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IDENTIFICATION AND EXPRESSION ANALYSIS OF TWO DEVELOPMENTALLY REGULATED MYOSIN HEAVY CHAIN GENE TRANSCRIPTS IN CARP (*CYPRINUS CARPIO*)

STEVEN ENNION[1], DAVID WILKES[1], LAURENT GAUVRY[1,*], HÉLENE ALAMI-DURANTE[2] AND GEOFFREY GOLDSPINK[1,‡]

[1]*Department of Anatomy and Developmental Biology, Royal Free and University College Medical School, Rowland Hill Street, London NW3 2PF, UK* and [2]*Unité Mixte INRA-IFREMER de Nutrition des Poissons, Station d'Hydrobiologie INRA, BP.3, 64310 Saint Pée sur Nivelle, France*

*Present address: Département Génie Biologie, Institut Universitaire de Technologie de Laval, 52 rue des docteurs Calmette et Guérin BP40, 53020 Laval, France

‡Author for correspondence (e-mail: goldspink@rfhsm.ac.uk)

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Summary

Whilst developmentally regulated genes for the myosin heavy chain (*MyoHC*) have been characterised in mammalian, avian and amphibian species, no developmental *MyoHC* gene has previously been characterised in a species of fish. In this study, we identify two developmentally regulated *MyoHC* gene transcripts (named *Eggs22* and *Eggs24*) in carp (*Cyprinus carpio*) and characterise their expression patterns during embryonic and larval development. The transcripts showed an identical temporal pattern of expression commencing 22 h post-fertilisation (18 °C incubation temperature), coincident with the switch from exclusive expression of genes for β-actin to expression of genes for both β- and α-actin, and continuing for 2 weeks post-hatching. No expression of these myosin transcripts was detected in juvenile or adult carp. Wholemount *in situ* hybridisation showed that both transcripts are expressed initially in the rostral region of the developing trunk and progress caudally. Both are expressed in the developing pectoral fin and protractor hyoideus muscles. However, the muscles of the lower jaw express only the *Eggs22* transcript. No expression of either transcript was detected in cardiac or smooth muscle. A distinct chevron pattern of expression was observed in the myotomal muscle. This was shown to be caused by localisation of the mRNAs to the myoseptal regions of the fibres, the sites of new sarcomere addition during muscle growth, suggesting transport of *MyoHC* mRNA transcripts. The 3′ untranslated region of the *Eggs24* transcript contains a 10 base pair motif (AAAATGTGAA) which is shown to be also present in the 3′ untranslated regions of *MyoHC* genes from a wide range of species. Possible reasons for the need for developmental isoforms of myosin heavy chain isoforms are discussed.

Key words: myosin heavy chain, muscle, development, *in situ* hybridisation, carp, *Cyprinus carpio*, muscle.

Introduction

The precursors of muscle fibres are mononucleated embryonic mesodermal cells which do not themselves synthesize any of the muscle-specific myofibrillar proteins. During muscle formation, these cells proliferate and differentiate into mononucleated myoblasts, which fuse to form multinucleated myotubes. In mammals, a biphasic process of muscle fibre formation is observed. The first myotubes to form are known as primary myotubes, and these provide a framework along which remaining myotubes orientate themselves longitudinally and subsequently fuse to form the secondary myotubes. This process initially leads to a 'rosette' arrangement of muscle fibres, with the larger primary myotubes surrounded by smaller secondary myotubes. The difference in size between primary and secondary myotubes gradually diminishes until the two populations are indistinguishable in the adult. In fish, this biphasic development of primary and secondary myotubes is not observed. However, the origins of different muscle fibres are both spatially and temporally segregated. In zebrafish, adaxial cells of the segmental plate migrate radially from a position adjacent to the notochord to the lateral surface of the myotome, where they differentiate into slow muscle fibres. In contrast, the fast muscle fibres arise from lateral presomatic cells that do not contact the notochord (Devoto et al., 1996). Subsequent to the formation of the inner white and superficial red zones of muscle in the embryo and yolk-sac larvae, a second stage of muscle development occurs in the free-swimming larvae when the adult red and pink fibre types form and a new phase of fibre hyperplasia begins in the white muscle zone. The newly formed small muscle fibres in these

later stages of development have been shown to be immunohistochemically different from more mature fibres (Rowlerson et al., 1985; Akster, 1983).

The molecular motors driving muscle contraction are the myosin heavy chains (MyoHC), and a complex pattern of MyoHC isoforms is expressed during muscle fibre development. In rat and human, the primary muscle fibres initially express the embryonic (Strehler et al., 1986; Karsch-Mizrachi et al., 1989; Eller et al., 1989a; Molina et al., 1987), neonatal (termed perinatal in human) (Weydert et al., 1987; Feghali and Leinwand, 1989; Periasamy et al., 1984) and the slow β cardiac (Narusawa et al., 1987; Barbet et al., 1991) MyoHC isoforms, but none of the fast MyoHC isoforms. The secondary fibre population of myotubes express embryonic, neonatal and fast MyoHC isoforms in a heterogeneous manner, but never express (at least in humans) the slow-twitch MyoHC isoform (Barbet et al., 1991). Later in embryonic development (at approximately 35 weeks of gestation in the human), embryonic isoform expression decreases, and shortly after birth expression of the neonatal isoform also disappears. Concomitantly with the elimination of the embryonic and neonatal isoforms, the adult slow and fast MyoHC isoforms begin to be expressed predominantly as the muscle takes on its adult phenotype. Also at this stage, certain fibres cease to express the slow β cardiac isoform and begin to express the adult fast isoforms (Barbet et al., 1991). The myosin heavy chain isoform transitions occurring during embryonic development in the rabbit have been quantified at the mRNA level by RNAase protection assay, and these changes are closely mirrored by changes in the corresponding proteins, suggesting that the control of isoform transitions occurs at the transcription level (McKoy et al., 1998). Immunological studies (Hughes et al., 1993) suggest that at least three different slow MyoHC isoforms are expressed during development in rat; however, there is no evidence for this at the gene level. A second embryonic MyoHC isoform has also been described in chicken skeletal muscle (Hofmann et al., 1988). *Xenopus laevis* has been shown to express at least two 'embryonic' isoforms, named E3 and E19 (Radice and Malacinski, 1989), with the E3 isoform being predominant in the larval type II fibres, whilst the E19 isoform is predominant in the type I fibres. However, a significant proportion of type I and type II fibres co-express both isoforms (Radice, 1995).

The family of sarcomeric MyoHC isoforms expressed in all vertebrate species studied to date has been shown to be large and complex, with the individual isoforms being very similar in size (about 220 kDa) and sharing many common epitopes. This has often hampered the distinction of separate isoforms at the protein level by electrophoretic or immunological means. To date, the most definitive way of characterising different MyoHC isoforms in a species has been to isolate the gene sequence for the separate isoforms because each individual isoform in vertebrate species is encoded by a separate gene. Whilst the genes coding for the mammalian and some avian MyoHC isoforms are well characterised, relatively few piscine *MyoHC* genes have been studied, of which none correspond to isoforms expressed exclusively during embryonic development. The aim of the present study was to characterise those *MyoHC* genes that are expressed in developing carp (*Cyprinus carpio*) embryos. The high levels of sequence homology which have been shown to exist between all MyoHC isoforms studied to date dictates that nucleotide probes to be used for the characterisation of the expression patterns of different isoforms within the same species have to be chosen with care. Probes covering the coding region of the gene have been shown to cross-hybridise to a number of MyoHC isoforms within the same species (Stedman et al., 1990; Eller et al., 1989b), making them of limited use in determining the expression patterns of individual isoforms. Therefore, the strategy adopted in the present study was to isolate the 3′ ends of *MyoHC* isogenes and use the 3′ untranslated region (3′UTR) as isogene-specific probes to map expression patterns using northern and *in situ* hybridisations.

Materials and methods

Animals

Carp eggs were fertilized at the INRA Station d'Hydrobiologie, Saint Pée sur Nivelle, France. Embryos were incubated and larvae reared at 18 °C according to the protocol described by Alami-Durante et al. (1997).

Oligonucleotides

The primers RoRidT17 (5′ATCGATGGTCGACGCATGC-GGATCCAAAGCTTGAATTCGAGCTCTTTTTTTTTTTTT-TTTT3′) and Ro (5′ATCGATGGTCGACGCATGCGGA-TCC3′) were synthesized according to the sequence designed by Harvey and Darlison (1991). The Ro primer corresponds to the first 25 nucleotides of the RoRidT17 primer that was used to prime cDNA synthesis. The oligonucleotide primer FG2EXN40 (5′AGGAAGGTCGAGCATGAACTGGAGG3′) was synthesised to correspond to part of the exon 40 sequence of the carp FG2 *MyoHC* gene (Ennion et al., 1995a). The expected size of the amplified polymerase chain reaction (PCR) product from the FG2 *MyoHC* gene is approximately 250 bp long when the FG2EXN40 and Ro primers are used. The oligonucleotides EGGS2224F (5′GACAAA-GCTTAGTCTCA3′), EGGS22R (5′TAGGAATTCAGATT-TATTTC3′) and EGGS24R (5′GATGAATTCAATGCTT-TAT3′) were designed on the basis of the sequence of myosin heavy chain containing PCR clones from FG2EXN40/Ro amplifications. EGGS2224F had a *Hin*dIII restriction site incorporated in the sequence, whereas EGGS22R and EGGS24R had *Eco*RI sites incorporated to facilitate subcloning into pBS$^{(+)}$ vector (Stratagene).

cDNA synthesis

Total RNA (20 μg) extracted from pre-hatched carp embryos by the method described by Chomczynski and Sacchi (1987) was resuspended in diethylpyrocarbonate (DEPC)-treated

water, heated to 65 °C for 5 min, and rapidly cooled on ice. To this was then added 40 units of RNAase inhibitor (Promega), 5 μl of 5× reverse transcriptase buffer (500 mmol l^{-1} Tris–HCl, 600 mmol l^{-1} KCl, 100 mmol l^{-1} $MgCl_2$, pH 8.15 at 42 °C), 5 μl of dNTP stock (5 mmol l^{-1} for each nucleotide), 500 ng of the oligonucleotide primer RoRidT17, 0.5 μl of 1 mol l^{-1} dithiothreitol (DTT), 0.5 μl (7.5 units) of Rous-associated-virus reverse transcriptase (Amersham) and DEPC-treated water to a final volume of 25 μl. Control reactions, identical to those described above but with no reverse transcriptase enzyme added, were also performed to rule out possible contamination of genomic DNA. Reaction mixtures were incubated at 42 °C for 2 h and stored at −20 °C.

Polymerase chain reaction

PCR amplifications of first-strand cDNA were performed using a recombinant *Taq* DNA polymerase (Boehringer Mannheim). Each reaction contained 1 μl of unpurified first-strand cDNA, 5 μl of 10× *Taq* polymerase buffer, 25 pmol of Ro primer, 25 pmol of FG2EXN40 primer, 2 μl of dNTP stock (5 mmol l^{-1} of each nucleotide in stock) and 5 units of *Taq* polymerase (added after a 'hot start') in a reaction volume of 50 μl. Control reactions, with no template added, were also performed to verify that contaminating DNA was absent. Reaction conditions were 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min for 25 cycles. After the last amplification cycle, samples were incubated at 72 °C for 5 min to extend incomplete products. PCR products were then analysed by gel electrophoresis on 2 % agarose gels made in TAE buffer (4 mmol l^{-1} Tris–Hcl, 4 mmol l^{-1} sodium acetate, 2 mmol l^{-1} EDTA). PCR products were isolated from agarose gels using a 'Prepagene' kit (Bio-Rad) and subcloned into Bluescript $pBS^{(+)}$ phagemid (Stratagene) using the T–A cloning method described by Marchuk et al. (1991). DNA sequencing was performed by the chain termination method (Sanger et al., 1977).

Probe synthesis

The plasmids EGGS22UTR and EGGS24UTR were constructed to facilitate production of isoform-specific probes that contained 3′ untranslated region sequence but no poly(A) tail. These plasmids contained PCR products amplified with EGGS2224F/EGGS22R (for EGGS22UTR) and EGGS2224F/EGGS24R (for EGGS24UTR) primers using EGGS22 and EGGS24 plasmid DNA as template and cloned into $pBS^{(+)}$ (Stratagene) vector at the *Eco*RI/*Hin*dIII restriction sites. For northern hybridisation probes, the 'UTR' plasmids were linearised with *Hin*dIII, and EGGS22R or EGGS24R was used for specific primer extension with [α-^{32}P]dATP (3000 Ci $mmol^{-1}$, Amersham International) as label. The carp actin probe FGA101 (Gerlach et al., 1990), which hybridises to both α- and β-actin, was labelled by random priming (Feinberg and Vogelstein, 1984). cRNA probes for *in situ* hybridisation were produced from linearised plasmid by T3 or T7 RNA polymerase (*Eco*RI linearised/T3 polymerase for the sense probe, *Hin*dIII/T7 polymerase for the antisense probe) using digoxigenin-11-UTP as label, according to the manufacturer's instructions (Boehringer Mannheim).

Northern hybridisation

Total RNA was extracted by the method described by Chomczynski and Sacchi (1987). Electrophoresis of RNA (30 μg) was performed in 1.5 % agarose gels prepared in MOPS buffer (0.02 mol l^{-1} MOPS, 5 mmol l^{-1} sodium acetate, 1 mmol l^{-1} EDTA, pH 7.0) with 0.66 mol l^{-1} formaldehyde. RNA was transferred to 'Zeta Probe' nylon membrane (Bio-Rad) in 10× standard saline citrate (SSC; 1×SSC is 0.15 mol l^{-1} NaCl, 0.015 mol l^{-1} sodium citrate) and fixed by baking at 80 °C for 2 h. Hybridisation and subsequent washes were carried out at 15 °C below the calculated melting temperature of the probe duplex (64 °C) according to the method of Church and Gilbert (1984) and at a probe concentration of 10^6 counts ml^{-1}. Hybridised probe was detected by exposure of the washed membrane to X-ray film (Du-Pont) at −70 °C using an intensifying screen.

In situ *hybridisation*

Carp embryos were anaesthetized in 75 mg l^{-1} MS222 (Thomson and Joseph Ltd) followed by fixation in 4 % paraformaldehyde at 4 °C overnight. Embryos were then dehydrated in methanol (30 %, 50 %, 80 %, 95 %, 100 % and 100 % for 10 min each) and stored at −20 °C until required. Embryos for hybridisation were dechorinated using fine forceps under 100 % methanol and washed sequentially (5 min each) in the following buffers: methanol/PBST 1:1, methanol/PBST 3:7, PBST, PBST (PBST is phosphate-buffered saline with 0.1 % Tween-20). Embryos were then refixed for 20 min in 4 % paraformaldehyde followed by two 5 min washes in PBST. Permeabilisation of the embryos was achieved by digestion with proteinase K (Boehringer Mannheim, 50 μg ml^{-1}) in PBST for 10 min at room temperature followed by a 5 min wash in PBST. Embryos were refixed for 20 min at room temperature in 4 % paraformaldehyde/0.2 % glutaraldehyde and washed twice in PBST (5 min each). After a 10 min incubation in 0.1 mol l^{-1} triethanolamine (pH 8.0) with 2.5 μl ml^{-1} acetic anhydride followed by two 10 min washes in PBST, embryos were incubated for 1 h in hybridisation buffer (50 % formamide 5×SSC, 0.1 % Tween-20, 50 μg ml^{-1} heparin, 10 μg ml^{-1} yeast tRNA) at 65 °C with one change of buffer. Embryos were hybridised overnight at 65 °C in preheated hybridisation buffer containing sense or antisense cRNA probe at a concentration of 1 ng μl^{-1}. Post-hybridisation washes consisted of the following: 50 % formamide/2×SSC for 30 s at room temperature (to remove the majority of unbound probe), 50 % formamide/2×SSC 1 h at 65 °C, three 10 min washes in 2×SSC at 37 °C and one 5 min wash in PBST at room temperature. Unbound probe was further removed by digestion with a mixture of RNAase A (20 μg ml^{-1}) and RNAase T1 (100 units ml^{-1}) in PBST for 1 h at 37 °C with shaking. Embryos were further washed in 2×SSC for 10 min at 37 °C, 2×SSC for 15 min at 55 °C, 0.2×SSC for 15 min at

55 °C (twice) and PBST for 5 min at room temperature. For detection of hybridised probe, alkaline-phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) was used as follows: incubation in solution B (PBS with 0.2 % Tween-20, 0.2 % Triton X-100 and 2 % sheep serum) for 1 h at room temperature followed by incubation with preabsorbed antibody in buffer B at a final antibody dilution of 1:4000 overnight at 4 °C (antibodies were preabsorbed by incubating for 1 h at a dilution of 1:400 in buffer B with embryos that had been treated as described above but with no probe in the hybridisation buffer). Unbound antibody was removed with two 10 s washes in solution B, three 30 min washes in solution B, one 30 min wash in solution B plus 1 mmol l^{-1} levamisol and three 10 min washes in detection buffer (100 mmol l^{-1} Tris–HCl, pH 9.0, 50 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} NaCl, 0.1 % Tween-20, 1 mmol l^{-1} levamisol), all at room temperature. Colour precipitation was performed with NBT/X-phosphate (Boehringer Mannheim) overnight at 4 °C in darkness. The reaction was stopped by incubation in PBS containing 1 mmol l^{-1} EDTA, and the embryos were visualised and photographed on an Olympus SZH10 microscope using darkfield illumination. Hybridised wholemount embryos were subsequently processed for wax embedding using standard histochemical techniques, and 10 μm sections were cut, stained with eosin and viewed with a Nikon eclipse E800 microscope using brightfield illumination.

Results

Two distinct MyoHC clones were obtained by PCR

PCR amplification using the FG2EXN40/Ro primers yielded a faint smear of PCR product with a prominent band at approximately 250 bp (Fig. 1). No bands were observed in the control reaction lacking template or in the control reaction lacking reverse transcriptase. The 250 bp band was cloned, and multiple colonies were sequenced. Two different types of clone were isolated from this band (multiple clones for each type), and these were named EGGS22 and EGGS24 (Fig. 2). The clones encoded the carboxy-terminal region and the 3′UTR of two *MyoHC* transcripts which were named *Eggs22* and *Eggs24* respectively. Both sequence types showed a high degree of sequence homology at the deduced amino acid level to equivalent regions of mammalian myosin heavy chain sequences (Fig. 3). The regions of the two transcripts that were used as probe (EGGS22UTR and EGGS24UTR plasmids, underlined sequence in Fig. 2) had 51.7 % sequence similarity determined using the CLUSTAL programme of Higgins and Sharp (1988) and did not hybridise to one another on Southern blots (data not shown).

Expression of the Eggs22 *and* Eggs24 MyoHC *transcripts is developmentally regulated*

To determine the temporal expression pattern of the two *MyoHC* transcripts, northern blots of RNA from successive developmental stages and from adult tissue were probed.

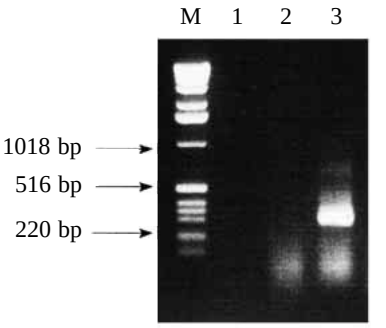

Fig. 1. Electrophoresis of polymerase chain reactions. Lane M, DNA size marker (1 kb ladder; Gibco BRL). Lane 1, control in the absence of template. Lane 2, carp fry cDNA control in the absence of reverse transcriptase. Lane 3, Carp fry cDNA. bp, base pair.

Both the EGGS24UTR and EGGS22UTR probes bound specifically to an RNA transcript of 6 kilobases (kb) in size (Fig. 4). The temporal expression patterns of the two myosin heavy chain transcripts *Eggs22* and *Eggs24* was identical at the northern hybridisation level. Both transcripts commenced expression at 22 h post-fertilisation. This was after somite formation and just before the first movements of the embryo were observed. Expression of the transcripts continued for over 2 weeks after hatching. No expression of the transcripts was detected at 21 or 28 days after hatching (Fig. 4), or in white or red muscle RNA of 12-month-old juvenile carp or adult carp (data not shown). The carp actin probe FGA101 bound to two RNA transcripts corresponding to α- and β-actin. Expression of the gene for β-actin was detected in all samples. Expression of the gene for α-actin, however, commenced at the same time as that for the *Eggs22* and *Eggs24* myosin heavy chain transcripts (after 22 h post

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AGGAAGGTCCAGCACGAACTGGAGGAGGCTGAGGAGCGTGCTGACATTGCTG Eggs22
|||||||||||||||||||||||||||||||||||||||||*||||||||||
AGGAAGGTCCAGCACGAACTGGAGGAGGCTGAGGAGCGTGCAGACATTGCTG Eggs24
R  K  V  Q  H  E  L  E  E  A  E  E  R  A  D  I  A
 R  K  V  Q  H  E  L  E  E  A  E  E  R  A  D  I  A

AGTCTCAAGTCAAGAAGCTCAGAGCCAAGAGCCGTGATGCTGGAAAGGCCAA Eggs22
|||||||*|||||*|||||||||||||||||||||||||||||||||*|||
AGTCTCAGGTCAACAAGCTCAGAGCCAAGAGCCGTGATGCTGGAAAGGGCAA Eggs24
E  S  Q  V  K  K  L  R  A  K  S  R  D  A  G  K  A
 E  S  Q   V  N  K  L  R  A  K  S  R  D  A  G  K  G

AGAAGAGTGAAACCCTCAAACCTGATGGAAGGCTCTACTGAAGTCATATAAT Eggs22
|||*|******|*****|**|*|*|******|**|*||******|***|**|
AGAGGCAGCTGAGTGAAATCCTTCACATTTAGTGCCACCTGCTCCCATTTGT Eggs24
K  E  E STOP
 K  E  A  A  E stop

ATGATTGTTCTGTAGATTTACATGTAATATTCCTTTTGAAATAAATCTGTCT Eggs22
||**********|*|||******|||*********|*****
ATCTGAAAATGTGAAATTGTTCAATAAAGCATTACATTCATCA(n)       Eggs24

 CACTAGA(n)                                           Eggs22
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Fig. 2. Nucleotide sequence of EGGS22 and EGGS24 and deduced amino acid sequence of *Eggs22* and *Eggs24*. The underlined sequence indicates the region used as probe for expression analysis. EMBL database accession numbers: EGGS22, AJ009735; EGGS24, AJ009734.

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EGGS22        RKVQHELEEAEERADIAESQVKKLRAKSRDAGKAK----EE
EGGS24        RKVQHELEEAEERADIAESQVNKLRAKSRDAGKGKEA--AE
(1) FG2       RKVQHELEEAQERADIAESQVNKLRAKSRDAGKSKD---EE
(2) Humanβ    RKVQHELDEAEERADIAESQVNKLRAKSRDIGTKGL--NEE
(3) Baboonβ   RKVQHELDEAEERADIAESQVNKLRAKSRDIGTKGL--NEE
(4) Ratβ      RKVQHELDEAEERADIAESQVNKLRVKSRDIGAKGL--NEE
(5) Rabbitβ   RKVQHELDEAEERADIAESQVNKLRAKSRDIGTKSL--NEE
(6) Humanα    RKVQHELDEAEERADIAESQVNKLRAKSRDIGAKKM--DEE
(7) Ratα      RKVQHELDEAEERADIAESQVNKLRAKSRDIGAKQKMHDEE
(8) Human2X   RRIQHELEEAEERADIAESQVNKLRVKSREVHTKIIS--EE
(9) Human2A   RKLQHELEEAEERADIAESQVNKLRVKSREVHTKVIS--EE
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Fig. 3. Amino acid comparison of the carboxy-terminal regions of myosin heavy chain isoforms. Amino acid sequences are given in the one-letter IUPAC code. Sequences and associated references are as follows: (1) carp FG2 (Ennion et al., 1995a); (2) human β cardiac (Jaenicke et al., 1990); (3) baboon β cardiac (Hixson and Britton, 1988); (4) rat β cardiac (Kraft et al., 1989); (5) rabbit β cardiac (Kavinsky et al., 1984); (6) human α cardiac (Matsuoka et al., 1991); (7) rat α cardiac (McNally et al., 1989); (8) human fast 2X (Saez and Leinwand, 1986); (9) human fast 2A (Ennion et al., 1995b). An asterisk indicates amino acid residues conserved in all the sequences.

fertilisation) and continued after the *Eggs22* and *Eggs24* transcripts had ceased to be expressed.

In situ *hybridisation*

Wholemount *in situ* hybridisation with digoxigenin-labelled 3′UTR probes allowed the spatial analysis of the two myosin heavy chain isoforms to be investigated. Staining was completely absent using the sense-labelled cRNA probes for both transcripts at all developmental stages (Fig. 5C and embryos not shown). Using the antisense-labelled probes, expression of both the *Eggs22* and *Eggs24 MyoHC* transcripts was detected in the developing myotomal muscle from approximately 25 h post-fertilisation. Expression of both isoforms commenced in the rostral region of the trunk and progressed caudally with development (Fig. 5A,B). Staining for the mRNA for both transcripts was stronger at the myoseptal regions, causing a distinct chevron pattern of staining through the trunk musculature (Fig. 5A,B,D). This stronger localisation of message to the myoseptal ends of the developing muscle fibres was also apparent in longitudinal wax-embedded sections (Fig. 5H, arrowheads). The *in situ* hybridisation protocol used produced complete penetration of the probe as demonstrated by transverse sections through the trunk showing staining throughout the whole of the muscle blocks (Fig. 5G). The hybridisation pattern observed from transverse sections was identical for both transcripts, with strong expression in the myotomal blocks of developing white muscle fibres but no expression in the peripheral layer of developing red muscle fibres (Fig. 5G).

Expression of both *MyoHC* transcripts could also be detected in the developing pectoral fin and protractor hyoideus muscles (Fig. 5E,F). Expression in the developing pectoral fin could be localised to two thin strips of muscle. Expression of the *Eggs22* transcript could also be detected in the muscles of the developing lower jaw, producing a distinctive triangular shape (Fig. 5F, arrowhead). The muscles of the lower jaw did not, however, express the *Eggs24* transcript (Fig. 5E). No expression of either transcript was detected in cardiac or smooth muscle.

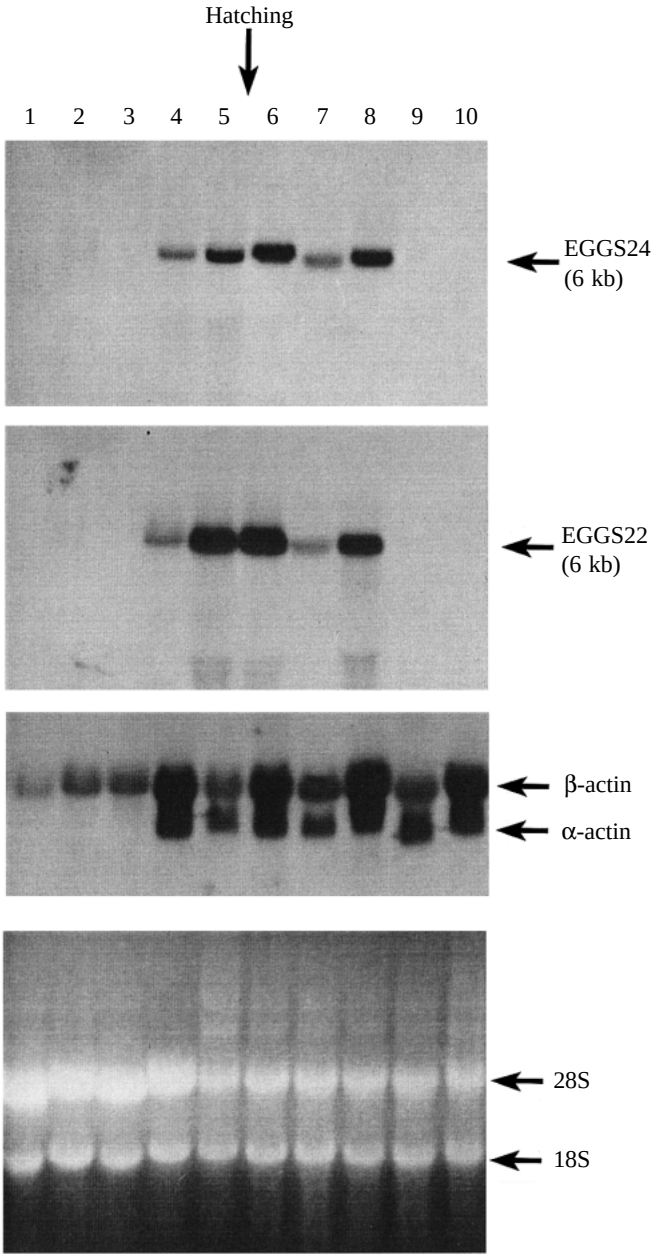
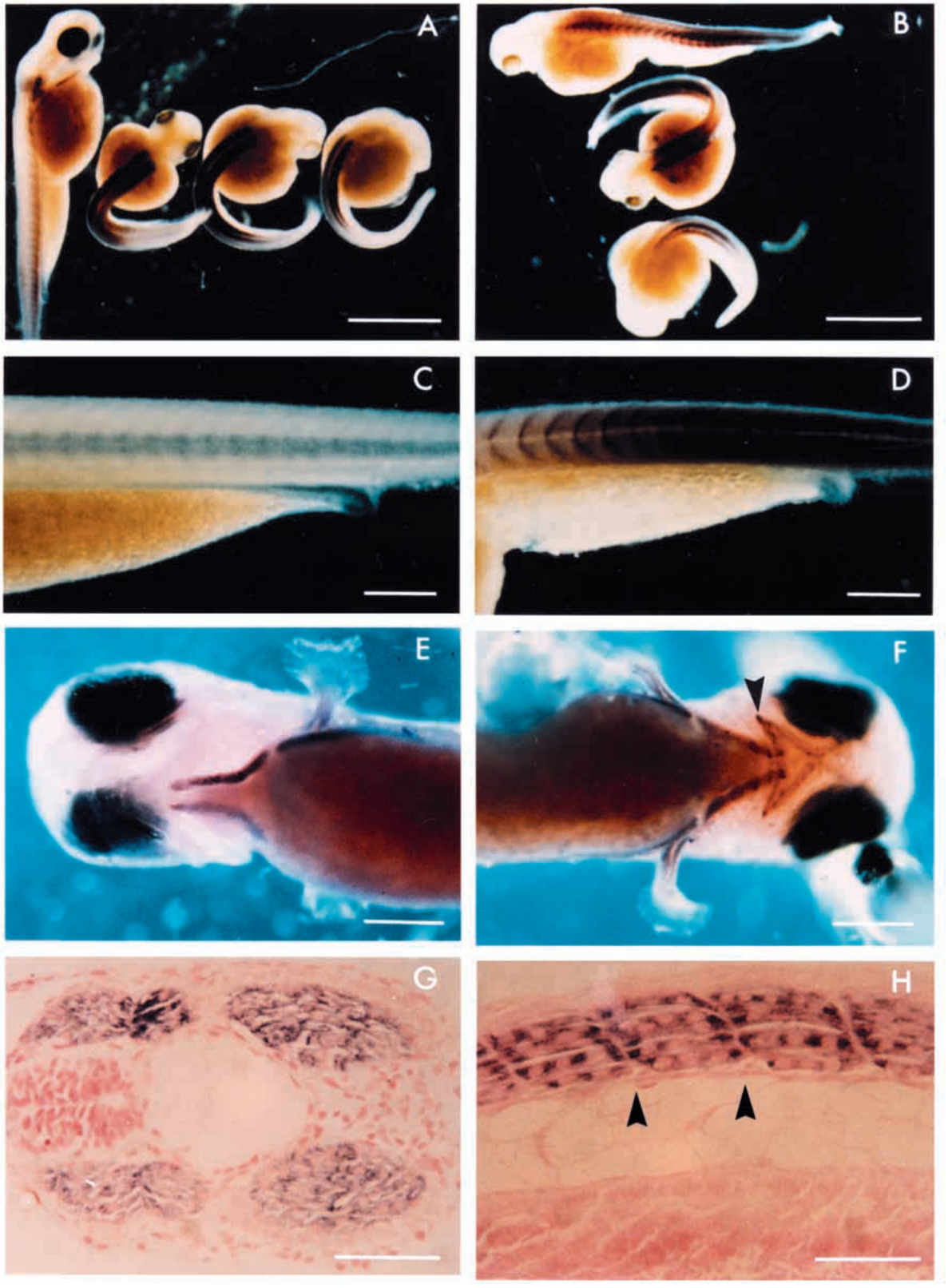


Fig. 4. Northern hybridisation. RNA extracted from carp embryos at different stages of development was probed sequentially with the probes EGGS24UTR (top panel), EGGS22UTR (second panel) and FGA101 (third panel). X-ray film exposure times are 24 h for each hybridisation. After each hybridisation, the membrane was stripped of probe and re-exposed to film for 48 h to ensure complete removal. The bottom panel shows the original RNA gel stained with ethidium bromide. Lanes are as follows: (1) 6 h, (2) 16 h, (3) 22 h, (4) 31 h, (5) 36 h (hatching), (6) 3 days, (7) 7 days, (8) 14 days, (9) 21 days, (10) 28 days. Times are in hours after fertilization and days after hatching. Incubation temperature for embryos and rearing temperature for larvae was 18 °C.

Discussion

Two distinct MyoHC cDNA clones, named EGGS22 and EGGS24, were isolated by random amplification of cDNA ends (RACE)-PCR in this study. The high degree of sequence divergence in the 3′UTRs between these two clones (42 % similarity) suggests that they correspond to two distinct *MyoHC* genes rather than to alleles of the same gene or to an evolutionarily recent duplication of the same gene. Duplicated



MyoHC genes have been described for *Xenopus laevis* (Radice and Malacinski, 1989) and trout (Gauvry and Fauconneau, 1996). The process of alternative exon splicing from the same gene cannot be ruled out for the *Eggs22* and *Eggs24* transcripts. However, this seems unlikely since no evidence for the alternative splicing of exons has been found in any of the numerous vertebrate myosin II heavy chain genes described to date. Mammalian species also express two distinct developmental MyoHC isoforms (the embryonic and neonatal isoforms), and two developmentally regulated MyoHC isoforms (E3 and E19) have been described for *Xenopus laevis* (Radice, 1995). It is tempting to draw analogies between isoforms across these species. However, the expression patterns of the two carp isoforms are more similar, both temporally and spatially, than those of the mammalian embryonic and neonatal isoforms and the two *Xenopus laevis* isoforms described to date. Furthermore, at approximately 28 isoforms (Gerlach et al., 1990), the family of MyoHC isoforms in carp is approximately twice the estimated sizes of the mammalian and *Xenopus laevis* MyoHC isoform families. Therefore, a direct analogy between MyoHC isoforms across species seems inappropriate.

The similarity between the expression pattern of the two carp *MyoHC* transcripts at both the northern and *in situ* hybridisation levels raises the question of whether the two probes used in this study cross-hybridise to one another. At the stringencies used in these experiments, the sequence similarity between the probes (51.7 %) is unlikely to result in any cross-hybridisation. Furthermore, the two probes did not cross-hybridise on Southern blots (data not shown), and the different hybridisation pattern observed in the muscles of the lower jaw (Fig. 5E,F) confirms that no cross-hybridisation occurred.

The appearance of transcripts for both the *Eggs22* and *Eggs24 MyoHC* transcripts coincided with the switch from the exclusive expression of β-actin to expression of both β- and α-actin (sarcomeric actin) and occurred just before the first movements of the embryo were observed. Expression of both *MyoHC* transcripts followed the normal progression of muscle development (Akster and Koumans, 1995), being initially expressed in the rostral region and progressing caudally. Such an expression pattern is also observed with the *Xenopus laevis* E3 *MyoHC* gene. The *Xenopus laevis* E19 *MyoHC* gene, however, is unusual in that it is expressed counter to this, starting caudally and progressing rostrally (Radice, 1995). Unlike the *Xenopus laevis* E3 and E19 *MyoHC* genes, no predominance in fibre type expression was observed for the carp *Eggs22* and *Eggs24* transcripts. Both carp transcripts were co-expressed at equivalent levels in developing white muscle fibres but not in the peripheral layer of developing red muscle fibres. Only the muscles of the lower jaw showed a differential expression pattern in that these muscles expressed the *Eggs22 MyoHC* transcript but not the *Eggs24* transcript. Why these muscles should differ in this respect is unknown.

A distinctive chevron pattern of expression was observed in the trunk musculature with both transcripts, and this is likely to be a consequence of the mechanism of longitudinal muscle fibre growth. In mammalian muscle, it has been shown that muscle fibres increase in length by the addition of new sarcomeres onto the ends of existing myofibrils (Griffin et al., 1971; Williams and Goldspink, 1971), and it is likely that the same mechanism of growth is adopted by other species, including fish. The appearance of the distinctive chevron pattern of staining can be explained by an accumulation of message at the myoseptal ends of the fibres, which are the sites of new protein synthesis for incorporation into the sarcomeres. Indeed, longitudinal wax-embedded sections (Fig. 5H) directly show this accumulation of message adjacent to the myosepta. This also suggests that transport of *MyoHC* mRNA to the site of new sarcomere production is occurring. Localisation of mRNA has previously been described for vimentin, actin and tubulin, which associate closely with the cytoplasmic actin filaments (Singer et al., 1989), and for myosin heavy chain, which accumulates at the myotendinous junction in stretch-hypertrophied rabbit skeletal muscle (Dix and Eisenberg, 1990). The 3′UTR has been implicated in the transport of mRNA to different cellular locations (Wiseman et al., 1997a,b) and, with this possibility in mind, the 3′UTR sequences of the two carp *MyoHC* transcripts isolated in the present study were aligned with corresponding sequences from all the *MyoHC* genes currently in the EMBL database. From this analysis, a conserved sequence motif of 10 base pairs (AAAATGTGAA) was found to be present in the 3′UTRs of *MyoHC* isoforms from a diverse range of species, including the carp *Eggs24* transcript (Table 1). Because this motif has been conserved over such a diverse range of species, whilst the flanking sequence as a whole shows no conservation across species, a biological function for this region seems very likely. It is possible that this sequence

Fig. 5. *In situ* hybridisation. Carp embryos were hybridised with digoxigenin-labelled cRNA probes corresponding to the 3′ untranslated regions of the *Eggs22* and *Eggs24 MyoHC* transcripts. (A) Carp embryos (left to right), 12 h post-hatching, 30 h post-fertilisation, 30 h post-fertilisation, 25 h post-fertilisation, hybridised with the *Eggs24* antisense cRNA probe. (B) Carp embryos (bottom to top), 12 h post-hatching, 30 h post-fertilisation, 25 h post-fertilisation, hybridised with the *Eggs22* antisense cRNA probe. (C,D) View of trunk musculature from larvae 12 h post-hatching hybridised with *Eggs22* sense cRNA (C) and antisense cRNA (D). (E,F) Underside view of the head of carp larvae (12 h post-hatching) hybridised with *Eggs24* antisense cRNA (E) and *Eggs22* antisense cRNA (F). The arrowhead highlights expression of *Eggs22* in the developing lower jaw. (G) Eosin-stained transverse section (10 μm) through the trunk of a 25 h post-fertilisation embryo which had previously been hybridised (wholemount) with antisense cRNA for the *Eggs24* gene (dorsal is to the left of the micrograph). Eosin staining is pink, hybridised probe is purple. (H) Longitudinal eosin-stained section through the trunk of a 12 h post-hatching larva which had previously been hybridised with antisense *Eggs22* cRNA. Note the muscle fibres running from myosept to myosept and the stronger hybridisation (purple) at the myoseptal ends of the fibres (arrowheads). Scale bars: A,B, 1 mm; C–F, 250 μm; G,H, 50 μm.

Table 1. *Conserved sequence motif in the 3′ untranslated regions of myosin heavy chain isoforms from a diverse range of species*

Isoform	Position	Sequence
Carp *Eggs24*	45–54	tttgtatctg **AAAATGTGAA** attgttcaat
Chicken fast	40–49	aggcatgcat **AAAATGTGAA** cctctgtgtt
Chicken embryonic 1	40–49	agaattgcac **AAAATGTGAA** attctatcac
Chicken embryonic 2	40–49	agaattgcac **AAAATGTGAA** attctatcac
Human IIX	41–50	agaaatgcac **AAAATGTGAA** aatctttgtc
Pig IIa	42–51	agagaggcac **AAAATGTGAA** gtctttgcgt
Rabbit IIb	40–49	agaaatgcac **AAAATGTGAA** gttcaaagtc
Rabbit IIX	40–49	agaaatgcac **AAAATGTGAA** actctttgtc
Rat IIa	42–51	agaaaggcac **AAAATGTGAA** gcctttggtc
Rat IIX	40–49	agagatgagc **AAAATGTGAA** gatctttgtc
Xenopus E19	38–47	tgaaatttgc **AAAATGTGAA** tttctcttcc
Xenopus E15	38–47	tgaaatttgc **AAAATGTGAA** tttcttccct

The sequence motif is given in bold uppercase.

Position indicates the number of nucleotides after the stop codon at which the sequence motif appears.

The source of the sequence data (EMBL accession numbers) is as follows: carp *Eggs24*, AJ009734; chicken fast, M16557; chicken embryonic 1, J00892; chicken embryonic 2, M12086; human IIX, X03740; pig IIa, S. Ennion unpublished results; rabbit IIb, Y1320; rabbit IIX, Y13202; rat IIa, X72589; rat IIX, X72591; *Xenopus* E19, M27235; *Xenopus* E15, M27237.

motif could be a localization signal involved in the transport of *MyoHC* mRNA. However, there are equally as many *MyoHC* 3′UTR sequences that do not contain this motif, including the *Eggs22* transcript characterised in the present study, and further studies are required to investigate this.

Expression of the *Eggs22* and *Eggs24 MyoHC* transcripts is undetectable 2 weeks post-hatching when these transcripts are replaced by mRNAs for adult/juvenile isoform types. Both transcripts in this study are expressed in the very early stages of muscle formation, and one possible explanation for the need for separate developmental and adult-type isoforms is that these embryonic isoform types are necessary for the initial myofibril structure to be laid down. This structure can subsequently be replaced by the adult isoforms by the law of mass action (Goldfine et al., 1991). Also, the need for separate developmental isoforms could be at the cross-bridge function level, with the developmental isoforms conferring more optimal actin binding or ATPase properties. Indeed the tailbeat frequency required for efficient locomotion changes dramatically throughout development in fish, and it is likely that different MyoHC isoforms would be required.

The muscle myosin/actin system is a very old molecular motor mechanism (for a review, see Warrick and Spudich, 1987) which is thought to have evolved first in primitive metazoan animals as a means of pumping haemolymph in circulatory systems. Even prior to this, the myosin/actin motors no doubt evolved in single-celled organisms in non-muscle cytoskeletal systems involved in ameboid movements (Hammer et al., 1987). The *MyoHC* gene family in present-day vertebrates includes individual genes that encode different isoforms which are developmentally regulated and which have different contractile characteristics. Skeletal muscle differs from cardiac muscle in that there are distinct embryonic and neonatal *MyoHC* genes expressed in this tissue that are not expressed in the heart. The heart develops very early and shows a gradual increase in size. However, skeletal muscle shows a rapid increase in mass just before or just after birth/hatching. Although it was known that embryonic *MyoHC* genes are expressed in developing mammalian muscle, it is interesting, from the point of view of developmental mechanisms, that the same initial steps in forming the myofibril infrastructure in lower vertebrates, such as fish, also involve the expression of a specific embryonic *MyoHC* gene or genes. In the carp, the *Eggs22* and *Eggs24* transcripts show more sequence homology with other carp *MyoHC* genes than with mammalian embryonic genes and it seems, therefore, that the system of sequential gene expression may have evolved more than once, indicating that the embryonic MyoHCs play an important role in early skeletal muscle development.

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