-459.

Martin, B. L. and Harland, R. M. (2001). Hypaxial muscle migration during primary myogenesis in *Xenopus laevis*. *Dev. Biol.* **239**, 270-280.

Martinez, I., Ofstad, R. and Olsen, R. L. (1991). Developmental transitions of myosin heavy-chains in the skeletal-muscle of the teleost *Salvelinus alpinus*. *J. Muscle Res. Cell Motil.* **12**, 74.

Mascarello, F., Rowlerson, A., Radaelli, G., Scapolo, P. A. and Veggetti, A. (1995). Differentiation and growth of muscle in the fish *Sparus aurata* (L): I. Myosin expression and organization of fibre types in lateral muscle from hatching to adult. *J. Muscle Res. Cell. Motil.* **16**, 213-222.

Matschak, T. W., Hopcroft, T., Mason, P. S., Crook, A. R. and C., S. N. (1998). Temperature and oxygen tension influence the development of muscle cellularity in embryonic rainbow trout. *J. Fish Biol.* **53**, 581-590.

McCubbin, W. D., Oikawa, K., Sykes, B. D. and Kay, C. M. (1982). Purification and characterization of troponin C from pike muscle: a comparative spectroscopic study with rabbit skeletal muscle troponin C. *Biochemistry* **21**, 5948-5956.

Meyer, A. and Schartl, M. (1999). Gene and genome duplications in vertebrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene functions. *Curr. Opin. Cell Biol.* **11**, 699-704.

Miyadzaki, J., Jozaki, M., Nakatani, N., Watanabe, T., Saba, R., Nakada, K., Hirabayashi, T. and Yonemura, I. (1999). The structure of the avian fast skeletal muscle troponin T gene: seven novel tandem-arranged exons in the exon x region. *J. Muscle Res. Cell Motil.* **20**, 655-660.

Moyes, C. D., Borgford, T., LeBlanc, L. and Tibbits, G. F. (1996). Cloning and expression of salmon cardiac troponin C: titration of the low-affinity $Ca^{(2+)}$-binding site using a tryptophan mutant. *Biochemistry* **35**, 11756-11762.

Mullen, A. J. and Barton, P. J. R. (2000). Structural characterization of the human fast skeletal muscle troponin I gene (TNNI2). *Gene* **242**, 313-320.

Ordahl, C. P., Evans, G. L., Cooper, T. A., Kunz, G. and Perriard, J. C. (1984). Complete cDNA-derived amino acid sequence of chick muscle creatine kinase. *J. Biol. Chem.* **259**, 15224-15227.

Parmacek, M. S., Bengur, A. R., Vora, A. J. and Leiden, J. M. (1990). The structure and regulation of expression of the murine fast skeletal troponin C gene. Identification of a developmentally regulated, muscle-specific transcriptional enhancer. *J. Biol. Chem.* **265**, 15970-15976.

Phillips, C. L., Yamakawa, K. and Adelstein, R. S. (1995). Cloning of the cDNA encoding human nonmuscle myosin heavy chain-B and analysis of human tissues with isoform-specific antibodies. *J. Muscle Res. Cell Motil.* **16**, 379-389.

Pownall, M. E., Gustafsson, M. K. and Emerson, C. P., Jr (2002). Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. *Annu. Rev. Cell Dev. Biol.* **18**, 747-783.

Reinach, F. C. and Karlsson, R. (1988). Cloning, expression, and site-directed mutagenesis of chicken skeletal muscle troponin C. *J. Biol. Chem.* **263**, 2371-2376.

Rescan, P. Y. and Gauvry, L. (1996). Genome of the rainbow trout (*Oncorhynchus mykiss*) encodes two distinct muscle regulatory factors with homology to MyoD. *Comp. Biochem. Physiol.* **113B**, 711-715.

Rescan, P. Y., Collet, B., Ralliere, C., Cauty, C., Delalande, J. M., Goldspink, G. and Fauconneau, B. (2001). Red and white muscle development in the trout (*Oncorhynchus mykiss*) as shown by in situ hybridisation of fast and slow myosin heavy chain transcripts. *J. Exp. Biol.* **204**, 2097-2101.

Robinson-Rechavi, M., Marchand, O., Escriva, H. and Laudet, V. (2001a). An ancestral whole-genome duplication may not have been responsible for the abundance of duplicated fish genes. *Curr. Biol.* **11**, 458-459.

Robinson-Rechavi, M., Marchand, O., Escriva, H. and Laudet, V. (2001b). Re: Revisiting recent challenges to the ancient fish-specific genome duplication hypothesis. *Curr. Biol.* **11**, 1007-1008.

Robinson-Rechavi, M., Marchand, O., Escriva, H., Bardet, P. L., Zelus, D., Hughes, S. and Laudet, V. (2001c). Euteleost fish genomes are characterized by expansion of gene families. *Genome Res.* **11**, 781-788.

Rubenstein, P. A. and Spudich, J. A. (1977). Actin microheterogeneity in chick embryo fibroblasts. *Proc. Natl. Acad. Sci. USA* **4**, 120-123.

Sabourin, L. A. and Rudnicki, M. A. (2000). The molecular regulation of myogenesis. *Clin. Genet.* **57**, 16-25.

Smillie, L. B., Golosinska, K. and Reinach, F. C. (1988). Sequences of complete cDNAs encoding four variants of chicken skeletal muscle troponin T. *J. Biol. Chem.* **263**, 18816-18820.

Soussi-Yanicostas, N., Whalen, R. G. and Petit, C. (1993). Five skeletal myosin heavy chain genes are organized as a multigene complex in the human genome. *Hum. Mol. Genet.* **2**, 563-569.

Stickland, N. C., White, R. N., Mescall, P. E., Crook, A. R. and Thorpe, J. E. (1988). The effect of temperature on myogenesis in embryonic development of the Atlantic salmon (*Salmo salar* L.). *Anat. Embryol.* **178**, 253-257.

Stolz, M. and Wallimann, T. (1998). Myofibrillar interaction of cytosolic creatine kinase (CK) isoenzymes: allocation of N-terminal binding epitope in MM-CK and BB-CK. *J. Cell Sci.* **111**, 1207-1216.

Sun, H. W., Hui, C. F. and Wu, J. L. (1998). Cloning, characterization, and expression in *Escherichia coli* of three creatine kinase muscle isoenzyme cDNAs from carp (*Cyprinus carpio*) striated muscle. *J. Biol. Chem.* **273**, 33774-33780.

Tan, X. and Du, J. (2002). Differential expression of two MyoD genes in fast and slow muscles of gilthead seabream (*Sparus aurata*). *Dev. Genes Evol.* **212**, 207-217.

Taylor, S. S., Buechler, J. A. and Yonemoto, W. (1990). cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. *Annu. Rev. Biochem.* **59**, 971-1005.

Taylor, J. S., Van de Peer, Y., Braasch, I. and Meyer, A. (2001a). Comparative genomics provides evidence for an ancient genome duplication event in fish. *Phil. Trans. R. Soc. Lond. B* **356**, 1661-1679.

Taylor, J. S., Van de Peer, Y. and Meyer, A. (2001b). Revisiting recent challenges to the ancient fish-specific genome duplication hypothesis. *Curr. Biol.* **11**, 1005-1008.

Taylor, J. S., Van de Peer, Y. and Meyer, A. (2001c). Genome duplication, divergent resolution and speciation. *Trends Genet.* **17**, 299-301.

Temple, G. K., Cole, N. J. and Johnston, I. A. (2001). Embryonic temperature and the relative timing of muscle-specific genes during development in herring (*Clupea harengus* L.). *J. Exp. Biol.* **204**, 3629-3637.

Tiso, N., Rampoldi, L., Pallavicini, A., Zimbello, R., Pandolfo, D., Valle, G., Lanfranchi, G. and Danieli, G. A. (1997). Fine mapping of five human skeletal muscle genes: alpha-tropomyosin, beta-tropomyosin, troponin-I

slow-twitch, troponin-I fast-twitch, and troponin-C fast. *Biochem. Biophys. Res. Commun.* **230**, 347-350.

van der Ven, P. F. M. and Furst, D. O. (1998). Expression of sarcomeric proteins and assembly of myofibrils in the putative myofibroblast cell line BHK-21/C13. *J. Muscle Res. Cell Motil.* **19**, 767-775.

Vandekerckhove, J. and Weber, K. (1978). At least six different actins are expressed in a higher mammal: an analysis based on the amino acid sequence of the amino-terminal tryptic peptide. *J. Mol. Biol.* **126**, 783-802.

Vandekerckhove, J. and Weber, K. (1979). The complete amino acid sequence of actins from bovine aorta, bovine heart, bovine fast skeletal muscle, and rabbit slow skeletal muscle. A protein-chemical analysis of muscle actin differentiation. *Differentiation* **14**, 123-133.

Venkatesh, B., Tay, B. H., Elgar, G. and Brenner, S. (1996). Isolation, characterization and evolution of nine pufferfish (*Fugu rubripes*) actin genes. *J. Mol. Biol.* **259**, 655-665.

Vieira, V. L. A. and Johnston, I. A. (1992). Influence of temperature on muscle fiber development in larvae of the herring *Clupea harengus*. *Mar. Biol.* **112**, 333-341.

Waddleton, D. M., Jackman, D. M., Bieger, T. and Heeley, D. H. (1999). Characterisation of troponin-T from salmonid fish. *J. Muscle Res. Cell Motil.* **20**, 315-324.

Wallimann, T., Wyss, M., Brdiczka, D., Nicolay, K. and Eppenberger, H. M. (1992). Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. *Biochem. J.* **281**, 21-40.

Wang, J. and Jin, J.-P. (1997). Primary structure and developmental acidic to basic transition of 13 alternatively spliced mouse fast skeletal muscle troponin T isoforms. *Gene* **193**, 105-114.

Warkman, A. S. and Atkinson, B. G. (2002). The slow isoform of *Xenopus* troponin I is expressed in developing skeletal muscle but not in the heart. *Mech. Dev.* **115**, 143-146.

Watabe, S., Hirayama, Y., Imai, J., Kikuchi, K. and Yamashita, M. (1995). Sequences of cDNA clones encoding alpha-actin of carp and goldfish skeletal muscles. *Fish. Sci.* **61**, 998-1003.

Weinberg, E. S., Allende, M. L., Kelly, C. S., Abdelhamid, A., Murakami, T., Andermann, P., Doerre, O. G., Grunwald, D. J. and Riggleman, B. (1996). Developmental regulation of zebrafish MyoD in wild-type, no tail and spadetail embryos. *Development* **122**, 271-280.

Wheeler, M. T., Snyder, E. C., Patterson, M. N. and Swoap, S. J. (1999). An E-box within the MHC IIB gene is bound by MyoD and is required for gene expression in fast muscle. *Am. J. Physiol. C* **276**, 1069-1078.

Wilkinson, D. G. (1992). Whole mount in-situ hybridisation of vertebrate embryos. In *In situ Hybridisation: A Practical Approach* (ed. D. G. Wilkinson), pp. 939-947. Oxford: IRL Press.

Wyzykowski, J. C., Winata, T. I., Mitin, N., Taparowsky, E. J. and Konieczny, S. F. (2002). Identification of novel MyoD gene targets in proliferating myogenic stem cells. *Mol. Cell. Biol.* **22**, 6199-6208.

Xie, S. Q., Mason, P. S., Wilkes, D., Goldspink, G., Fauconneau, B. and Stickland, N. C. (2001). Lower environmental temperature delays and prolongs myogenic regulatory factor expression and muscle differentiation in rainbow trout (*Onchrhynchus mykiss*) embryos. *Differentiation* **68**, 106-114.

Xu, Y. F., He, J. Y., Wang, X. K., Lim, T. M. and Gong, Z. Y. (2000). Asynchronous activation of 10 muscle-specific protein (MSP) genes during zebrafish somitogenesis. *Dev. Dyn.* **219**, 201-215.

Yao, Y., Nakamura, M., Miyazaki, J. I., Kirinoki, M. and Hirabayashi, T. (1992). Expression pattern of skeletal muscle troponin T isoforms is fixed in cell lineage. *Dev. Biol.* **151**, 531-540.

Yoshimi, T., Nakamura, M. and Asano, A. (1995). Expression of several muscle-specific genes during differentiation of cultured quail pineal body cells under artificial (high NaCl) conditions. *Differentiation* **59**, 307-319.

Yuasa, H. J., Cox, J. A. and Takashi, T. (1998). Diversity of the troponin C genes during chordate evolution. *J. Biochem* **123**, 1180-1190.

Zar, J. H. (1999). *Biostatistical Analysis*. London: Prentice-Hall Inc.