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Molecular cloning and mRNA expression analysis of carp embryonic, slow and cardiac myosin heavy chain isoforms

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Summary

Three embryonic class II myosin heavy chains (MYHs) were cloned from the common carp (*Cyprinus carpio* L.), MYH_{emb1}, MYH_{emb2} and MYH_{emb3}. MYH DNA clones were also isolated from the slow muscle of adult carp acclimated to 10°C (MYH_{S10}) and 30°C (MYH_{S30}). Phylogenetic analysis demonstrated that MYH_{emb1} and MYH_{emb2} belonged to the fast skeletal muscle MYH clade. By contrast, the sequence of MYH_{emb3} was similar to the adult slow muscle isoforms, MYH_{S10} and MYH_{S30}. MYH_{emb1} and MYH_{emb2} transcripts were first detected by northern blot analysis in embryos 61 h post-fertilization (h.p.f.) at the heartbeat stage, with peak expression occurring in 1-month-old juveniles. MYH_{emb1} continued to be expressed at low levels in 7-month-old juveniles when MYH_{emb2} was not detectable. MYH_{emb3} transcripts appeared at almost the same stage as MYH_{emb1} transcripts did (61 h.p.f.), and these genes showed a similar pattern of expression. Whole mount *in situ* hybridization analysis revealed that the transcripts of MYH_{emb1} and MYH_{emb2} were expressed in the inner part of myotome, whereas MYH_{emb3} was expressed in the superficial compartment. MYH_{S10} and MYH_{S30} mRNAs were first detected at hatching. In adult stages, the expression of slow muscle MYH mRNAs was dependent on acclimation temperature. MYH_{S10} mRNA was expressed at an acclimation temperature of 10 and 20°C, but not at 30°C. In contrast, MYH_{S30} mRNA was strongly expressed at all acclimation temperatures. The predominant MYH transcripts found in adult slow muscle and in embryos at hatching were expressed in adult fast muscle at some acclimation temperatures but not others. A MYH DNA clone was isolated from the cardiac muscle of 10°C-acclimated adult fish (MYH_{card}). MYH_{card} mRNA was first detected at 61 h.p.f., but strong signals were only observed in the adult myocardium. The present study has therefore revealed a complex pattern of expression of *MYH* genes in relation to developmental stage, muscle type and acclimation temperature. None of the skeletal muscle MYHs identified so far was strongly expressed during the late juvenile stage, indicating further developmentally regulated members of the MYH II gene family remain to be discovered.

Key words: carp, *Cyprinus carpio*, myosin heavy chain, fast skeletal muscle, slow skeletal muscle, myocardium.

Introduction

Skeletal muscle is composed of different muscle fiber types. Differential expression of muscle structural proteins, including the class II myosin heavy chain (MYH) is a major contributing factor to the diversity of skeletal muscle fibers (for a review, see Schiaffino and Reggiani, 1996). The fiber type composition of individual muscles has been shown to vary dramatically during ontogeny. In the mouse a transition of MYH isoforms has been reported during embryogenesis (Lyons et al., 1990; Whalen et al., 1981) and in response to unloading and exercise (Linderman et al., 1996; Sugiura et al., 1993; Talmadge et al., 1996). β-Cardiac and embryonic skeletal MYHs were first detected between 9 and 10 days post fertilization (d.p.f.) in the myotomes of the most rostral somites. Perinatal MYH transcripts started to accumulate at 10.5 d.p.f. By 12.5 d.p.f., both embryonic and perinatal MYH mRNAs were found to an equal extent, whereas by 15.5 d.p.f., perinatal MYH mRNA was predominantly expressed and mRNA levels of embryonic

MYH had decreased. The mRNAs of adult fast and slow MYHs become segregated according to fiber types at later stages. β-Cardiac MYH transcripts are always present as a minor component in trunk muscle of embryonic stages (Lyons et al., 1990). At least three forms of slow twitch MYH accumulate sequentially during mouse fetal development. Slow MYH maturation occurs in slow fibers before expression of the adult fast MYHs in fast fibers (Maggs et al., 2000). Allen and Leinwand (2001) analyzed the expression of seven sarcomeric MYHs in the hindlimb muscles of wild-type mice at several time points from 1 day of postnatal life (d.p.n.) to 20 d.p.n. In early postnatal life, the developmental isoforms (embryonic and perinatal) constitute >90% of the total MYH expression, whereas three adult fast isoforms (IIa, IIb, and IId) constitute <1% of the total MYH protein. However, between 5 and 20 d.p.n. their expression increases to be >90% of the total MYH. In developing cardiac muscle, there is a small but significant decrease in the level of MYH alpha in the developing human ventricle between 7 and 12 weeks of gestation (Reiser et al., 2001).

Three distinct-type skeletal muscle fibers, slow, fast and intermediate are formed during ontogeny in the zebrafish *Danio rerio* (van Raamsdonk et al., 1978; Devoto et al., 1996; Weinberg et al., 1996). The embryonic slow and fast muscle fibers express tissue-specific MYH isoforms (Devoto et al., 1996). Blagden et al. (1997) suggested that Sonic hedgehog (Shh) acts to induce myoblasts committed to slow muscle differentiation from uncommitted presomitic mesoderm. Mutant fish lacking Shh expression failed to form slow muscle but did form fast muscle. Ectopic expression of Shh, either in wild-type or mutant embryos, lead to ectopic slow muscle at the expense of fast muscle. In rainbow trout *Oncorhynchus mykiss*, there is a developmental reduction in the number of MYH isoforms present in red, white and ventricular muscle between smaller parr and older juveniles (smolts; Weaver et al., 2001).

Many teleosts can modify their swimming performance following several weeks acclimation to a new temperature regime (Fry and Hart, 1948). The mechanisms underlying the plasticity of locomotory performance are complex and vary between species (Johnston and Temple, 2002). In the goldfish *Carassius auratus* (Johnston et al., 1975) and common carp *Cyprinus carpio* L. (Heap et al., 1985) changes in muscle myofibrillar ATPase activity constitute an important element of the acclimation response. The common carp has become a model species for the study of temperature acclimation in fish and the subject of a large number of investigations (Watabe, 2002). Studies with fully activated skinned fibers found that maximum tension and shortening speed increased at low temperatures in both fast and slow muscles following a period of cold acclimation (Johnston et al., 1985). We have shown that changes in the expression of MYH isoforms play a key role in the plasticity of myofibrillar ATPase activity and contractile properties with temperature acclimation (Hwang et al., 1990; Watabe et al., 1992, 1995; Guo et al., 1994). Three distinct MYH DNAs were cloned from the fast myotomal muscle of carp acclimated to either 10°C or 30°C for a minimum of 6 weeks (Imai et al., 1997; Hirayama and Watabe, 1997). The nomenclature used for these clones was based on the acclimation temperature at which the corresponding mRNAs were most strongly expressed. Thus, the 10°C-type MYH (MYH_{F10}) and the 30°C-type MYH (MYH_{F30}) cDNAs were the predominant transcripts in 10°C- and 30°C-acclimated fish, respectively. The relative proportions of each isoform varied with acclimation temperature (Imai et al., 1997). A third cDNA, which was named the intermediate-type because of its intermediate DNA nucleotide and deduced amino acid sequences between those of the 10°C- and 30°C-type MYH isoforms, was also isolated from fast muscle, which was expressed over a relatively broad temperature range (MYH_{Fint}). Studies with chimeric myosins constructed with *Dictyostelium discoideum* myosin heavy chain backbone indicate that amino acid substitutions in the surface loops 1 and 2, involved in ATP-binding and actin-binding, respectively, are responsible for the different temperature-dependent properties of myosin ATPase (Hirayama et al., 2000; Watabe, 2002).

Adult common carp can overwinter in frozen ponds and survive 35°C in the summer, however, embryonic and early larval stages are not viable below 15°C (Rothbard and Yarron, 1995). In fast muscle, the ability to change myofibrillar ATPase activity is acquired gradually during ontogeny, concomitant with the development of cold tolerance (Cole and Johnston, 2001). Peptide mapping studies have shown that the MYH composition characteristic of 10°C-acclimated carp appears in juveniles after several months at around 37 mm total length (Wakeling et al., 2000). Studies of expressed MYH have shown a complex developmental-stage-specific expression of isoforms in different tissue types that continues throughout much of the life cycle (Huriaux et al., 1991; Martinez et al., 1991; Mascarello et al., 1995; Johnston et al., 1998). In common carp, two developmentally regulated MYH isoform genes (Eggs22 and Eggs24) have been described (Ennion et al., 1999). These genes were expressed from 22 h.p.f. until 2 weeks after the larvae hatched. It is therefore likely that further developmental stage-specific MYH genes remain to be discovered. Furthermore, nothing is known about changes in slow muscle MYH gene expression with temperature acclimation.

The aim of the present study was to comprehensively clone *MYH*s from embryonic muscle, adult slow muscle and adult cardiac muscle in the common carp and to characterize their expression patterns in relation to developmental stage.

Materials and methods

Fish

Common carp *Cyprinus carpio* L. were reared at the Yoshida Research and Training Station, Tokyo University of Fisheries, Japan. Embryos and larvae were cultured in freshwater aquaria at approximately 20°C and experimental samples were collected during ontogeny as described in our previous paper (Kobiyama et al., 1998). Somite formation was

observed with a light microscope to assess the developmental stages of embryos. The whole embryos (3-somite, 15-somite, first heart-beat and eyed-stage) and the whole larvae at hatching were frozen in liquid N_2 and stored at –80°C until use. Larvae aged 2 days were transported to the laboratory of the University of Tokyo and further cultured by feeding commercial pellets twice daily for 7 months under a 14 h:10 h L:D photoperiod at approximately 20°C. The whole trunk muscle was isolated from juveniles aged 1 and 7 months, frozen in liquid N_2, and stored at –80°C until use. Fast and slow skeletal muscles and myocardium were taken from adult carp aged 2 years acclimated to 10°C, 20°C and 30°C for a minimum of 6 weeks.

Reverse-transcription PCR

Total RNAs were prepared by the method of Chomczynski and Sacchi (1987) and first strand cDNAs were synthesized using the 3′RACE system (rapid amplification of cDNA ends; Invitrogen, Carlsbad, CA, USA). RNA was extracted from embryos at hatching and from the slow and cardiac muscle of adult fish acclimated to either 10°C or 30°C for a minimum of 6 weeks. 3′RACE was performed using gene-specific primers for embryonic MYH (5′-ACCTGCAGCACCGTCTGGAT-3′) or for cardiac/slow MYH (5′-CCATGATGGCTGAGGAGC-TG-3′) with abridged universal amplification primer (AUAP; 5′-GGCCACGCGTCGACTAGTAC-3′). PCR amplifications were carried out for 3 min at 94°C, followed by 30 cycles of denaturation for 30 s at 94°C, 1 min of annealing at 58°C and 1 min of extension at 72°C with the final extension step for 5 min, using a model 2400 DNA thermal cycler (Applied Biosystems, Foster City, CA, USA). Forward and reverse primers (25 pmol) and 20 ng of the first strand cDNA template were added to 8 μl of a solution containing 2 mmol l^{-1} dNTP mixture and 10 μl 10× PCR buffer in 100 mmol l^{-1} Tris-HCl (pH 8.3), 500 mmol l^{-1} KCl and 15 mmol l^{-1} $MgCl_2$. The final volume was adjusted to 99 μl with sterilized water, and a 1 μl aliquot containing 1 U *Taq* DNA polymerase was added to start the reaction. Amplified DNA fragments were ligated into pBluescript SKII$^-$ (Stratagene, La Jolla, CA, USA) for sequence analysis. DNA sequencing was performed using a Dye-terminator Cycle Sequencing Ready Reaction Kit with model 373A and 310 DNA sequencers (Applied Biosystems).

Phylogenetic tree construction

The deduced amino acid sequences obtained in this study contained part of a myosin rod region corresponding to amino acid residues 1792–1924 from the N terminus of MYH_{F10} (BAA22067; Hirayama and Watabe, 1997). These sequences together with those for the corresponding regions in the fast and cardiac/slow type MYHs reported from human *Homo sapiens*, zebrafish *Danio rerio* and torafugu pufferfish *Takifugu rubripes* were used for phylogenetic analysis following paired alignment of the sequences with CLUSTAL W (Thompson et al., 1994). The neighbor-joining (NJ) and maximum parsimony (MP) methods were employed to obtain phylogenetic trees using the software Mega3 (Kumar et al., 2004). Bootstrap resampling analysis from 1000 replicates was used to evaluate internal branches.

Northern blot analysis

Samples for northern blot analysis were: the whole carp embryos, the whole larvae at hatching, the whole trunk muscle from juveniles aged 1 and 7 months (Kobiyama et al., 1998), and pure samples of slow and cardiac muscle isolated from adult carp acclimated to 10°C, 20°C and 30°C. Total RNAs were fractionated in 1% (w/v) agarose gels containing 18% (v/v) formamide and transferred onto the nylon membrane Hybond N$^+$ (Amersham Bioscience, Buckinghamshire, UK). The membranes were prehybridized at 65°C for 1 h in

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emb1       LQHRLDEAENLAMKGGKKQLQKLESRVRELEAEIEAEQRRGTDAVKGVRK   50
emb2       ......................................V.......A.....   50
emb3       .........QI........V....A..H...N.V.L...KASES......   50
slow10     .........QI........V....A......S.V.I...KSS.S...I..   50
slow30     .........QI........V....A......N.V.L...KASES...I..   50
cardiac    .........QI........V....A......C.V....KKSSESI..I..   50
10°C fast  .........S..................H.....V.......A..... 1842
I fast     .........S..................H.....V.......A..... 1841
30°C fast  .........S.....................V.......A..... 1844

emb1       YERRVKELSYQTEEDKKNINRLQDLVDKLQLKVKAYKRQAEEAEEQANSH  100
emb2       ........T.........L.....................S........T.  100
emb3       ....I...T......R..LA..............S...A.........AN  100
slow10     ....I...T......R..LA..............S...V..........S  100
slow30     ....I...T......R..LA..............S...A..........N  100
cardiac    ....I...T......R...A..................A.........I.  100
10°C fast  ........T.........VT.......N.....................T. 1892
I fast     ........T.........SVT.............................T. 1891
30°C fast  ........T.........VI..............V............T.T. 1894

emb1       LSKLRKVQHELEEAEERADIAESQVNKLRAKSRDAGKAKEE           141
emb2       ....................................G..AAE           143
emb3       .GRF..I....D..............M......S.SK.GHDAE         144
slow10     .G.F..I....D..................V....T.SK.GADEE        144
slow30     .T.F..I....D.....................T.SK.GHDEE         144
cardiac    .G.F..L....D.....................V.PK.GFDEE         144
10°C fast  ..RY.........SH..................E...T.VAE         1934
I fast     ..RY.........SH..................E...T.D.          1933
30°C fast  ..RY..........Q....V..................S.DAE         1936
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Fig. 1. Comparison of the partial deduced amino acid sequences of carp embryonic (emb), slow skeletal and cardiac types of myosin heavy chain (MYH) with those of corresponding regions of carp fast skeletal MYH isoforms. The data on adult fast muscle MYH sequences were taken from Imai et al. (1997). Slow 10, slow 30 and 10°C fast, 30°C fast refer to the isoforms predominantly expressed at 10°C and 30°C, respectively. I fast is an intermediate type, which is expressed over a broad range of temperature. Amino acid residues identical to those of carp MYH_{emb1} are indicated by dots. Numbers in the right margin represent amino acid residues from the N terminus. Primer binding regions of *MYH$_{emb1}$*, *MYH$_{emb2}$* and *MYH$_{emb3}$* are boxed.

Table 1. *Percentage comparison of amino acid sequences of myosin heavy chains from carp*

	emb2	10°C fast	I fast	30°C fast	emb3	slow10	slow30	cardiac
emb1	95	88	88	89	78	79	78	79
emb2		90	90	90	78	78	79	78
10°C fast			97	92	75	74	75	75
I fast				92	75	75	76	75
30°C fast					76	75	77	75
emb3						90	96	88
slow10							91	87
slow30								88

a solution containing 0.5 mol l^{-1} Church buffer (Church and Gilbert, 1984), 1 mmol l^{-1} EDTA and 7% SDS, and then hybridized at 65°C for 20 h in the same solution used for prehybridization but containing ^{32}P-labelled probe. The membranes were washed sequentially with 2× SSC (1× SSC is 0.15 mol l^{-1} sodium chloride, 0.015 mol l^{-1} sodium citrate) plus 0.1% SDS at room temperature for 20 min, 1× SSC plus 0.1% SDS at 65°C for 30 min and 0.1× SSC plus 0.1% SDS at 65°C for 10 min, and then exposed to X-ray films.

The DNA fragments used as probes encoding MYH_{emb1}, MYH_{emb2}, MYH_{emb3}, MYH_{S10}, MYH_{S30} and MYH_{card} isoforms (refer to Results) were amplified by PCR and labeled with [α-^{32}P]dCTP using a Random Primer DNA labeling kit Ver.2 (Takara, Otsu, Japan). The probes correspond to nucleotides (nt) 397–494 for MYH_{emb1}, nt 414–505 for MYH_{emb2}, nt 411–506 for MYH_{emb3}, nt 417–539 for MYH_{S10}, nt 417–539 for MYH_{S30} and nt 184–581 for MYH_{card} cDNAs, and these have been registered in the DDBJ/EMBL/GenBank databases with the accession numbers of AB104622, AB104623, AB104624, AB104625, AB104626 and AB104627, respectively. Alternatively, a probe was amplified from the DNA sequence encoding residues 534–717 from the N terminus for MYH_{F10} of carp fast skeletal muscle, which is thought to react with all known skeletal muscle-type MYH mRNAs of carp (consensus) (Hirayama and Watabe, 1997). The oligonucleotide probes specific to MYH_{F10} and MYH_{F30} mRNA were 5′-TCCTTTCTTTCCAGCGTCCTC-TGCT-3′ and 5′-GCCCTCAGCTTCAGCTCCATGAGTGG-3′, respectively, which were labeled with [γ-^{32}P]dATP using a Megalabel DNA end labeling kit (Takara) (Hirayama and Watabe, 1997).

The specificity of probes used for northern blot analysis was examined by dot blot analysis. Plasmid DNAs encoding partial sequences of eight MYH isoforms were spotted onto a nylon membrane and allowed to react with the same probes as those used in northern blot analysis (Hirayama and Watabe, 1997), which were derived from MYH_{emb1}, MYH_{emb2}, MYH_{emb3}, MYH_{S10}, MYH_{S30} and MYH_{card} cDNAs.

Whole-mount in situ hybridization

Whole-mount *in situ* hybridization (WISH) was performed according to the method of Westerfield (1993). RNA probes derived from nt 357–527 for MYH_{emb1}, nt 357–512 for MYH_{emb2} and nt 1–512 for MYH_{emb3} were synthesized from plasmids incorporating *MYH_{emb1}*, *MYH_{emb2}* and *MYH_{emb3}*. Sample tissues were reacted with the probes and cut at a thickness of 18 μm using a cryostat.

Results

cDNA cloning of embryonic MYH isoforms

We employed 3′ RACE for identification of embryonic-type MYH cDNAs including the untranslated region. PCR amplification of RNA from larvae at hatching using universal primer for fast skeletal MYHs with AUAP yielded a single band with high staining intensities in agarose gel electrophoresis at approximately 600 bp (data not shown). The DNA fragments in this band were subcloned into pBluescript SK II$^-$, and the resulting clones were sequenced. As a result, three different clones encoding carp embryonic MYHs were

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carp cardiac LQHRLDEAEQIAMKGGKKQVQKLEARVRELECEVEAVQKKSSESIKGIRK   50
zebrafish    .....................................E..R....V.....  114
rat-α        ............L......L...........N.L..E..RNA..V..M.. 1844
rat-β        ............L......L...........N.L..E..RNA..V..M.. 1843
chicken      ..K.........L......I....S......N.L.NELRRN.DAQ..A.. 1009

carp cardiac YERRIKELTYQTEEDRKNIARLQDLVDKLQLKVKAYKRAAEEAEEQANIH  100
zebrafish    ..........................................S.....V.  164
rat-α        S..............K..LV.................Q.........TN 1894
rat-β        S..............LL..................Q.........TN 1893
chicken      F..........S...K..L..M...I........S..HQ.....A...LY 1059

carp cardiac LGKFRKLQHELDEAEERADIAESQVNKLRAKSRDVGPKKGFDEE       144
zebrafish    ....................................A....         208
rat-α        .S....V.........................I.A.QKMHD.E       1939
rat-β        .S....V.........................I.A.GLNE.         1936
chicken      .S.Y..Q..D..D.....E......D...S....I.M..VDA..       1103
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Fig. 2. Comparison of the partial deduced amino acid sequence of carp cardiac myosin heavy chain (MYH) with the corresponding regions of zebrafish ventricular, rat α and β cardiac, and chicken atrial MYHs. Comparative data are from the zebrafish (Yelon et al., 1999), rat α (McNally et al., 1989), rat β (Kraft et al., 1989) and chicken (Yutzey et al., 1994). Amino acid residues identical to those of carp are indicated by dots. Numbers in the right margin represent amino acid residues from the N terminus.

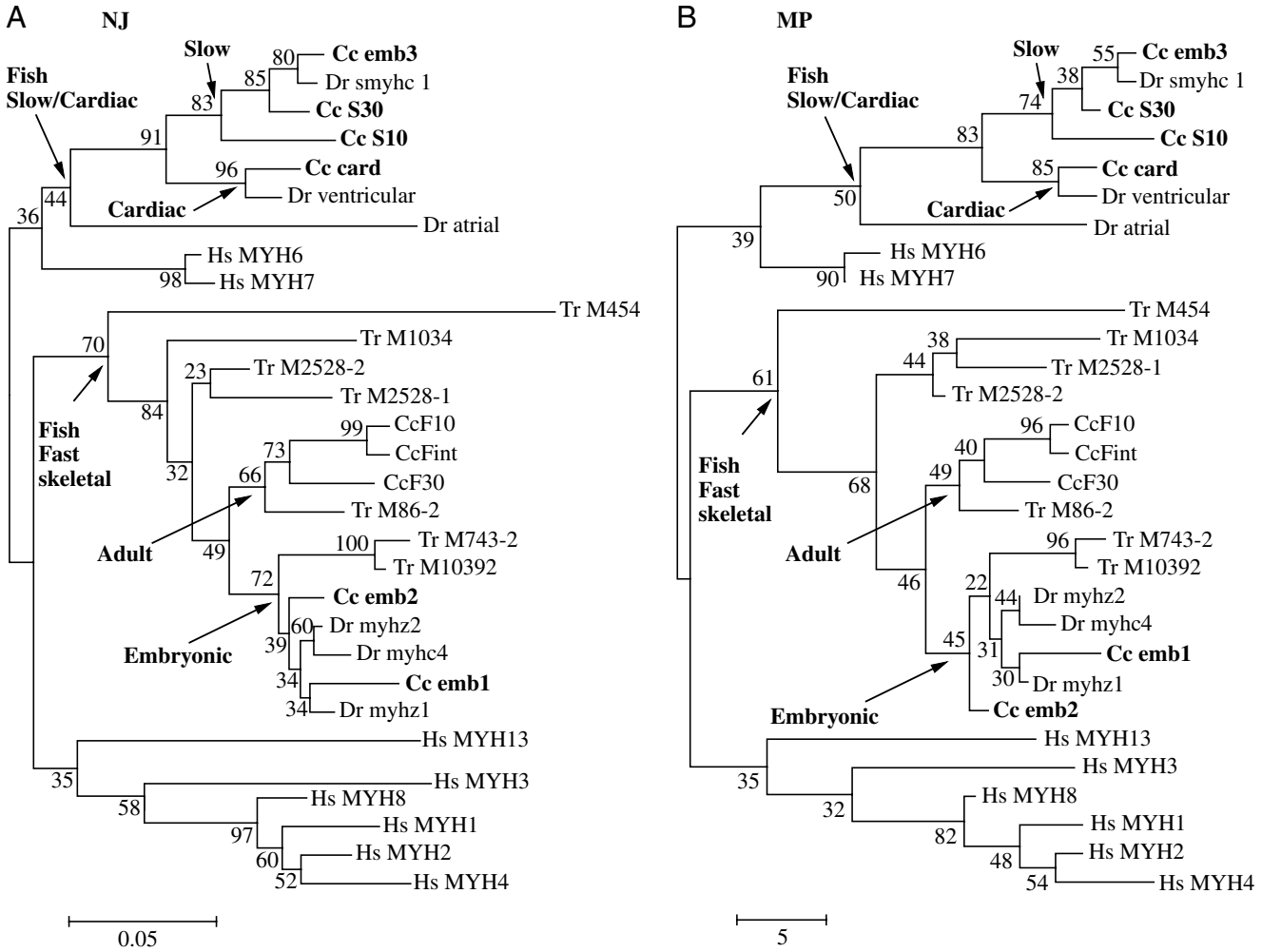

Fig. 3. The neighbor-joining (A, NJ) and maximum parsimony (B, MP) tree based on deduced amino acid sequences in partial rod domains of MYHs. Carp MYHs identified in this study (in bold) were compared with *H. sapiens*: Hs MYH3 (embryonic), NM_002470; Hs MYH2 (adult fast IIa), AF111784; Hs MYH1 (adult fast IId/x), AF11785; Hs MYH4 (adult fast IIb), AF11783; Hs MYH8 (perinatal), NM_002474; Hs MYH13 (extraocular), AF11782; Hs MYH6 (alpha-cardiac), NM_002471; Hs MYH7 (beta-cardiac), NM_000257; *C. carpio*: Cc F10 (adult 10°C-acclimated fast), D89990; Cc Fint (adult 20°C-acclimated fast), D89991; Cc F30 (adult 30°C-acclimated fast), D89992; *D. rerio*: Dr myhz1 (embryonic fast), AF180893; Dr myhz2 (embryonic fast), NM_152982; Dr myhc4 (embryonic fast), AY921650; Dr artial (atrial), AY138982; Dr ventricular (ventricular), AF114427; Dr smyhc1 (embryonic slow), AY921649. MYH sequences of *T. rubripes* (Tr) were constructed by using Fugu genomic sequence assembly data version 3.0 (Ikeda et al., 2004).

isolated and named MYH_{emb1} (AB104622), MYH_{emb2} (AB104623) and MYH_{emb3} (AB104624). The corresponding mRNAs of these three genes were predominantly expressed at early developmental stages of carp as revealed by northern blot analysis (see below) and, therefore, we refer to them as embryonic. Two independent clones were sequenced for each gene.

The nucleotide sequences of MYH_{emb1} and MYH_{emb2} cDNAs showed 95% and 98% identity to those of Eggs22 and Eggs24 from carp embryos previously characterized by Ennion et al. (1999). However, MYH_{emb3} cDNA showed a low sequence identity compared to MYH isoforms reported previously. All embryonic cDNA clones encoded a part of the carboxy-terminal region of L-meromyosin (Fig. 1) and contained polyadenylation signals (AATAAA) and poly(A) tails (data not shown). The deduced amino acid sequences of MYH_{emb1} and MYH_{emb2} showed about 90% identity with those of the MYH_{F10}, MYH_{Fint} and MYH_{F30} isoforms from adult carp fast skeletal muscle (Imai et al., 1997) (Table 1). By contrast, the amino acid sequence of MYH_{emb3} had only 75% identity with those of the three carp fast skeletal MYH isoforms (Table 1).

cDNA cloning of slow MYH isoforms

Starting with RNA extracted from adult slow muscle, PCR using universal primer for cardiac/slow MYHs with AUAP, amplified a single band of about 700 bp from first strand cDNA. The PCR products were cloned into a plasmid vector, randomly selected and sequenced. Sequencing demonstrated the presence of two types of slow muscle MYH, MYH_{S10}

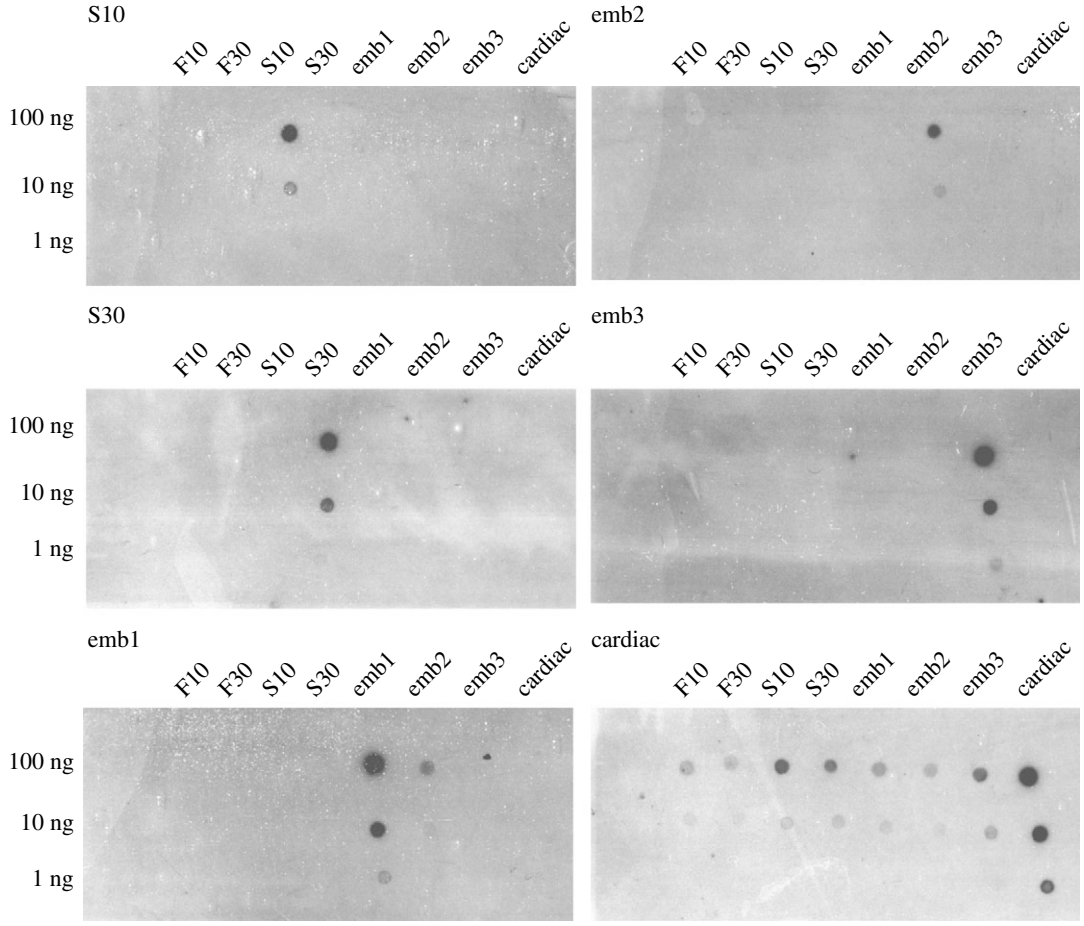

Fig. 4. Specificity of probes for northern blot hybridization from eight types of cDNA clones encoding MYH isoforms of carp. Nylon membranes incorporating MYH_{F10}, MYH_{F30}, MYH_{S10}, MYH_{S30}, MYH_{emb1}, MYH_{emb2}, MYH_{emb3} and MYH_{card} cDNA clones were hybridized with probes specific to *MYH*$_{S10}$, *MYH*$_{S30}$, *MYH*$_{emb1}$, *MYH*$_{emb2}$, *MYH*$_{emb3}$ and *MYH*$_{card}$ used for northern blot analysis. S10, S30, slow cDNA, emb1–3 embryonic 1–3 cDNA, cardiac, cardiac cDNA (all at 1, 10 or 100 ng).

(AB104625) and MYH_{S30} (AB104626), which were predominantly expressed in carp acclimated to 10°C and 30°C, respectively. We sequenced three independent clones for each gene. The slow muscle from adult carp acclimated to 20°C contained both MYH isoforms. MYH_{S10} and MYH_{S30} cDNAs had polyadenylation signals and poly(A) tails, and their deduced amino acid sequences encoded part of L-meromyosin (Fig. 1), showing 74–96% identity with the MYH isoforms found in the present study and those reported previously (Imai et al., 1997) (Table 1). It was noted that the MYH_{emb3} isoform was more similar to MYH_{S10} and MYH_{S30} (90–96% identity) than to other carp embryonic and adult fast skeletal MYH isoforms (74–79% identity).

cDNA cloning of cardiac MYH

We synthesized first strand cDNA from total RNAs of the myocardium from carp acclimated to 10°C and 30°C. PCR using universal primer for cardiac/slow MYHs with AUAP gave an intense single band of about 700 bp for carp acclimated to both 10°C and 30°C. Sequencing of PCR products revealed that the clone isolated was that of the cardiac type, MYH_{card} (AB104627), since its deduced amino acid sequence was different from any fast skeletal MYHs from carp (75–79% identity; Imai et al., 1997), but resembled carp slow skeletal MYHs in the present study (87–88% identity) and cardiac types from other vertebrates (72–96% identity; Yelon et al., 1999; McNally et al., 1989; Kraft et al., 1989; Yutzey et al., 1994; Figs 1 and 2). We sequenced two independent clones for this gene.

Phylogenetic analysis

The topology in NJ and MP trees were almost the same, implying that the trees obtained were highly reliable (Fig. 3). MYH_{S30} and MYH_{S10} as well as MYH_{emb3} were located in the same clade with zebrafish slow type MYH encoded by *smyhc1* (Bryson-Richardson et al., 2005). However, MYH_{card} formed monophyly with zebrafish MYH expressed in ventricular muscle, but was different from zebrafish MYH expressed in atrial muscle (Berdougo et al., 2003).

MYH_{emb1} and MYH_{emb2} were monophyletic with zebrafish MYHs encoded by *myhz1*, *myhz2* and *myhc4* expressed in embryos (Bryson-Richardson et al., 2005; Peng et al., 2002; Xu et al., 2000). This group also contained torafugu MYH_{M743-2}

and MYH_{M10392} (Ikeda et al., 2004). MYH_{F10}, MYH_{Fint} and MYH_{F30} isolated from carp adult fast muscle (Imai et al., 1997; Hirayama et al., 2000; Watabe, 2002) were located in the same clade with torafugu MYH_{M86-2} and different from other four torafugu fast skeletal MYHs (Ikeda et al., 2004) (Fig. 3). It was noted that human slow/cardiac MYHs and fast skeletal MYHs formed separate groups from fish counterparts, respectively.

Northern blot analysis

Although the specificities of probes for MYH_{F10} and MYH_{F30} had been previously confirmed (Hirayama and Watabe, 1997), it was not clear whether or not the DNA fragments used as probes for MYH_{emb1}, MYH_{emb2}, MYH_{emb3}, MYH_{S10}, MYH_{S30} and MYH_{card} were specific to their respective genes. Dot blot analysis revealed that all probes except MYH_{card} were highly specific to each target mRNA (Fig. 4). Only MYH_{card} gave faint positive signals with MYH_{emb3}, MYH_{S10} and MYH_{S30} (Fig. 4).

We then performed northern blot analysis to determine the accumulated mRNA levels of MYH DNAs for carp at various developmental stages as well as for adult carp acclimated to 10°C, 20°C and 30°C (Fig. 5). The expression pattern of MYH_{emb1} was similar to that of MYH_{emb2}, and both species of mRNA were first detected in embryos 61 h.p.f. at the heartbeat stage. The peak expression of these mRNAs occurred in juveniles 1 month post-hatching. Faint expression of MYH_{emb1}, but not MYH_{emb2} was found 7 months post-hatching. Signals for MYH_{emb1} mRNA were also detected in fast skeletal muscle from adult carp acclimated to 10°C and 20°C. MYH_{emb3} was also first detected in embryos 61 h.p.f., and faintly detected in fast and slow skeletal muscles of adult carp acclimated to 10°C and 20°C as well as in fast skeletal muscle of adult carp acclimated to 30°C. MYH_{S10} and MYH_{S30} mRNAs were faintly expressed at hatching and the transcripts increased in abundance between 1 month and 7 months post-hatching. The transcripts of MYH_{S10} and MYH_{S30} were detected in slow skeletal muscles of adult carp acclimated to 10°C and 20°C, whereas 30°C-acclimated fish only expressed MYH_{S30}. The transcripts of MYH_{card} were first detected in embryos 61 h.p.f. and were found in juvenile and in fast and slow skeletal muscles from adult fish.

The transcripts of MYH_{emb1} and MYH_{emb2} were not found, even at very low levels, in 7-month-old juveniles, by which time the MYH_{F10} gene was showing strong expression. In adult stages, the fast skeletal muscle of fish acclimated to 10°C expressed the MYH_{F10}, MYH_{emb1}, MYH_{emb3}, MYH_{S10}, MYH_{S30} and MYH_{card} mRNAs, while 30°C-acclimated fish expressed MYH_{F30}, MYH_{S10}, MYH_{S30}, MYH_{emb3} and MYH_{card} mRNAs. The transcripts of MYH_{S10} and MYH_{S30} were found in embryos at hatching and juveniles of 1-month and 7-months old. It was noted that fast skeletal muscle from adult carp acclimated to 20°C expressed MYH_{S10}, MYH_{S30} and MYH_{card} mRNA. The slow skeletal muscle of adult carp acclimated to 10°C and 20°C contained both MYH_{S10} and MYH_{S30}. By contrast, 30°C-acclimated fish predominantly expressed MYH_{S30}.

As a next step in the analysis a universal probe was adopted for northern blot analysis. This DNA probe encoded residues

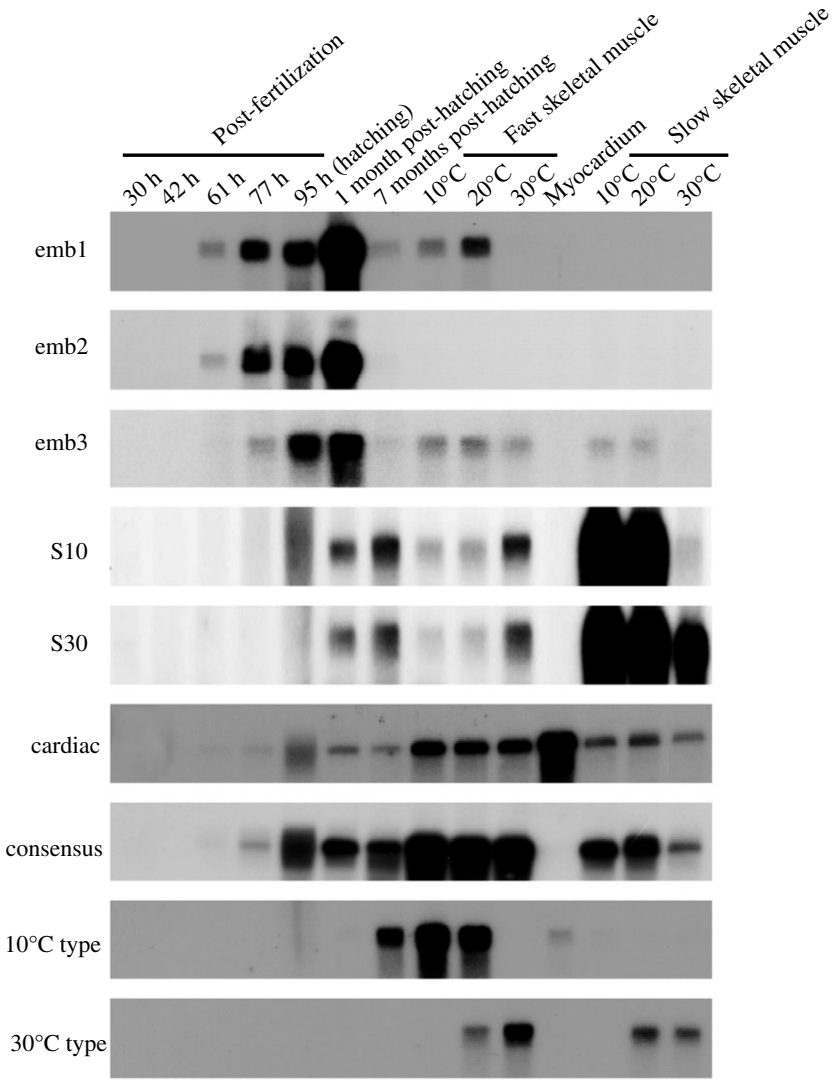

Fig. 5. Changes in the accumulated mRNA levels of carp MYH isoforms during ontogeny. Total RNAs (20 μg) were electrophoresed in a 1% agarose gel and transferred on to the nylon membranes, which were then hybridized with ^{32}P-labeled probe. Lanes contain total RNAs from the whole embryos at 30 h, 42 h, 61 h, and 77 h.p.f., from the whole larvae at hatching (96 h), from the muscle of juveniles aged 1 and 7 months post-hatching and from the fast and slow skeletal muscles of thermally acclimated adult fish. An ethidium bromide-stained gel shows 28S and 18S rRNA. Consensus indicates the membrane hybridized with the probe, which is supposed to react with all skeletal MYH mRNA of carp. Each lane contains 5 μg of total RNA.

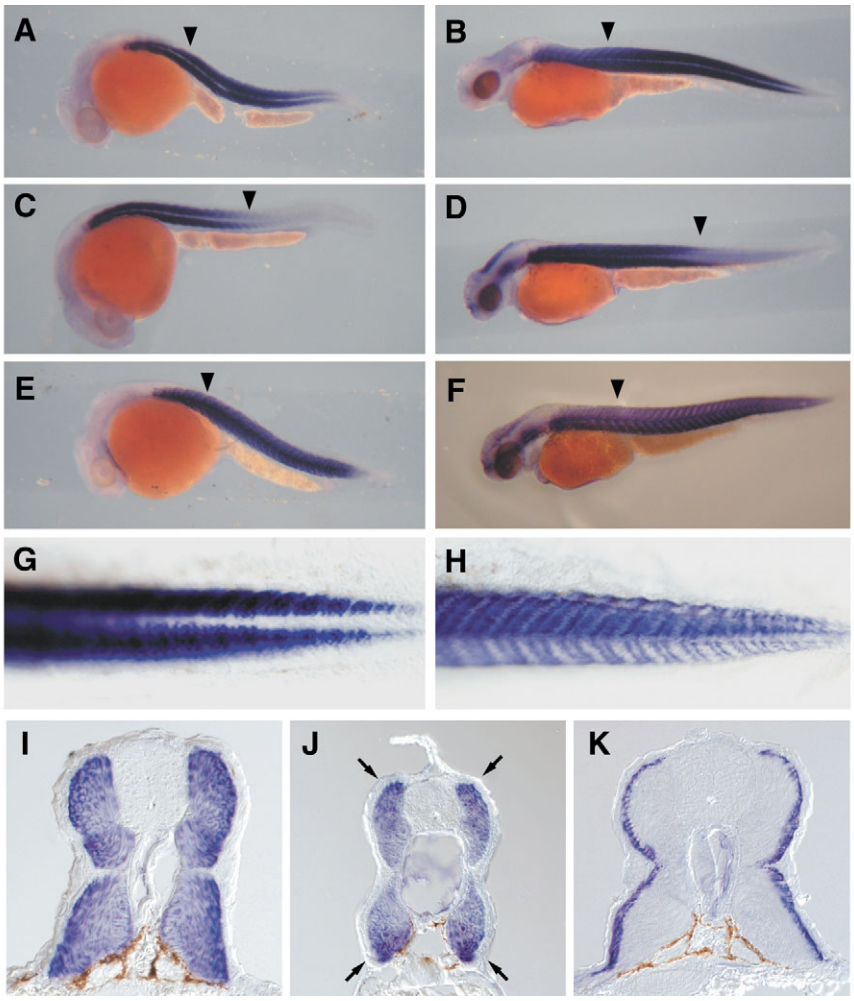

Fig. 6. Localization of the transcripts of MYH_{emb1}, MYH_{emb2} and MYH_{emb3} in carp embryos. Carp embryos were hybridized with probes specific to MYH_{emb1} (A,B), MYH_{emb2} (C,D) and MYH_{emb3} (E,F) at 77 (A,C,E) and 95 (B,D,F) h.p.f. G and H are higher magnifications of the caudal region shown in B and F, respectively. (I,J,K) Transverse sections of B,D,F, respectively, in the regions indicated by arrowheads. Arrows in J indicate the transcripts of MYH_{emb2} in the four 'corners' of the trunk.

534–717 from the 3′ region of the MYH_{F10} cDNA, which is thought to react with all known skeletal MYH isoforms of carp (Hirayama and Watabe, 1997). The signals to this probe were first detected in embryos 61 h.p.f., and then in fast and slow skeletal muscles from adult fish (Fig. 5).

Whole-mount in situ hybridization

Whole-mount *in situ* hybridization (WISH) was carried out for MYH_{emb1}, MYH_{emb2} and MYH_{emb3} to localize their transcripts in 77 h.p.f. (eyed-stage) and 95 h.p.f. (hatching stage) fish. Probes used for northern blot analysis consisting of about 100 nt gave no signals (data not shown), therefore, the size of probes was enlarged to about 150 nt as described in the Materials and methods. The transcripts of MYH_{emb1} (Fig. 6G,I) and MYH_{emb2} (Fig. 6J) were expressed in the inner part of the myotome, whereas that of MYH_{emb3} was expressed in the superficial compartment (Fig. 6H,K). The superficial compartment of the myotome largely comprises slow muscle fibers (Devoto et al., 1996), thus MYH_{emb3} is probably predominantly expressed in slow embryonic muscle. Whereas the transcripts of MYH_{emb1} were detected in almost the whole trunk muscle (Fig. 6A,B), those of MYH_{emb2} were observed in the region of anterior to middle trunk muscle (Fig. 6C,D) at 77 and 95 h.p.f. Observation of this region revealed that the transcripts of MYH_{emb2} were localized only in four corners of trunk (Fig. 6J, arrows).

Discussion

In the present study, we isolated six types of MYH DNA from common carp, the deduced amino acid sequences of which were classified into two groups (Table 1 and Fig. 3). One was fast skeletal muscle type, which contained MYH_{emb1}, MYH_{emb2}, MYH_{F10}, MYH_{Fint}, MYH_{F30} and the other, slow/cardiac muscle type, which consisted of MYH_{emb3}, MYH_{S10}, MYH_{S30} and MYH_{card}. The slow/cardiac muscle group of *MYH*s was further divided into slow and cardiac (MYH_{card}) types (Fig. 3). Carp *MYH*s belonging to the fast skeletal muscle clade were divided into two types, one expressed in adult fast muscle (MYH_{F10}, MYH_{Fint} and MYH_{F30}) and the other in embryonic stages (MYH_{emb1} and MYH_{emb2}). The two carp embryonic type *MYH*s formed the other group with zebrafish *myhz1*, *myhz2* and *myhc4* expressed in embryos (Xu et al., 2000; Peng et al., 2002; Bryson-Richardson et al., 2005) and also with torafugu $MYH_{M743\text{-}2}$ and MYH_{M10392} (Ikeda et al., 2004) (Fig. 3).

The expression patterns of various MYHs of carp identified in relation to tissue type and developmental stage are summarized in Fig. 7. MYH_{emb1} was not exclusively expressed in the embryonic and larval stages, since transcripts were also detected in the fast skeletal muscle of adult fish acclimated to 20°C. This may reflect its expression in newly formed myotubes. Small amounts of embryonic and neonatal *MYH* mRNAs are known to be expressed in adult rat *plantaris* and *soleus* muscles during hypertrophic growth (Periasamy et al., 1989). In addition, Ennion et al. (1995) isolated a *MYH* clone (FG2) from carp that hybridized exclusively to small diameter fibers in warm-acclimated fish. Ennion et al. (1999) isolated two developmentally regulated *MYH*s, *Eggs22* and *Eggs24*, from the carp. These transcripts were first detected in embryos 22 h.p.f. and continued to be expressed over 2 weeks post-hatching, but were not detected in juveniles 21 and 28 d post-hatching or in adult stages. In contrast, the mRNA levels of

MYH_{emb1} and MYH_{emb2}, homologues of *Eggs22* and *Eggs24*, respectively, were still high in 1-month-old juveniles. MYH_{emb1} transcripts were also detected at low levels in 7-month-old juveniles, and in the fast skeletal muscle of adult carp acclimated to 10°C and 20°C (see Fig. 4). Although both MYH_{emb1} and MYH_{emb2} had nucleotide sequences very similar to each other, their expression patterns were markedly different. WISH analysis demonstrated no obvious differences in expression patterns between MYH_{emb1} and MYH_{emb2} (Fig. 6). Wakeling et al. (2000) demonstrated, by peptide mapping, that the expression of carp fast type MYHs was only altered with temperature acclimation in fish greater than 37 mm total length. Furthermore, larval MYH isoforms were replaced by juvenile isoforms when larvae reached 20 mm. In the present study, only weak signals for MYH_{emb1} were detected at 7 months post-hatching (Fig. 7), which suggests that an as yet uncharacterized MYH isoform(s) is expressed in juvenile fish.

MYH_{emb3} had DNA nucleotide and deduced amino acid sequences very similar to those of MYH_{S10} and MYH_{S30} isolated from slow muscle in adult fish and these three isoforms were located on the same clade in the phylogenetic tree (Fig. 2). The transcripts of MYH_{emb3} were first detected in 61 h.p.f. embryos by northern blot analysis and in the superficial layer of myotomes by WISH (Figs 5 and 6). It has been demonstrated, using immunohistochemistry, that expression of the slow type *MYH* gene is followed by expression of the fast type in zebrafish (van Raamsdonk et al., 1978; Devoto et al., 1996; Weinberg et al., 1996). The initial slow muscle fibers in zebrafish are formed from an array of adaxial cells in response to hedgehog signaling from the notochord (Blagden et al., 1997). The adaxial cells elongate to span the somite width and migrate through the somite to form a superficial layer of slow muscle fibers (Devoto et al., 1996). However, these adaxial-derived slow muscle fibers account for about half the number of slow fibers present at the time the embryo hatches. In the late embryo, additional slow muscle fibers are added from discrete germinal zones by stratified hyperplasia (Barresi et al., 2001). Northern blot analysis in the present study revealed that in common carp MYH_{S10} and MYH_{S30} transcripts were not expressed until hatching.

We previously cloned Myf-5, MyoD, myogenin, MEF2A and MEF2C from carp and showed that these genes were expressed in carp embryos, larvae and juveniles (Kobiyama et al., 1998). It was noted that no differences in the timing of expression of Myf-5, MyoD and myogenin mRNAs were seen between different temperature groups of carp embryo (Cole et al., 2004). The signals of carp MEF2C and MEF2A mRNA were first detected in carp embryos at 30 and 42 h.p.f., respectively (Kobiyama et al., 1998). The MEF2 family is a key regulator of cardiac muscle lineage (Gossett et al., 1989; Olson, 1995). In zebrafish, the transcripts of MEF2C were detected in primordial cardiac cells from embryos at 16 h.p.f., whereas those of MEF2A and α-tropomyosin were first observed in embryos at 10 h.p.f. (Ticho et al., 1996). Yelon et

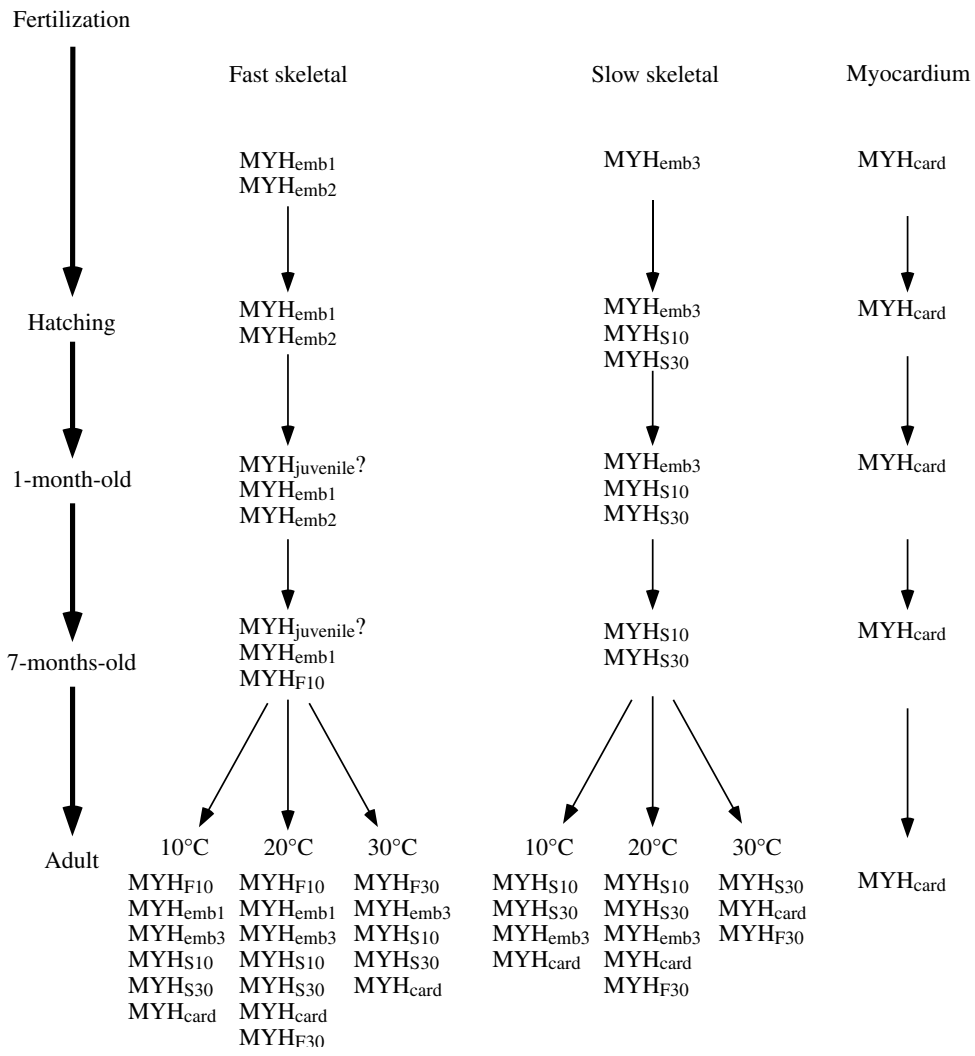

Fig. 7. Schematic representation of the mRNA expression patterns of the various MYHs identified in relation to muscle type, developmental stage and acclimation temperature. Since northern blot analysis was only performed on pure muscle types in adults, the tissue-specific expressions in embryonic and juvenile fish were based on inference (see text for discussion), and therefore remain a hypothesis. Putative MYH possibly expressed in juveniles are indicated by question marks.

al. (1999) showed that ventricular *MYH* was first expressed in zebrafish embryos at 30 h.p.f., and that of cardiac myosin light chain 2 mRNA appeared at the same stage and was expressed only in the heart tube. In the present study MYH_{card} was first expressed in embryos at 61 h.p.f. and it continued to be expressed during subsequent embryonic stages. The transcripts of MYH_{card} were detected not only in myocardium of adult carp, but also in adult slow and fast skeletal muscles. Since the present probe for MYH_{card} showed weak signals against MYH_{emb3}, MYH_{S10} and MYH_{S30}, a new specific probe for MYH_{card} is required to distinguish between the expression patterns of MYH_{card}, $MYH_{emb3}MYH_{S10}$ and MYH_{S30}. However, Allen and Leinwand (2001) showed that αMYH, which is expressed almost exclusively in the heart, is expressed in scattered fibers in all the hindlimb muscles of the mouse during postnatal development. Single skinned fibers from slow and fast muscle of adult rats contained MYHI/β and MYHIIa, although the frequency of this co-expression was very low (Bottinelli et al., 1994). Bisaha and Bader (1991) showed that a type of cardiac MYH, VMHC 1, expressed exclusively in the chicken heart during embryogenesis, was also transiently expressed in all embryonic skeletal muscles.

In summary, we isolated six MYH DNAs from carp, three embryonic, two slow skeletal and one cardiac. According to the deduced amino acid sequence, each MYH isoform was classified into either fast or slow type using phylogenetic analysis. Our initial hypothesis that there would be multiple slow muscle MYH genes that are expressed at different acclimation temperatures was accepted although we do not rule out the possibility that some of these genes might correspond to the alternatively spliced transcripts from the same gene. However, we have revealed a complex pattern of expression of *MYH*s in relation to developmental stage, muscle type and acclimation temperature. For example, *MYH*s predominantly associated with slow muscle or early developmental stages were expressed in the fast muscle of adult fish at some acclimation temperatures but not others. Since none of the nine skeletal muscle *MYH*s, including the three previously reported, were strongly expressed in 7-month-old juveniles it is likely that further members of the *MYH* family remain to be identified.

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