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Temperature influences the coordinated expression of myogenic regulatory factors during embryonic myogenesis in Atlantic salmon (*Salmo salar* L.)

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Summary

Potential molecular mechanisms regulating developmental plasticity to temperature were investigated in Atlantic salmon embryos (*Salmo salar* L.). Six orthologues of the four myogenic regulatory factors (MRFs: individually: smyf5, smyoD1a/1b/1c, smyoG and sMRF4), the master transcription factors regulating vertebrate myogenesis, were characterised at the mRNA/genomic level. *In situ* hybridisation was performed with specific cRNA probes to determine the expression patterns of each gene during embryonic myogenesis. To place the MRF data in the context of known muscle fibre differentiation events, the expression of slow myosin light chain-1 and Pax7 were also investigated. Adaxial myoblasts expressed *smyoD1a* prior to and during somitogenesis followed by *smyoD1c* (20-somite stage, ss), and *sMRF4* (25–30 ss), before spreading laterally across the myotome, followed closely by the adaxial cells. *Smyf5* was detected prior to somitogenesis, but not in the adaxial cells in contrast to other teleosts studied. The expression domains of *smyf5*, *smyoD1b* and *smyoG* were not confined to the *s-smlc1* expression field, indicating a role in fast muscle myogenesis. From the end of segmentation, each MRF was expressed to a greater or lesser extent in zones of new muscle fibre production, the precursor cells for which probably originated from the *Pax7* expressing cell layer external to the single layer of *s-smlc1*$^+$ fibres. *SmyoD1a* and *smyoG* showed similar expression patterns with respect to somite stage at three different temperatures investigated (2°C, 5°C and 8°C) in spite of different rates of somite formation (one somite added each 5 h, 8 h and 15 h at 8°C, 5°C and 2°C, respectively). In contrast, the expression of *smyf5*, *sMRF4* and *s-smlc1* was retarded with respect to somite stage at 2°C compared to 8°C, potentially resulting in heterochronies in downstream pathways influencing later muscle phenotype.

Key words: *Salmo salar*, teleost fish, myogenesis, myogenic regulatory factors, adaxial cells, temperature, developmental plasticity.

Introduction

Variations in embryonic temperature can produce both time-limited and persistent alterations to skeletal muscle phenotype in teleost fish (reviewed in Johnston, 2006). For example, embryonic incubation temperature changes mitochondrial abundance (Vieira and Johnston, 1992), the timing of expression of developmental-stage specific myofibrillar proteins (Johnston et al., 1997; Johnston et al., 1998) and the number and size distribution of myotomal muscle fibres in larval and juvenile stages (Stickland et al., 1988; Johnston et al., 2000). In amniotes, the myogenic progenitor cells responsible for embryonic and postnatal muscle growth express the transcription factors Pax3/Pax7 (Relaix et al., 2005) and are derived from the dermomyotome (Gros et al., 2005), a transient compartment of the dorsal somite of embryonic stages. In zebrafish, Pax7-expressing cells of the anterior part of the epithelial somite migrate laterally to form a layer of cells external to the myotome (the external cell layer), as the entire somite rotates through 90° from its starting position (Hollway et al., 2007). Self-renewing, undifferentiated Pax7-expressing cells persist in the external cell layer in adult zebrafish and provide myogenic precursors utilised during larval and possibly adult muscle growth (Hollway et al., 2007; Stellabotte et al., 2007; Devoto et al., 2006). Additionally, the external cell layer is the source of quiescent Pax7$^+$ muscle progenitors found under the basal lamina of muscle fibres in adult zebrafish (Hollway et al., 2007), reminiscent of the satellite cells that are required for muscle growth and repair in mammals (Mauro, 1961). The anterior somite of the zebrafish also supplies progenitors used in the growth of the dermis and pectoral and dorsal fin muscles, supporting a functional role for this region equivalent to the amniote dermomyotome (Hollway et al., 2007).

Teleosts produce new myotubes throughout larval, juvenile and adult stages, a reflection of the large increase in body size that occurs during ontogeny. For example, in Atlantic salmon (*Salmo salar* L.) there were ~5000 fast muscle fibres per myotomal cross-section in hatched embryos and fibre number expanded to around 850 000 by the time recruitment stopped in adult fish (Johnston et al., 2003). The final fibre number in adult salmon (FN_{max}) can be modified by around 20%

according to the temperature experienced during the early life history stages (Johnston et al., 2003). If temperature affects the number of post-embryonic myogenic precursors originating from the external cell layer, this could provide a plausible explanation for later changes in FN_{max} in adult fish. The ecological significance of the developmental plasticity of fibre number is unknown, but a higher fibre number would be expected to increase the potential for fast growth (Johnston et al., 2003) at the expense of higher routine maintenance costs (Johnston et al., 2005).

The MRFS are a conserved family of four proteins (myf5, myoD, myoG and MRF4), related by ancient gene duplication (Atchley et al., 1994). The MRFs are potent transcriptional activators of muscle-specific genes, owing to two domains conserved in each family member: the basic region and helix-loop-helix (HLH) domain (Weintraub et al., 1991). The ubiquitously expressed E-proteins share these regions and dimerize to MRFs *via* the HLH and the resulting complexes then bind *via* the basic regions to a specific motif (CANNTG, the e-box) conserved in the regulatory region of most muscle genes (Murre et al., 1989; Lassar et al., 1989). The MRFs share partial redundancy and *in vitro*, can each convert several cell lines to differentiated skeletal muscle (Weintraub et al., 1989). However, each gene has evolved a unique expression pattern and specialist function in initiating or maintaining myogenesis. Mouse double knockouts have shown that myoD and myf5 are critical for myogenic specification, as indicated by a lethal phenotype lacking myoblasts and skeletal muscle (Rudnicki et al., 1993). In contrast, *myoG* knockout mice have myoblasts, but die from a lack of differentiated muscle (Hasty et al., 1993). MRF4 plays a double role in muscle specification/differentiation since myogenesis occurs normally in *myf5*:*myoD*$^{-/-}$ mice, when MRF4 is not compromised (Kassar-Duchossoy et al., 2004).

Heterochronies in the expression of myoD family members might be expected if they are involved in the developmental plasticity of myogenesis, including effects on the number of muscle fibres formed. To test this hypothesis, the expression of myoD family members has been investigated by *in situ* hybridisation in fish embryos reared at different temperatures. The majority of studies have found no difference in the relative timing or intensity of *myoD* or *myoG* expression with respect to somite stage in embryos reared at a range of temperatures [Atlantic cod *Gadus morhua* (Hall et al., 2003); Atlantic herring *Clupea harengus* (Temple et al., 2001); common carp *Cyprinus carpio* (Cole et al., 2004) and Atlantic halibut *Hippoglossus hippoglossus* (Galloway et al., 2006)]. However, in rainbow trout *Oncorhynchus mykiss* it was reported that *myoD* and *myoG* expression was more intense at the mRNA and protein levels and also more advanced with respect to somite stage in embryos incubated at 12°C *versus* 4°C (Xie et al., 2001).

MyoD in the rainbow trout was shown to occur as two paralogues, which was thought to reflect the tetraploidization of the salmonid genome (Rescan and Gauvry, 1996). Subsequently two *myoD* paralogues of lower percentage identity were identified in five Percomorpic teleost fish (Tan and Du, 2002; Galloway et al., 2006; Macqueen and Johnston, 2006; Fernandes et al., 2007). A third *myoD* paralogue was recently characterised in Atlantic salmon, rainbow trout and brown trout (*Salmo trutta*) and a phylogenetic analysis showed that each salmonid *myoD* paralogue was orthologous to a universal teleost *myoD* gene (named *myoD1*) and distinct from the second *myoD* paralogue (named *myoD2*) found in some fish (Macqueen and Johnston, 2006). The three salmonid *myoD* paralogues, which were named *myoD1a*/*1b*/*1c*, had distinct expression patterns during embryonic development and probably represent a whole genome duplication followed by a more recent local gene duplication event (Macqueen and Johnston, 2006). In the light of these recent discoveries it was thought worthwhile to re-examine potential developmental plasticity of *myoD* expression with temperature in Atlantic salmon. Furthermore, in order to thoroughly test the hypothesis that developmental plasticity to temperature is associated with heterochronies in MRF expression, it is necessary to extend the study to include the other myoD family members not so far investigated. *MRF4* is of particular interest since to our knowledge its expression during embryonic development has not previously been described in fish.

Materials and methods

Embryos

Salmo salar (L.) embryos were reared by Akvaforsk (Sunndalsora, Norway) at 2°C, 5°C and 8°C. Embryos were sampled based on the staging system of Gorodilov (Gorodilov, 1996), which accounts for the rate of embryonic Atlantic salmon development at different temperatures. The following seven stages were selected: (1) the end of gastrulation, (2) 1–3-somite stage (ss), (3) 10–15 ss, (4) 30–40 ss, (5) 45–50 ss, (6) toward the end of segmentation (60–65 ss), (7) post-segmentation (the ‘eyed stage’). Embryos were fixed in 4% (m/v) paraformaldehyde/PBS and then dehydrated by consecutive washes in increasingly concentrated methanol (until 100% m/v) and stored at –80°C until later use.

Cloning new myoD family members and smlc1

Juvenile Atlantic salmon (N=6; mean mass=291±36 g, mean fork length=263±27 mm), obtained from EWOS innovation (Lonningdal, Norway), were sampled for fast muscle, which was dissected from the dorsal epaxial myotome and flash frozen in liquid nitrogen. For total RNA extraction, 100 mg of muscle was added to FastRNA Pro Green Beads (MP Biomedicals, Stretton, Cheshire, UK) with 1 ml of Tri Reagent (Sigma, Gillingham, Dorset, UK) and then homogenised with a Fast Prep instrument (MP Biomedicals). Genomic DNA was removed from the RNA sample using the TURBO DNA-free kit (Ambion, Huntingdon, Cambs, UK). RNA quality was confirmed by assessing the integrity of 28S and 18S ribosomal RNA by gel electrophoresis. Digested RNA was quantified using the fluorescent nucleic acid dye Ribogreen (Invitrogen, Paisley, Scotland, UK). First strand cDNA was synthesised using 1 μg of total RNA and a RETROscript kit (Ambion). Genomic DNA was extracted from 50 mg of spleen tissue (Dneasy Tissue Kit, Qiagen, Crawley, W. Sussex, UK). The primers shown in Table 1 were then used to amplify Atlantic salmon full coding sequences of *smyoG*, *smyf5* and *s-smlc1*, and a partial *sMRF4* sequence, using several standard PCR reactions with gDNA (MRFs) and cDNA (MRFs and *s-smlc1*). Additionally, to obtain the 3′ of the *sMRF4* gene (plus full coding sequence), a BD Smart™ RACE cDNA

Table 1. *Experimental primer sequences*

Primer name	Product	Related accession no.	Primer sequence (5′-3′)
smyf5 F1	Whole coding sequence	DQ452070	f: ATGGATGTCTTCTCCCAGTCC
smyf5 R1	Whole coding sequence	DQ452070	r: TCACAATACGTGGTACACAGGTC
sMRF4 F1	Nucleotides 1–648	DQ479952	f: ATGATGGACCTTTTTGAGACC
sMRF4 R1	Nucleotides 1–648	DQ479952	r: GATTGATGACAGGCGAAGAAG
sMRF4 RACE	3′ cds/UTR MRF4	DQ479952	f: GAGTCTTCAGCGTCCACCAGCCTTCTTCG
smyoG F1	Whole coding sequence	DQ294029	f: CTAGCGTCGACCAGTATGGAG
smyoG R1	Whole coding sequence	DQ294029	r: CTCTGGGTTTATTTGGGAATG
s-smlc1 F1	Whole coding sequence	DQ916288	f: CTGTCCTCCTGTGGCTCCTG
s-smlc1 R1	Whole coding sequence	DQ916288	r: TTAAGATGCCATGACGTGTTTTAC
Pax7 F1	Nucleotides 502–1119	AJ618975	f: CTGTGAGTTCCATCAGCCGAG
Pax7 R1	Nucleotides 502–1119	AJ618975	r: TGGGGTTACTCAGGATGCTC

amplification kit was used (BD Biosciences, Oxford, Oxon, UK) (primer in Table 1). PCR products were separated using, and isolated from 1.1% (m/v) agarose gels, purified using a QIAquick gel extraction kit (Qiagen) and then ligated into a pCR4-TOPO T/A vector (Invitrogen) before transformation into chemically competent *Escherichia coli* cells (Invitrogen). At least two clones per gene fragment were then sequenced in sense/antisense directions by the University of Dundee sequencing service.

Bioinformatics and phylogenetic analyses

A consensus nucleotide and amino acid (AA) translation of each gene was constructed from each sequencing result. The identity of putative genes was confirmed against the complete non-redundant NCBI database using BLAST and TBLASTN searches (http://www.ncbi.nlm.nih.gov/blast/). Subsequently, each gene was submitted to the GenBank public database (http://www.ncbi.nlm.nih.gov). The intron–exon structure of each MRF was assessed by aligning cDNA and gDNA sequences in the program Spidey (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/). For sequence alignments, Clustal X (Thompson et al., 1997) was used with the Gonnet 250 matrix for pairwise comparisons, and Gonnet series parameter for alignments. Amino acid translations of the following mRNA sequences were aligned: *tmyoD1a* (X75798), *tmyoD1b* (Z46924), *smyoD1a* (AJ618978), *smyoD1b* (AJ557150), *smyoD1c* (DQ317527), *btmyoD1c* (DQ366710), *smyf5* (DQ452070), *tmyf5* (AY751283), *sMRF4* (DQ479952), *smyoG* (DQ294029), *tmyoG* (Z46912) and *amphi-myoD1* (AB092415). Maximum likelihood was then performed on this alignment using PHMYL (Guindon and Gascuel, 2003) and the WAG model (Whelan and Goldman, 2001), with 500 pseudoreplicate bootstraps. For comparison, a Neighbour Joining (NJ) analysis was performed on the same alignment in Mega 3.1 (Kumar et al., 2004) using the JTT model and 1000 bootstrap iterations for branch support. Trees produced by both methods were reconstructed in Mega 3.1

Probe transcription and in situ hybridisation

To make DNA templates for RNA probe synthesis, PCR was used with T3/T7 primers (Invitrogen) and as a template, a pCR4-TOPO T/A plasmid (Invitrogen) containing the cDNA products of *smyf5*, *smyoG*, s-*smlc1* and *sMRF4* (Table 1) excluding the *sMRF4* RACE product. The *smyoD1a/1b/1c* probe templates were as described previously (Macqueen and Johnston, 2006). Finally, nucleotides 502–1119 of the Atlantic salmon *Pax7* gene previously reported (Gotensparre et al., 2006) were amplified, cloned and sequenced as described above (primers in Table 1).

Each cRNA probe was synthesised in sense and antisense directions using T3/T7 RNA polymerases (Roche, Lewes, E. Sussex, UK) with concurrent incorporation of digoxigenin (DIG) or fluorescein (FLU) labelling (both Roche). *In situ* hybridisation was based on a standard procedure (Jowett, 2001) and all hybridisation and stringency washes were performed at 70°C. Probes were detected with alkaline-phosphotase-conjugated antibodies (Roche) using NBT/BCIP (Roche) for DIG and Fast Red (Invitrogen) for FLU. Different temperature treatments were incubated in each solution for identical time periods. This ensured that differences recorded between temperature groups in the colour development step were attributable to differences in gene expression rather than unequal sample treatment.

Processing embryos and figure construction

All embryos from each temperature treatment and stage were studied using both a DMRB compound microscope and a Leica MZ7.5 binocular microscope (Leica Microsystems Ltd., Milton Keynes, Bucks, UK). When DIC optics was used, embryos were flat-mounted with a coverslip on a clear microscope slide and orientated to a dorsal or lateral perspective. Embryos were staged by counting the somite number and photographs were recorded on a Nikon P4500 camera. Subsequently, representative embryos were mounted in cryomatrix (Thermo Electron Corp., Waltham, MA, USA), orientated and then frozen in isopentane cooled to its freezing point (–159°C) by liquid nitrogen. Serial 18 μm cryosections (–20°C) were cut on a Leica cryostat (Leica Microsystems, CM1850). Differences in gene expression patterns between temperature treatments were considered reliable when replicated in each embryo at each stage (N=6). When figures were constructed, representative images of embryos from equivalent somite stages were selected from each temperature treatment. This meant that differences in temperature groups were not considered in developmental windows when embryos could not be accurately staged, i.e. prior to somite formation and after the completion of segmentation.

Results

Characterisation of Atlantic salmon myoD family members

In salmonids, myoD has a complex evolutionary history and is represented by three paralogues (*myoD1a*, *1b* and *1c*) (Macqueen and Johnston, 2006). In the current study, we have also obtained full coding sequences of other Atlantic salmon myoD related genes: *myf5* (*smyf5*), *myoG* (*smyoG*) and *MRF4* (*sMRF4*). Using primers designed from *tmyf5*, a complete coding sequence (cds) of *smyf5* (DQ452070) was obtained incorporating 720 bp that translated into a protein of 239 AA. The percentage identity conserved between *smyf5* and other vertebrate *myf5* orthologues at the respective nucleotide/protein level was 97.9/96.2% with rainbow trout (AY751283), 75.9/73.0% with pufferfish *Takifugu rubripes* (NM_001032770), 71.7/76.3% with zebrafish *Danio rerio* (AF253470), 59.2/54.8% with frog *Xenopus leavis* (AJ579311), 60.7/56.3% with chicken *Gallus gallus* (NM_001030363) and 63.4/54.5% with human *Homo sapiens* (NP_005593).

Using primers designed from *tmyoG* (Z46912), a complete cds corresponding to *smyoG* (DQ294029) was obtained, which was 789 bp long, and translated into an open reading frame (ORF) of 254 AA. The percentage identity conserved between *smyoG* and other vertebrate *myoG* orthologues at the respective nucleotide/protein level was 97.9/98.4% with rainbow trout, 76.2/77.6% with pufferfish (AY566282), 72.7/73.5% with zebrafish (NM_131006), 61.9/58.1% with frog (NM_001016725), 64.1/56.4% with chicken (D90157), and 65.4/53.2% with human (NM_002479).

MRF4 had not previously been cloned in any salmonid fish. For this reason, primers used to amplify *MRF4* were initially based on an expressed sequence tag (DN165140) obtained from a TBLASTN search of the salmon genome project (http://www.salmongenome.no/cgi-bin/sgp.cgi#Blast) using the translated *D. rerio MRF4* mRNA (NM_001003982) as a probe. A reverse primer was designed from this sequence and was used with a forward primer designed in the start region of *MRF4* based on alignments with several vertebrate *MRF4* sequences (Table 1) to amplify nucleotides 1–649 of the salmon coding sequence. Finally, a 3′ RACE primer was designed at the 3′ of the confirmed *sMRF4* sequence (Table 1) and this was used in a 3′ RACE reaction to obtain a whole coding sequence for *sMRF4* and a complete 3′ untranslated region, with a poly-A tail and one polyadenylation signal (AATAAA) (not shown). *SMRF4* shared closest homology to its orthologue in the knifefish *Sternopygus macrurus* (DQ059552) with 75.0/76.9% nucleotide/AA identity. The percentage identity between

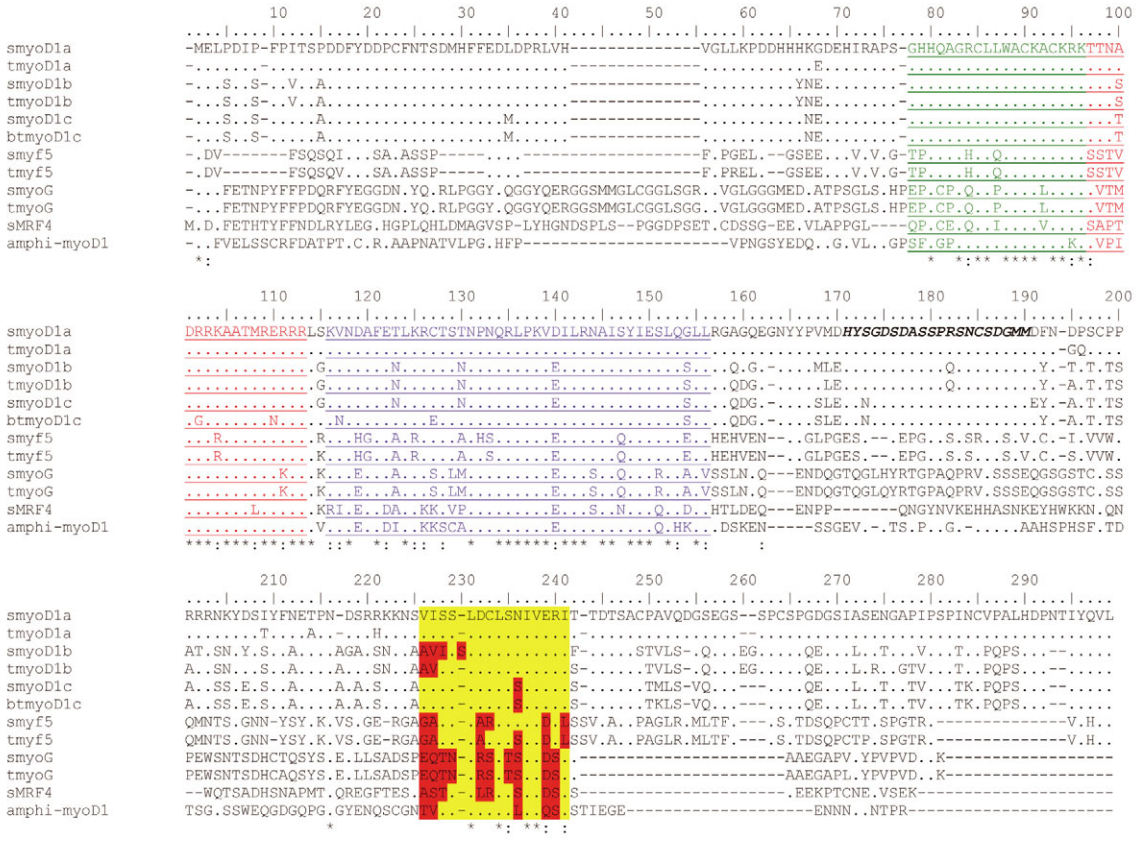

Fig. 1. Amino acid sequence alignment of all known salmonid myoD family members with a myoD orthologue in the cephalochordate-amphioxus (amphi-myoD1). Accession numbers are identical to those described in phylogeny methodology. Within the alignment, dots mark residues identical to smyoD1a and dashes indicate a gap. Below the alignment, asterisks show residues conserved globally and colons highlight conserved amino acid substitutions. The basic (red underlined) and helix-loop-helix (blue underlined) regions are highly conserved. Also shown is the cysteine–histidine rich region (green underlined) and helix-III domain, where residues identical and different to smyoD1a are respectively highlighted yellow and red. A highly conserved region present in vertebrate *myoD* genes, (but not other MRFs and less so with amphi-myoD) is shown in bold italics on smyoD1a.

sMRF4 and other vertebrate orthologues at the coding nucleotide/protein level was 70.9/71.6% with pufferfish (AY445320), 73.9/76.0% with zebrafish, 63.8/59.3% with frog (S84990), 62.8/60.9% with chicken (D10599), and 62.7/62.1% with human (NM_002469).

Fig. 1 shows an alignment of all the known salmonid MRFs with an ancient myoD homologue in the cephalochordate *Branchiostoma belcheri*. The bHLH domain and cis–his-rich region (just N-terminal to the basic region) are strongly conserved in all salmonid MRFs and with the ancient myoD gene. Additionally, the helix-III domain of myoD1 paralogues (AAs 206–221 of smyoD1a) is most similar to cephalochordate *myoD* (5/15 substitutions *vs smyoD1a*) >salmonid-myf5 genes (6/15 substitutions *vs smyoD1a*) >sMRF4 (8/15 substitutions *vs smyoD1a*) >salmonid myoG genes (10/15 substitutions *vs myoD1a*). Additionally a highly conserved motif is present in salmonid myoD paralogues and other vertebrate myoD proteins (not shown), that is not conserved in other MRFs, but partially conserved in amphi-myoD1 (Fig. 1). This motif has not been assigned any function at present. The NH_2- and COOH- terminals are the least conserved regions of the salmonid MRF proteins.

Characterisation of Atlantic salmon smlc1

Primers to amplify a complete coding sequence of Atlantic salmon *smlc1* (s-*smlc1*) were designed from the rainbow trout sequence previously reported [EST (BX076946) (Chauvigne et al., 2005)]. The coding sequence of *s-smlc1* (DQ916288) was 561 bp that translated into an ORF of 185 AA. The percentage identity conserved between *s-smlc1* and other vertebrate *smlc1* orthologues at the respective nucleotide/protein level is 99.1/99.5% with rainbow trout, 78/81% with the pufferfish *Tetraodon nigroviridis* (putative: predicted within CAAE01014556), 80/83% with zebrafish (NP_956810), 65/67% with frog (EST: AAI28964), 66/69% with chicken (P02606) and 64/67% with human (NP_002467).

Genomic organisation and phylogeny of salmonid MRFs

The exon–intron structure of all known Atlantic salmon MRFs is presented in Fig. 2. Common to all vertebrate MRFs, each salmonid *myoD* family gene is represented as three exons and two introns. For each gene, exon 1 is the largest, incorporating the NH_2-terminal activation domain, basic and HLH motifs, and in vertebrate *myoD* genes, a highly conserved region that has no assigned function currently. Exon 2 is the smallest for each MRF, and exon 3 incorporates the helix-III domain.

A maximum likelihood analysis was

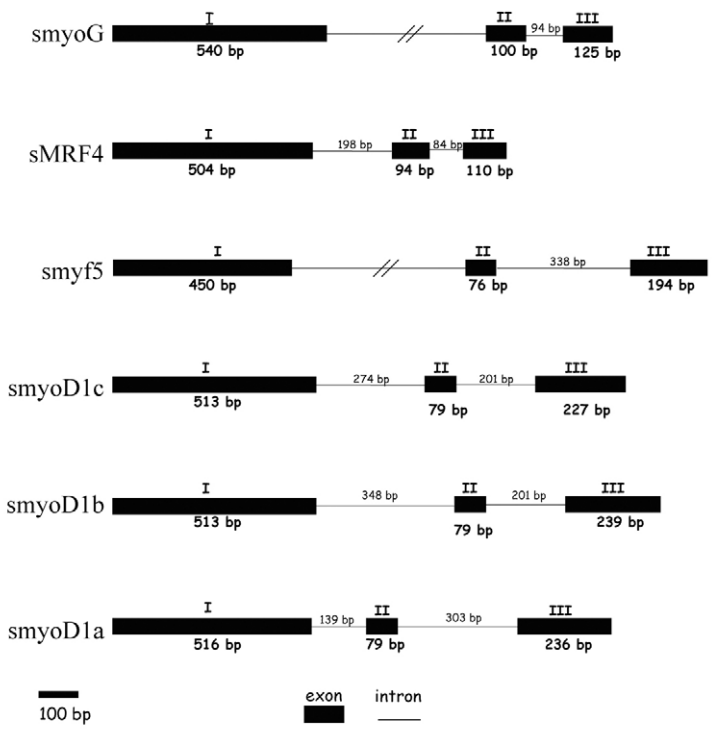

Fig. 2. Intron–exon structures of all known Atlantic salmon myoD family member genes. Each gene is represented by three exons (black boxes) and two introns (lines). The known sizes of exons and introns are shown. Introns with a double line are of unknown size (but in each case are greater than 1 kb). However, all intron–exon boundaries are supported experimentally.

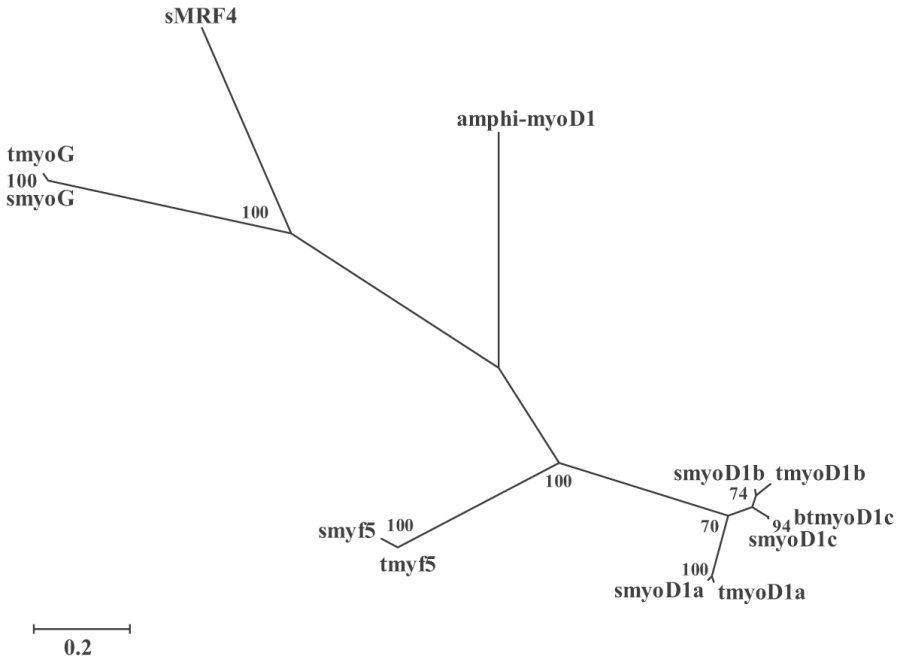

Fig. 3. Maximum likelihood tree produced in PHYML (see Materials and methods) displaying the phylogenetic relationships of all known salmonid myoD family proteins. The cephalochordate myoD homologue amphi-myoD1 is included as an outgroup. All confidence values are shown and were obtained using 500 bootstrap psuedoreplicates. The scale bar shows the number of substitutions per site.

performed on an alignment of all known salmonid myoD family members (Fig. 3) using amphi-*myoD1* as an outgroup. A NJ analysis was performed on the same alignment, producing a tree entirely consistent with Fig. 3 (not shown). Salmonid MRFs cluster distinctly into four groups, representing the four *myoD* family members. The three myoD1 proteins clustered together, representing their close paralogy. This is also reflected in their highly conserved genomic organisation (Fig. 2). As expected, smyoD1/smyf5 and smyoG/sMRF4 separately branch from amphioxus myoD (100% and 93% bootstrap support). This is consistent with the established evolutionary scenario where an ancestor *myoD* homologue (represented in sub-vertebrate taxa as a single gene) duplicated twice to produce the ancestor lineages to *myoD*/*myf5* and *myoG*/*MRF4* and then the individual MRFs (Atchley et al., 1994).

MRF expression co-ordinated by fibre-type differentiation in the maturing somite

We have recorded the mRNA expression patterns of six MRF genes throughout salmon embryogenesis. To place the expression of each MRF in the context of known muscle fibre differentiation events we also studied the expression of *s-smlc1*, which is expressed in rainbow trout adaxial cells as they differentiate (Chauvigne et al., 2005), and *Pax7*, which in zebrafish is expressed in the myogenic precursors of the external layer (Stellabotte et al., 2007; Hollway et al., 2007).

To simplify the expression data we present our findings in two formats. Firstly, a schematic diagram shows the progressive expression of each gene in the most anterior somite of salmon embryos during segmentation and post-segmentation stages of embryogenesis (Fig. 4). This excludes the complexity generated when considering the embryos rostral–caudal axis and associated gradient in expression patterns due to changing somite maturity. Fig. 4 enables the reader to quickly establish the spatio-temporal correlations between the expression patterns of the six MRFs with *s-smlc1* and *Pax7* in a single maturing somite. Next, we produced a detailed inventory of expression images for *smyoD1a*, *smyf5*, *smyoG*, *sMRF4* and *s-smlc1* from

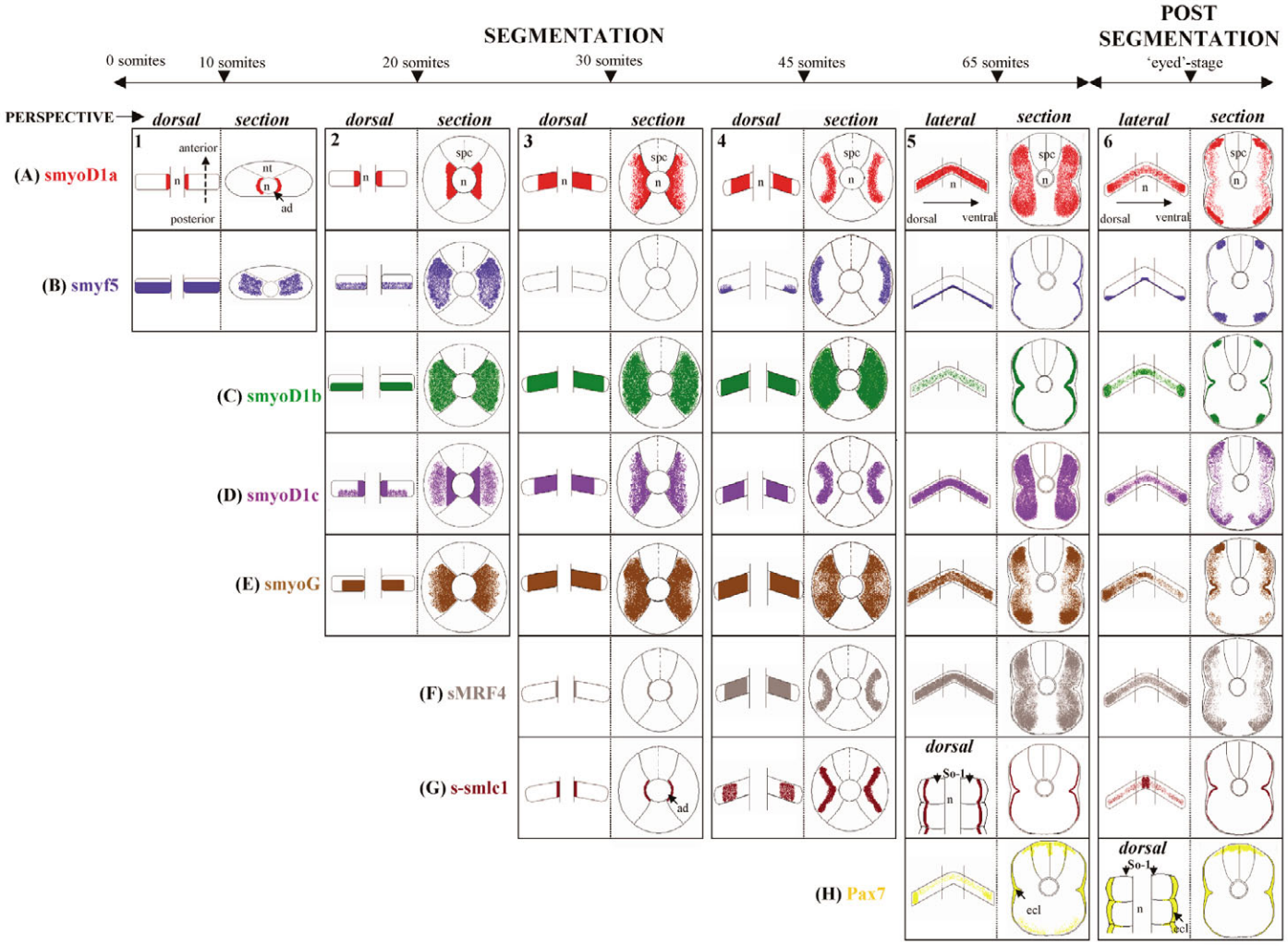

Fig. 4. Schematic diagram illustrating the mRNA expression patterns recorded for six MRF genes, *s-smlc1* and *Pax7* in the most anterior somite of the Atlantic salmon developmental stages numbered 1–6 (shown at the top of the figure). (A) *smyoD1a*, (B) *smyf5*, (C) *smyoD1b*, (D) *smyoD1c*, (E) *smyoG*, (F) *sMRF4*, (G) *s-smlc1*, (H) *Pax7*. The left of each box shows an expression field as viewed from either a dorsal of lateral perspective (indicated) of the most anterior somite using DIC optics. The right of each box shows a corresponding cross section through the region of expression in that somite. ad, adaxial cells; ecl, external cell layer; n, notochord; nt, neural tube; spc, spinal cord; So-1, somite 1.

the 30–45 ss (Fig. 5) and these genes plus *Pax7* at the end of segmentation and in a post-segmentation stage (Fig. 6). Only one *myoD1* paralogue (*1a*) is represented in this figure considering the recent detailed description of the expression of *smyoD1a*/*1b*/*1c* (Macqueen and Johnston, 2006). Each of the descriptions has been written for independent use, and thus a degree of overlap exists between them.

MRF expression in a single maturing somite (Fig. 4)

The first somite (So-1) is the oldest at any given stage of development and is used here to describe the progression in MRF, *s-smlc1* and *Pax7* expression during its maturation. When So-1 arose from the unsegmented mesoderm, *smyoD1a* and *smyf5* were, respectively, expressed in the adaxial myoblasts flanking the notochord (A1) and throughout its entire lateral width, excluding the most anterior quarter (B1). *Smyf5* was not expressed in So-1 adaxial cells during any period of embryogenesis (B1–6) in contrast to other teleosts studied to date (e.g. Coutelle et al., 2001; Cole et al., 2004). At the point when there were around 20 newer somites caudal to So-1, *smyoD1a* and *smyf5* did not change significantly (A2 and B2). However at this time three other MRFs were turned on in So-1. *SmyoD1b* expression was similar to *smyf5*, extending through the entire posterior domain of So-1, including the undifferentiated adaxial cells (C1 *vs* B1). *SmyoD1c* had a comparable expression pattern to *smyoD1a* and *1b*: transcripts were detected in the adaxial myoblasts (D2 *vs* A2) and diffusely in the posterior-lateral region of So-1, although less extensively than *smyoD1b*/*smyf5* (D2 *vs* C2, B2). At this time, *smyoG* was also expressed across So-1 but it expression extended further anteriorally than *smyoD1b* or *smyf5* (E2 *vs* C2/B2).

The first expression of *sMRF4* was present in So-1 at the 25 ss in the adaxial cells adjacent to the notochord (F3), just before an identical expression field was recorded for *s-smlc1* at the 30 ss marking the onset of adaxial cell differentiation (G3). At this time *smyf5* expression was downregulated in So-1 (B3), whilst *smyoD1b* and *smyoG* extended anteriorally (C3 and E3), coinciding with the first differentiation of fast muscle fibres. In contrast *smyoD1a*/*1c* expression spread laterally whilst maintaining a signal to the medial *s-smlc1* expressing adaxial cells (A3 and D3). This phase of expression preceded the lateral migration of the adaxial cells that was marked by an inward facing triangular wave of *s-smlc1* expression throughout the middle of So-1 at the 45 ss (G4). At this point *smyoD1a*/*1c* and *sMRF4* expression was comparable, but not identical to *s-smlc1* and was no longer present in the medial myotome of So-1 (A4, D4 and F4). In contrast, *smyoD1b* and *smyoG* were detected in the entire length and width of So-1 at this time (C4 and G4). Additionally, *smyf5* expression re-accumulated at the superficial edge of the posterior region of So-1, before the completion of adaxial cell migration (B4).

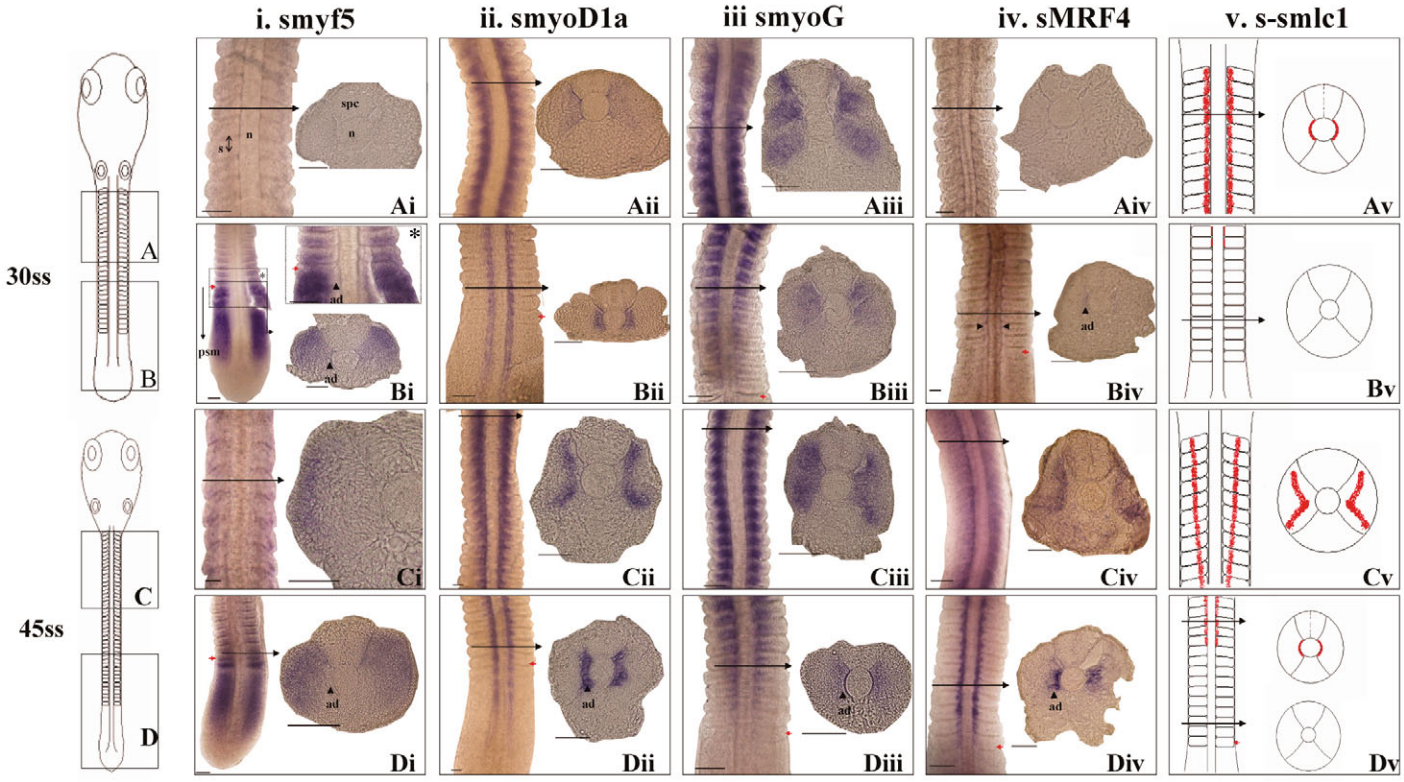

Fig. 5. mRNA expression patterns of myoD family member genes and a schematic representation of *s-smlc1* expression during the 30–45 ss. Numbers i-v represent the following cRNA probes: (i) *smyf5*, (ii) *smyoD1a*, (iii) *smyoG*, (iv) *sMRF4* and (v) *s-smlc1*. Letters A–D represent specific regions marked on schematic drawings of whole embryos from different stages (diagram on left side of figure). Images on the left of each box are dorsal perspective flat-mounts. Images on the right of each box are 18 μm cryosections from the region identified by a black arrow. Red arrows show the position of the last somite. *Magnified flat-mount of *smyf5* expression to show the lack of expression in adaxial myoblasts. Abbreviations are as in Fig. 4 with the addition of s, somite; psm, presomitic mesoderm. Scale bars, 50 μm.

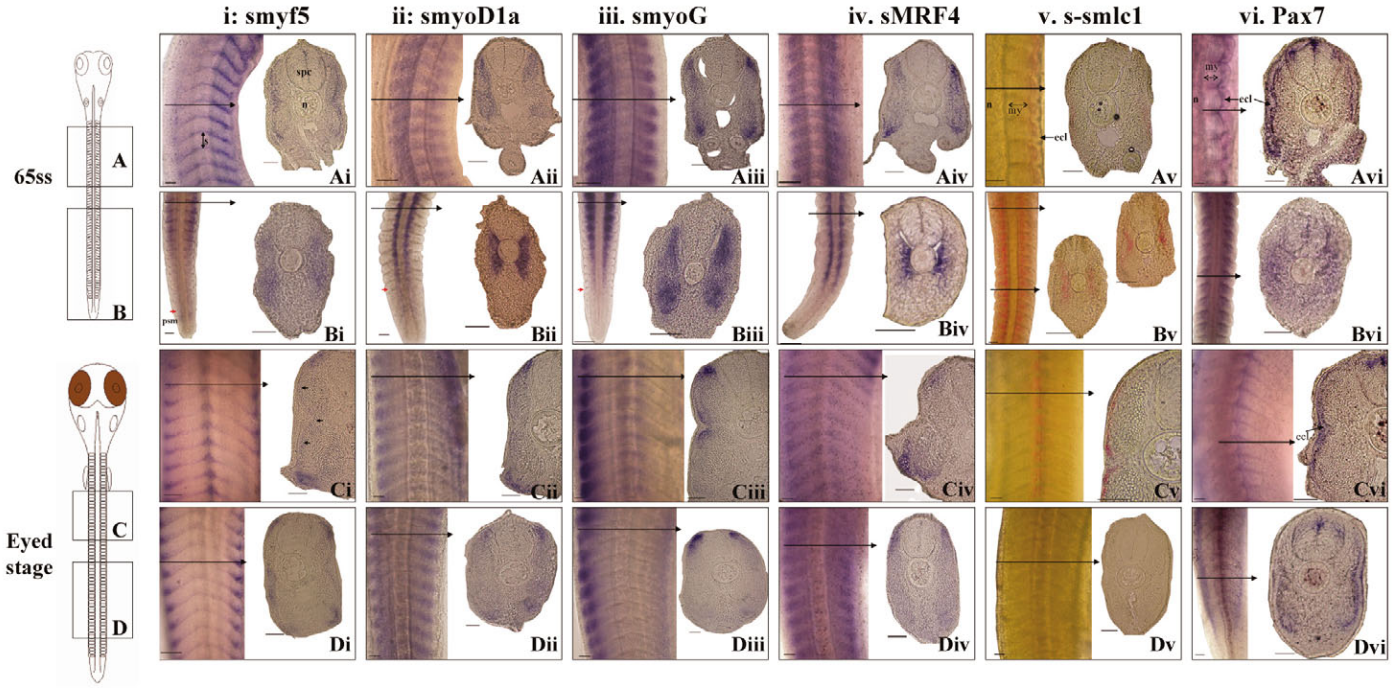

Fig. 6. mRNA expression patterns of myoD family member genes, *s-smlc1* and *Pax7* at the 65 ss and during the eyed stage. The numbering and lettering system is equivalent to that used in Fig. 5, except that (vi) represents the *Pax7* cRNA probe. Images on the left of each box are lateral perspective flat-mounts, except for Av–Avi and Bv–Bvi, which are mounted from the dorsal perspective. Images on the right of each box are 18 μm cryosections from the region identified by a black arrow. Abbreviations are as in Figs 4 and 5 and red arrows as in Fig. 5. Scale bars, 50 μm.

At the end of segmentation, So-1 had fully acquired the chevron-shaped phenotype, and the adaxial cells had spanned the myotome to form a single layer of slow-fibres, indicated by *s-smlc1* expression (G5). *Pax7* expression was present external to this layer, presumably marking myogenic progenitors of the external cell layer (H5). At the end of segmentation, *smyoD1a*, *smyoD1c*, *smyoG* and *sMRF4* were each expressed throughout the bulk of the myotome of So-1 (A5, D5, E5, F5). Conversely *smyf5* expressed was limited to the lateral edge of the myotome, in the posterior domain of So-1 (B5). From the 45 ss-end of segmentation *smyoD1b* was rapidly downregulated in all but the superficial region of the So-1 myotome (C5).

As So-1 matured further, *s-smlc1* and *Pax7* expression respectively remained in the single-slow layer and external cell layer (G6 and H6). At this time, each MRF was expressed most strongly in superficial regions of the So-1 myotome, particularly in dorsal and ventral regions and at the level of the horizontal septum (A6-E6). *Smyf5* staining was still restricted to the posterior region of So-1, faintly along the whole superficial edge of the myotome, and more strongly in the dorsal-ventral-zones (B6). *SmyoD1b* expression was very similar to *smyf5* in any cross-section, although the staining was present throughout the length of So-1 (C6). *MyoD1a*/*1c*, *sMRF4* and *smyoG* expression was not restricted to dorsal-ventral regions and each was also present in the deeper fast muscle fibres (A6, D6, E6, F6), although *smyoG* expression was comparatively fainter in ventral regions of the myotome (E6).

The dynamics of rostral-caudal expression of MRFs during embryogenesis (Figs 5 and 6)

Several MRFs were expressed in the adaxial myoblasts before *s-smlc1*. *SmyoD1a* was expressed in a bilateral strip flanking the nascent notochord of some pre-somitic embryos, although more often in adaxial progenitors of the presomitic mesoderm (PSM)/somites from the 0–10 ss and then maintained here in the newest somites/PSM throughout segmentation (Fig. 5Bii,Dii, Fig. 6Bii). *Smyf5* was expressed before or contemporaneously to *smyoD1a*, in two triangular fields of the PSM either side of the notochord, but did not colocalise with *smyoD1a* in pre-somitic adaxial myoblasts at this stage (not shown). During segmentation, *smyf5* was expressed throughout the mid-posterior of the newest somites, and in the anterior PSM, displaying a pattern of interspersed strong and reduced signal where the newest two somites arose (Fig. 5Bi,Di). Expression continued moving down the tailbud, terminating adjacent to the notochord's end (Fig. 5Bi,Di), but unlike other teleosts (e.g. Coutelle et al., 2001; Cole et al., 2004), *smyf5* was not expressed in the adaxial myoblasts of the anterior PSM or caudal somites (Fig. 5Bi,Di) until the end of segmentation when a residual PSM remained (Fig. 6Bi).

As somitogenesis progressed, other MRFs were expressed in adaxial myoblasts of somites, but not the PSM as for *smyoD1a*. At the ~20 ss *smyoD1c* colocalised with *smyoD1a* in somite adaxial cells (see Macqueen and Johnston, 2006), but also with *smyf5*/*smyoD1b* in the posterior domain of the newest somites (not shown). As somites matured, *smyoD1b* spread anteriorally to encompass the whole myotome (not shown) whereas *smyf5* was initially downregulated and barely detected in the rostral somites at the 30 ss (Fig. 5Ai). *SmyoG* mRNA was also detected at the 20 ss and was present in the adaxial myoblasts of the final few caudal somites (Fig. 5Biii,Diii, Fig. 6Biii), before rapidly spreading to encompass the whole myotome of more anterior

somites (Fig. 5Aiii,Ciii, Fig. 6Biii). The final myoD family member detected before the expression of *s-smlc1* was *sMRF4* at ~25 ss, in a faint transient wave of rostral–caudal expression in adaxial cells (30 ss stage, mid-somites shown: Fig. 5Biv).

S-smlc1 marks the differentiation of adaxial cells to slow muscle myocytes, which started in the rostral somites of ~30 ss embryos and progressed in a caudal direction as newer somites matured (Fig. 5Av). The progression of *s-smlc1* expression could be correlated with that of some *myoD* family members, whereas others seemed independent. For example, at the 25 ss, *sMRF4*, expression was present in adaxial cells of the rostral somites, immediately before *s-smlc1* expression at the 30 ss (not shown and Fig. 5Av) and similarly progressed caudally. However, the rostral–caudal progression of *sMRF4* was initially transient, disappearing in more rostral somites as it accumulated in newer somites. The timing of *sMRF4* preceded *s-smlc1*, so at the 30 ss, *s-smlc1* was maintained in rostral somites (Fig. 5Av), *sMRF4* had been downregulated at this site (Fig. 5Aiv), but was expressed in the mid-caudal somites (Fig. 5Biv), prior to *s-smlc1* expression here (Fig. 5Bv). In the rostral somites at the 30 ss, *smyoD1a*/*1c* transcripts had spread laterally away from the medial somite but this domain still overlapped with *s-smlc1* expression in differentiating medial adaxial cells (*smyoD1a* shown: Fig. 5Aii). As somites matured, the adaxial cells migrated laterally, indicated by a wave of *s-smlc1* transcripts in the rostral somites of 45 ss embryos (Fig. 5Cv). By the end of segmentation, this migration was occurring from around the tenth most caudal somite (Fig. 6Bv) and was completed in the rostral somites (Fig. 6Av). During adaxial cell migration, *smyoD1a*/*1c* and *sMRF4* transcripts moved away from the notochord and at the 45 ss, mRNA of each gene was present in a broad v-shaped domain similar to *s-smlc1* expression (e.g. *smyoD1a*: Fig. 5Cii, *sMRF4*: Fig. 5Civ). During this time, each of these MRFs remained in the adaxial myoblasts of the caudal somites, co-expressed with *smyoG*, before *s-smlc1* expression (e.g. Fig. 5Dii,Diii,Div,Dv, Fig. 6Bii,Biii,Biv).

In contrast to the 30 ss, where *smyf5* was downregulated in maturing somites (Fig. 5Ai), by the 45 ss, *smyf5* had accumulated in the rear quarter of the rostral somites at the superficial myotome, before the adaxial cells had completed their migration (Fig. 5Ci,Cv). This pattern was maintained, so that at the end of segmentation (65 ss), *smyf5* was expressed along the entire outer edge of the myotome at the rear border of the rostral-mid somites (Fig. 6Ai). *Smyf5* transcripts were present at this site before the adaxial cells had completed migrating, making it unlikely that this domain was limited to the slow layer. Instead, we suggest that *smyf5* expression marked the earliest production of muscle fibres sourced from the external cell layer. In support of this, at this time *Pax7* was clearly expressed specifically in the external cell layer of the rostral somites outside of the single slow layer (Fig. 6Avi). In more caudal somites, where *smyf5* had not reached the myotome border (Fig. 6Bi), *Pax7* was distributed throughout the somite, and particularly strongly at the anterior border (Fig. 6Bvi). Thus, the migration of *Pax7* mRNA to a position external to the myotome occurred at a similar time as the restriction of *smyf5* mRNA to the posterior border of the myotome.

The expression domains of *smyoG* and *smyoD1b* from the 30–65 ss also suggest a role for these transcription factors that is independent of adaxial cell migration. For example, both genes were unchangingly present across the width/length of the myotome in all but the most caudal somites at the 20–65 ss, irrespective of the migration state of adaxial cells [*smyoG* shown: Fig. 5Aiii,Ciii, Fig. 6Aiii, see also *myoD1b* (fig. 3B,F in Macqueen and Johnston, 2006)]. Additionally, the extension of *smyoD1b*/*smyoG* transcripts occurred in an anterior direction during somite maturation; adaxial cells migrated laterally.

After segmentation, when the eyes became pigmented, the fin buds lengthened and all somites developed the chevron shape (the eyed stage), *s-smlc1* expression was present as a single superficial layer of slow-twitch fibres in rostral-mid (Fig. 6Cv) but not caudal somites (e.g. Fig. 6Dv) and *Pax7* was expressed in the external cell layer and dorsal spinal cord along the embryos rostral–caudal axis (Fig. 6Cvi,Dvi). At this time, *smyf5*-expressing cells were present in the rear portion of all somites, mainly in the dorsal and ventral superficial fast myotome, adjacent to the horizontal septum and more faintly adjacent to the single slow muscle layer (Fig. 6Ci,Di). *SmyoD1b* was also expressed in similar regions at the superficial myotome, but was maintained along each somites length (not shown). Conversely, *smyoD1a*/*1c* and *sMRF4* transcripts were detected to a greater or lesser extent throughout the entire myotome (as in Fig. 6Aii,Aiv). As embryos (and somites) matured further, staining for these genes was reduced in the medial myotome but increasingly maintained in more superficial regions of the myotome, particularly in dorsal/ventral regions (*smyoD1a*: Fig. 6Cii,Dii; *sMRF4*: Civ,Div). Similarly, *smyoG* expression was present to a greater or lesser extent throughout the myotome, but as embryos matured, expression was reduced in the medial myotome but maintained at the dorsal (and faintly at the ventral) edge of the myotome and adjacent to the horizontal myoseptum (Fig. 6Ciii,Diii).

Embryonic temperature and somitogenesis

Fig. 7 shows the relationship between the rate of somitogenesis and embryonic temperature. Segmentation proceeded from around 750–1700, 425–960 and 250–600 h post-fertilization (h.p.f.) at 2°C, 5°C and 8°C, respectively. A first order linear regression was fitted to data of developmental

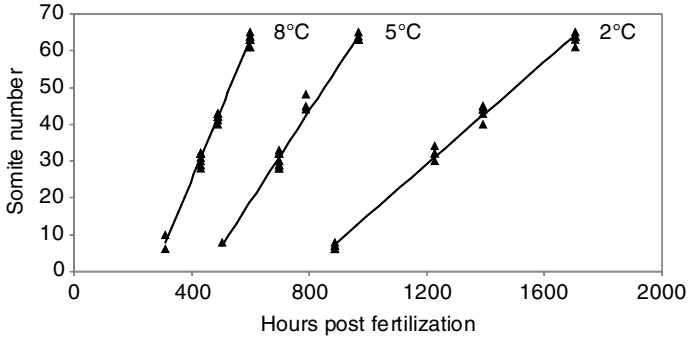

Fig. 7. Rate of somitogenesis in Atlantic salmon reared at three embryonic temperatures, 2°C, 5°C and 8°C. First order linear regressions were fitted to each group and the following equations were obtained: 2°C: somite number=–54.32+0.0696×h.p.f., R^2=99.5% (N=23). 5°C: somite number=–54.7+0.123×h.p.f., R^2=98.2% (N=21). 8°C: somite number=–51.8+0.192×h.p.f., R^2=99.3% (N=28).

time (h.p.f.) *versus* somite number during the linear phase of somitogenesis, which occurs from the 0 ss until the last few somites are added as segmentation is completed (Gorodilov, 1996). Using the regression equation from each plot, it was calculated that somitogenesis proceeded at a respective rate of one somite added each 15 h, 8 h and 5 h at 2°C, 5°C and 8°C.

Embryonic temperature affected the co-ordinated expression of MRFs

The expression of *smyoD1a*, *smyoG*, *smyf5*, *sMRF4* and *s-smlc1* was investigated at three embryonic temperatures (2°C, 5°C and 8°C). *SmyoD1a* and *smyoG* expression showed no variation between temperature treatments for corresponding somite stages (not shown). In contrast, at several equivalent somite stages, replicated differences (in six embryos per stage) were recorded in the mRNA expression profiles of *smyf5*, *sMRF4* and *s-smlc1* with respect to somite stage. The expression pattern of each gene at 5°C was approximately intermediate between that observed at 2°C and 8°C (not shown). *In situ* hybridisation cannot be used as a quantitative tool for comparative analysis and therefore we only highlight cases in which differences in staining intensity bordered on the presence or absence of transcripts in all embryos examined.

At the 30 ss and 45 ss, *smyf5* staining was intense in the newly formed caudal somites, presomitic mesoderm and tailbud at 8°C, but faint at 2°C (45 ss shown, Fig. 8A). In embryos approaching the end of segmentation (with ~63 out of 65 somites), *smyf5* staining had reached somite number 58 at 8°C, but was almost absent from somites 59–63 (Fig. 8B). In contrast, at 2°C, an *smyf5* mRNA signal was detected in somites 58–63 and within the residual presomitic mesoderm (Fig. 8B, see arrows in corresponding transverse sections). We interpret these results to show that *smyf5* expression was retarded with respect to somite stage at 2°C, with staining in the caudal somites and PSM peaking and subsequently retracting earlier at 8°C compared to lower temperatures.

In somites 30–45 of 45 ss embryos, *sMRF4* transcripts were detected in the medial somite at both temperatures (not shown), but as somites matured staining was more advanced at 8°C. For example, in somites 20–25, *sMRF4* transcripts were starting to migrate laterally away from the notochord at 8°C but not 2°C (Fig. 9B). Furthermore, *sMRF4* staining had advanced into somites 1–15 at 8°C, but not 2°C (Fig. 9A, see arrow on transverse sections). Towards the end of segmentation, while the most caudal somites (53–63) had *sMRF4* transcripts in adaxial cells at both temperatures (Fig. 9D), in more rostral somites (numbers 43–50) the medial compartment showed a strong *sMRF4* signal at 8°C, but was virtually unstained at 2°C (Fig. 9C, see arrowheads on transverse sections). These results indicate that the wave of *sMRF4* expression in maturing somites was retarded with respect to somite stage at lower temperatures.

As segmentation reached completion, the most newly formed somite with *s-smlc1* expression in the adaxial cells at 2°C was number 52–53, compared to 56–57 at 8°C (Fig. 10A). Thus at an equivalent somite stage, *s-smlc1* expression was delayed by 4–5 somites at 2°C (illustrated by blue arrowhead in Fig. 10A: also see arrowhead on transverse sections through equivalent somite number of 2 and 8°C embryos). In more rostral somites, a clear wave of *s-smlc1* transcripts could be seen migrating laterally away form the notochord between s43-s48 at 8 but not 2°C (not shown). In rostral somites (numbers 1–20) an *s-smlc1*

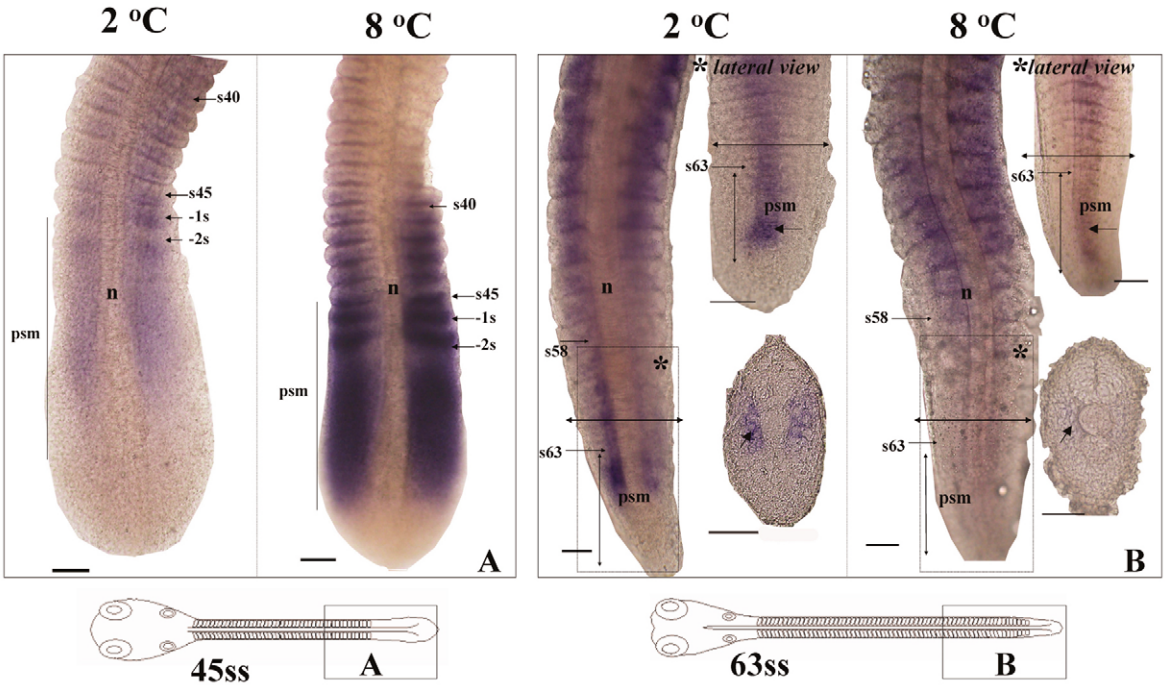

Fig. 8. Representative images showing the temperature associated heterochronies observed in *smyf5* expression. Images A and B correspond to the boxed regions labelled A and B on the schematic embryos below and temperatures (2°C and 8°C) are identified above each panel. Flat-mount images are viewed from the dorsal perspective except where otherwise indicated (by an asterisk) in boxes in B. Somite number is shown as s(*n*) where s=somite, *n*=number and the most caudal somite is the last numerically. Abbreviations are as in Fig. 5. Scale bars, 50 μm.

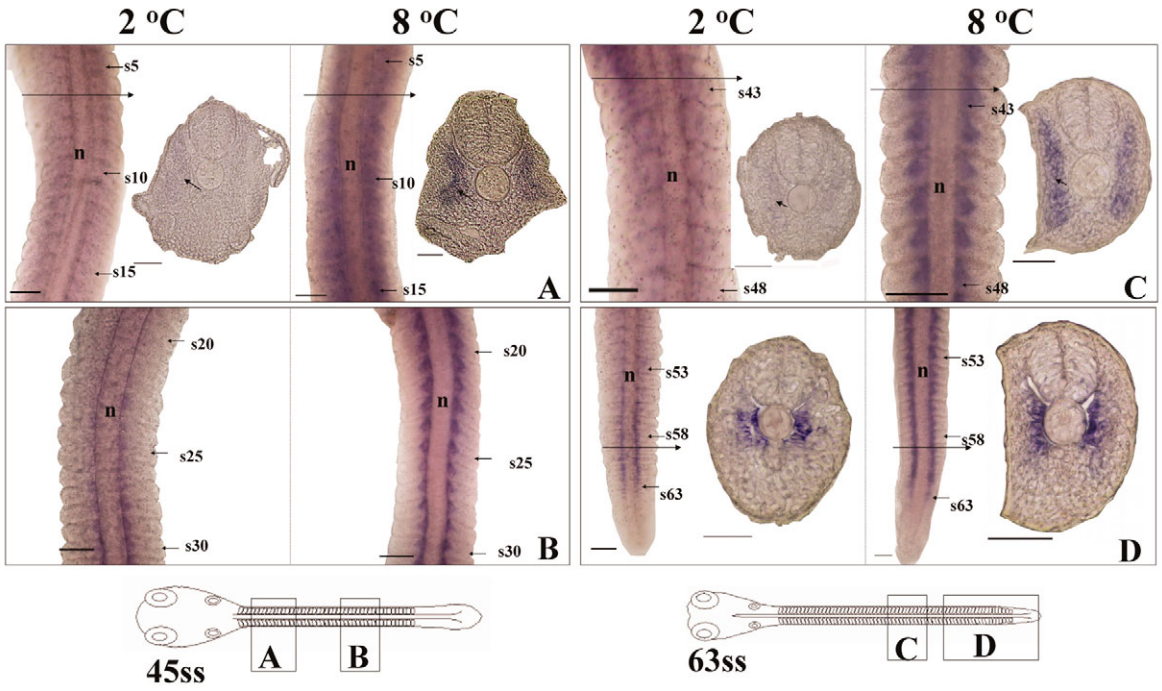

Fig. 9. Representative images showing the temperature associated heterochronies observed in *sMRF4* expression. Lettering and numbering system is the same as in Fig. 8. Flat-mount images are viewed from the dorsal perspective. Scale bars, 50 μm.

signal was detected in the superficial slow layer at 8°C, but not 2°C (not shown). These results suggest that like *sMRF4*, *s-smlc1* expression was strongly retarded at 2°C compared to 8°C.

Discussion

Expression patterns of myogenic regulatory factors (MRFs) in Atlantic salmon

We studied the coordinated expression of six Atlantic salmon MRFs during embryonic myogenesis. In mice, myoD and myf5 have mainly redundant roles in myogenic specification (Rudnicki et al., 1993). In the zebrafish, *myoD* and *myf5* are the first MRFs expressed in adaxial myoblasts (Weinberg et al., 1996; Coutelle et al., 2001) and while the morpholino-knockdown of either gene has no effect on slow muscle formation, when both are nulled, slow muscle formation is ablated (Hammond et al., 2007). In salmon, *myf5* and *myoD1a* are the first MRFs to be expressed in myogenic precursor cells of the segmental plate followed by *myoG* as differentiated muscle is formed. Although the order that MRF transcripts appear in salmon myotomes is similar to that described in zebrafish (Coutelle et al., 2001; Weinberg et al., 1996) there are some notable differences in expression patterns, which are probably related to the tetraploid nature of the salmonid genome (Allendorf and Thoorgard, 1984). The lineage leading to modern salmonids has undergone two whole genome duplications relative to the common tetrapod ancestor (Jaillon et al., 2004; Allendorf and Thoorgard, 1984). The salmonid-specific genome duplication is thought to have occurred 10–25 million years ago (Allendorf and Thoorgard, 1984). Approximately 50% of the duplicated genes have subsequently been lost from the genome and are represented by a single paralogue (Bailey et al., 1978). For example, only one *myoG* gene has been described in zebrafish (Weinberg et al., 1996), common carp *Cyprinus carpio* (Cole et al., 2004), rainbow trout (Delalande and Rescan, 1999) and Atlantic salmon (present study). The highly conserved expression pattern of myoG during embryonic myogenesis in these species suggests that myoG is retained as a single gene in salmonids. In other cases, duplicated genes have

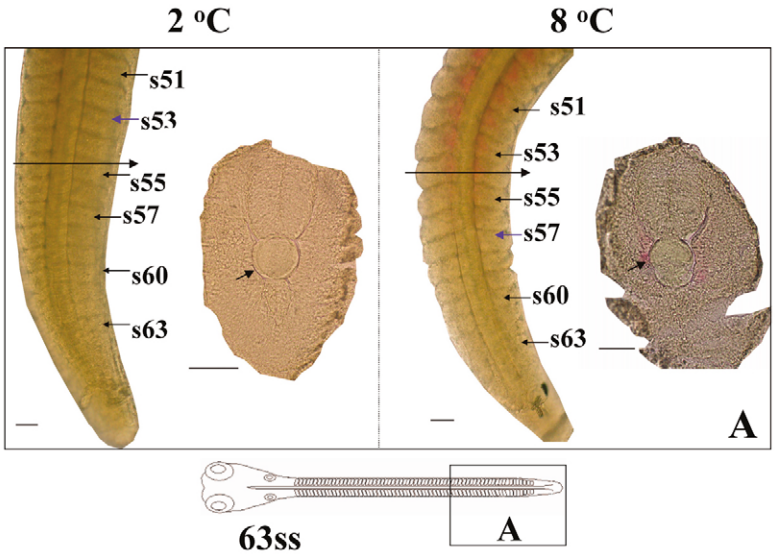

Fig. 10. Representative images showing the temperature-associated heterochronies observed in *s-smlc1* expression. Lettering and numbering system is the same as in Fig. 8. Flat-mount images are viewed from the dorsal perspective. The blue arrow shows the last somite considered to have *s-smlc1* expression. Abbreviations are as in Fig. 5. Scale bars, 50 μm.

been retained. For example, salmonid fish have three myoD paralogues (*myoD1a*/*1b*/*1c*), which, based on phylogenetic evidence (see Fig. 3), are thought to have arisen from a single gene orthologous to zebrafish *myoD1 via* a whole genome and subsequent local duplication (Macqueen and Johnston, 2006). During the segmentation period, *smyoD1a* and *smyoD1c* are sequentially expressed in adaxial cells and their progenitors as they differentiate in overlapping domains whereas *smyoD1b* is expressed exclusively in the lateral somite. The expression patterns of *smyoD1a*/*1c* and *smyoD1b* correspond to two embryonic phases of expression of the single *myoD1* gene in zebrafish in the adaxial cells and posterior-lateral somite (Weinberg et al., 1996). These expression domains mark the progenitors of the embryonic slow and fast muscle fibres (Devoto et al., 1996), respectively, which arise earlier than and are distinct from those muscle progenitors arising from the anterior somite (Hollway et al., 2007; Stellabotte et al., 2007). The myogenic precursors of the posterior somite and the adaxial cells are respectively regulated by hedgehog and fgf8 signals (Barresi et al., 2000; Groves et al., 2005). Our working hypothesis is that the role of the teleost *myoD1* gene was sub-functionalised in Atlantic salmon according to the model of Force et al. (Force et al., 1999), with each paralogue regulated by a sub-set of the *cis*-acting elements found in the promoter region(s) of the single *myoD1* gene (Macqueen and Johnston, 2006). A second myoD gene, *myoD2*, has been retained in five percomorphic teleosts (Tan and Du, 2002; Galloway et al., 2006; Macqueen and Johnston, 2006; Fernandes et al., 2007). To date *myoD2* paralogues have not been identified in salmonids or zebrafish. Since hundreds of skeletal muscle genes are regulated by myoD (Bean et al., 2005), the presence of multiple paralogues may provide additional levels of control and complexity of expression patterns providing some selective advantage leading to their retention in the genome.

In Atlantic salmon, *myf5* was expressed in the posterior domain of recently formed somites, the anterior PSM and tailbud (Fig. 5Bi,Di), in a similar pattern to that described in zebrafish (Coutelle et al., 2001) and common carp (Cole et al., 2004). However, in contrast with the other teleosts studied, *smyf5* was not expressed in adaxial cells during most of segmentation (Fig. 5Bi,Di), until a small residual PSM was present at the 65 ss. This finding is consistent with the presence of two possible *myf5* paralogues. To examine this further, we designed primers in conserved regions of *smyf5* to amplify intron 2. A single band was obtained by PCR using a gDNA template and, despite multiple sequencing, the identical sequence was represented in all clones. Furthermore, a single *myf5* orthologue was retrieved when BLAST searches were performed at the salmon genome project (http://www.salmongenome.no/cgi-bin/blast.cgi), TGI (Atlantic salmon/rainbow trout databases at: http://compbio.dfci.harvard.edu/tgi/) and GRASP (http://web.uvic.ca/cbr/grasp/) databases using *smyf5* as a probe. An interesting alternative possibility is that the two *myf5* genes produced during the tetraploidisation of the salmonid genome became sub-functionalised before one paralogue (expressed in adaxial myoblasts) was lost, perhaps because of the abundance of transcribed myoD1 paralogues in adaxial cells (Macqueen and Johnston, 2006) and known redundancy of myf5/myoD proteins in myogenic specification in vertebrates (Rudnicki et al., 1993; Hammond et al., 2007). If a second Atlantic salmon *myf5* paralogue does not fulfil the known role of the single zebrafish *myf5* orthologue in adaxial cell specification (Coutelle et al., 2001), then slow muscle development in salmonids is likely to vary significantly to other teleosts. A morpholino-based knock-down of individual salmonid MRFs would be informative in this respect.

No previous MRF4 expression pattern has been described in fish embryos for comparison with our results and nothing is currently known about its regulation. In wild-type mouse embryos, *MRF4* is the third myoD family member to be expressed within the hypaxial region of each thoracic somite whilst it is also expressed contemporaneously with *myf5* in the undifferentiated dermomyotome (Summerbell et al., 2002). When MRF4 expression was not compromised in *myoD*/*myf5* double null mice, normal myogenesis occurred, indicating that MRF4 can substitute for myf5/myoD in initiating muscle growth (Kassar-Duchossoy et al., 2004). It is unknown whether this dual role of MRF4 also occurs in teleost fish or is specific to mammals. In Atlantic salmon, MRF4 is first expressed in a transient rostral to caudal wave in somitic adaxial progenitors just prior to their differentiation and the expression of *s-smlc1*, suggesting it acts downstream of smyoD1a. It is interesting to note that that the Helix-III of sMRF4 is more distinct from smyoD1a (8/15 substitutions: Fig. 1), than a comparable alignment of mouse MRF4 *vs* myoD [6/15 substitutions in MRF4 compared to AA 245–258 of myoD (see Bergstrom and Tapscott, 2001)]. Substituting the helix-III of mouse myoD with the equivalent MRF4 region resulted in a chimera that efficiently activated endogenous muscle-specific genes (Bergstrom and Tapscott, 2001). The equivalent region of mouse myoG (with 8/15 substitutions, i.e. the same as sMRF4) could not replace the original myoD motif. It is possible that the increased number of substitutions in the helix-III of sMRF4 compared to mammalian MRF4 and/or differences in regulatory elements that have arisen during evolution have resulted in a reduced potency for myogenic specification whilst maintaining its role in differentiation. It is currently unknown whether multiple MRF4 paralogues are conserved in salmonids. Again, *in silico* searches in the same resources as described above for *myf5* lead us to retrieve *MRF4* cDNA sequences likely originating from the single gene described here (Figs 1, 3).

We have shown that following the end of segmentation, each MRF is expressed in zones of new myotube production that occur at the lateral edge of the fast myotome (stratified hyperplasia), particularly in dorsal and ventral areas and adjacent to the horizontal myoseptum. *Smyf5* was present at the superficial edge of the myotome in rostral somites from the 45 ss, initially prior to the completion of adaxial cell migration and was thus independent of the first wave of slow muscle differentiation. It is possible that *smyf5* marked the onset of stratified hyperplasia, which began at a similar stage of development in the closely related salmonid, *S. trutta*, evidenced by *myoD*/*myoG* expression (Steinbacher et al., 2007). *SmyoD1a*/*1c*, *sMRF4* and *smyoG* expression in the bulk of the myotome was reduced from the end of segmentation onwards, but maintained (or upregulated) at the lateral edge of the fast myotome at either the dorsal and/or ventral extremes and/or adjacent to the horizontal myoseptum. The source of additional embryonic fast muscle

fibres is likely to be the external cell layer, which is marked by *Pax7* expression (Fig. 4H5,H6 and Fig. 6Avi,Cvi,Dvi) (Hollway, 2007; Stellabotte et al., 2007; Steinbacher et al., 2007; Devoto et al., 2006).

Heterochronies in MRF expression at different temperatures

We have shown that altering egg incubation temperature produces heterochronies in the expression of some myogenic regulatory factors but not others. Thus, whereas *smyoD1a* and *smyoG* expression showed no consistent differences with temperature with respect to developmental-stage, the expression of *sMRF4* and *smyf5* and the slow muscle differentiation marker *s-smlc1* were retarded at 2°C compared to 8°C. Our finding that the relative timing of *smyoD1a* and *smyoG* expression was independent of temperature parallels observation in Atlantic cod (Hall et al., 2003), Atlantic herring (Temple et al., 2001), common carp (Cole et al., 2004) and Atlantic halibut (Galloway et al., 2006), but differs from the result reported in rainbow trout (Xie et al., 2001). As a consequence of the heterochronies in *sMRF4* and *smyf5* expression, the ratio of the individual myoD family members at each developmental stage was a function of environmental temperature. It is known that the different MRF proteins vary in their intrinsic abilities to initiate myogenesis or promote muscle differentiation (Bergstrom and Tapscott, 2001; Ishibashi et al., 2005). For example, whilst myf5 and myoD targeted a similar array of genes involved in myogenic specification, myoD was markedly more efficient at inducing muscle differentiation genes (Ishibashi et al., 2005). Functional analysis in mouse has shown that myoD strongly upregulates capn2, a protease required for myoblast–myotube fusion, whereas myoG has a weak effect and myf5 no effect (Dedieu et al., 2003). Using a combination of genome-wide transcriptional factor binding and expression profiling in the mouse a total of 126 genes were identified that bound myoD (Blais et al., 2005). Many of these genes were transcription factors that propagate and amplify signals initiated by the MRFs (Blais et al., 2005). MyoD and myoG occupied 91 and 137 promoters in differentiating myotubes, indicating the MRFs recognise distinct, but overlapping, targets (Blais et al., 2005). Of particular interest was the finding that MRFs bind a set of genes involved in synapse specification and the function of the neuromuscular junction (Blais et al., 2005). In Atlantic herring, embryonic temperature has been shown to produce major changes to the timing of development of neuromuscular junctions in the myotomal and fin muscles (Johnston et al., 1997; Johnston et al., 2001). Herring were reared at 12°C and 5°C until just after hatching and then transferred to a common ambient temperature. The development of dorsal and anal fin ray muscles and their neuromuscular junctions occurred at shorter body lengths in the 12°C-group, resulting in improved fast-start swimming performance relative to the 5°C-group (Johnston et al., 2001).

Morpholino knock-down experiments of *myoD* and *myf5* in the zebrafish resulted in an increase in the number of *Pax3/7*-expressing external cells on the lateral surface of the somite (Hammond et al., 2007). Since these cells are a source of fast muscle growth throughout post-embryonic zebrafish growth (Hollway et al., 2007; Stellabotte et al., 2007), heterochronies in MRF expression provide a potential mechanism that could explain some of the major changes in muscle phenotype that occur with variations in developmental temperature, including changes in muscle fibre number.

Note added in proof

Since this paper was submitted the expression pattern of the zebrafish orthologue of MRF4 has been published (Hinits et al., 2007). This work showed that zebrafish MRF4 has a comparable expression pattern to sMRF4, being initially expressed in differentiated slow muscle precursors of somites after *myoD1* (regulated by hedgehog signalling, and ablated by the morpholino antisense knockdown of *myf5* and *myoD1*) and later in differentiated fast muscle fibres subsequent to the expression of *myoD1* and *myoG* in the lateral somite.

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