The Journal of Experimental Biology 201, 2801–2813 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JEB1625

MOLECULAR CLONING AND DEVELOPMENTAL EXPRESSION PATTERNS OF THE MyoD AND MEF2 FAMILIES OF MUSCLE TRANSCRIPTION FACTORS IN THE CARP

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Accepted 5 August; published on WWW 22 September 1998

Summary

cDNA clones encoding the myogenic regulatory factors (MRFs) myogenin, MyoD and myf-5 were isolated by reverse-transcription polymerase chain reaction from larvae and embryos of the common carp (*Cyprinus carpio* L.). Myocyte-specific enhancer factor 2 (MEF2) cDNAs were identified from a cDNA library from adult carp. Northern blot analysis showed that MyoD, myf-5 and MEF2C transcripts were present in three-somite embryos, whereas myogenin and MEF2A transcripts were not detected until the 15-somite stage. Intense signals of myogenin and MyoD transcripts were observed even in 1-month-old juveniles. Levels of MyoD, myogenin and MEF2A transcripts declined between 1 and 7 months after hatching, and myf-5 gave only a weak signal in the oldest fish. In contrast, levels of MEF2C transcripts were considerably higher in 7-month-old juveniles than in 1-month-old larvae. mRNAs encoding carp myosin heavy chain and α-actin were first detected at approximately the time of the first heartbeat, and levels were maximal in juveniles 1 month post-hatching. The relatively high levels of MRF mRNA in juvenile fish probably reflect the recruitment of new muscle fibres from the satellite cell population. It was concluded that the relative importance of the different members of the MyoD and MEF2 families of transcription factors for muscle differentiation changes during ontogeny in the carp.

Key words: carp, *Cyprinus carpio*, myogenin, MyoD, myf-5, MEF2A, MEF2C, myogenesis, development, cDNA cloning, myosin heavy chain, α-actin, muscle.

Introduction

The trunk musculature of fish is derived from the segmental plate mesoderm flanking the notochord and lying underneath the presumptive spinal cord. Studies in zebrafish (*Brachydanio rerio*) have shown that the most medial cells in the segmental plate, called adaxial cells, commit to becoming myoblasts with a specific slow muscle lineage (Devoto *et al.* 1996) under the influence of the glycoprotein Sonic hedgehog (Shh) secreted from the notochord (Blagden *et al.* 1997). In zebrafish, the adaxial cells are also distinguished from the smaller, more numerous, lateral segmental plate cells by their unique expression of *snail1* (Thisse *et al.* 1993) and *MyoD* (Weinberg *et al.* 1996) prior to somite formation. Somites are formed from the segmental plate in a rostral to caudal sequence as paired blocks of mesoderm that rapidly differentiate into a ventral sclerotomal compartment and an epithelial dermomyotome, which contributes to the dermis and gives rise to the muscle tissue (Morin-Kensicki and Eisen, 1997). The rows of adaxial cells become incorporated within each somite, and injection studies with vital fluorescent dyes have shown that these cells migrate through the somite to become the superficial layer of slow muscle fibres (Devoto *et al.* 1996). A small subset of the adaxial muscle cells elongates to span the entire width of the somite, and these cells form the mononuclear muscle pioneer fibres present at the horizontal septum (Devoto *et al.* 1996). The pioneer cells are distinguished by their high levels of expression of the *engrailed* gene (Hatta *et al.* 1991) and they are the first muscle fibres to become innervated (Hanneman, 1992; Johnston *et al.* 1997). The fast muscle fibres derived from the lateral presomitic mesoderm are formed from the fusion of several myoblasts to form multi-nucleated myotubes. Sonic hedgehog appears not to be required for the commitment of the lateral presomitic cells to a fast muscle lineage because the *boz* mutant lacks Shh and yet contains fast muscle fibres throughout the somite (Blagden *et al.* 1997).

Members of the MyoD family of basic helix–loop–helix (bHLH) transcription factors have a central role in the

determination and differentiation of vertebrate skeletal muscle (see Weintraub, 1993). Collectively referred to as myogenic regulatory factors (MRFs), they include the genes for MyoD and myogenin, which are not closely linked, and the closely linked genes for myf-5 and MRF4 (myf-6), the coding sequences of which are separated by only 8500 base pairs (8.5 kb) on human chromosome 12 (Braun *et al.* 1990). In mammals, forced expression of each of the MRFs can convert non-muscle cells to myoblasts, suggesting that they may be functionally interchangeable (Weintraub *et al.* 1991), although 'knock-out' and expression studies indicate that each has distinct but overlapping functional roles (Rudnicki *et al.* 1992; Smith *et al.* 1994). Double-mutant knock-out mice deficient in the genes for both MyoD and myf-5 are lethal and lack all myogenic progenitor cells (Rudnicki *et al.* 1992). There is evidence that *cis*-acting interactions are important in regulating the expression of the genes for myf-5 and MRF4 (Yoon *et al.* 1997). MRFs form heterodimers with E protein products, such as E12 and E47 of the ubiquitiously expressed *E2A* gene family, and bind to the E box DNA sequences (CANNTG) present in the control regions of many muscle-specific genes (see Olson, 1992). MRF activity is controlled through both positive and negative regulatory pathways involving, among other factors, LIM protein (MLP) (Kong *et al.* 1997) and Id proteins (Benezra *et al.* 1990), respectively.

Another important regulator of skeletal muscle differentiation is the myocyte-specific enhancer factor 2 (MEF2) family of transcription factors which bind to an A/T-rich sequence present in many muscle-specific promotors and enhancers (Gossett *et al.* 1989). Multiple isoforms of MEF2 have been identified in vertebrates, including fish (Ticho *et al.* 1996), all of which contain the DNA-binding sequence characteristic of the MADS gene family and an adjacent highly conserved MEF2-specific domain. MEF2 isoforms regulate myogenic bHLH genes and cooperates with MRFs in activating skeletal-muscle-specific transcription (for a review, see Olson, 1992).

In zebrafish, MyoD (Weinberg *et al.* 1996) and MEF2D (Ticho *et al.* 1996) transcripts are first detected at mid-gastrulation and are present in the adaxial cells adjacent to the notochord in the presomitic mesoderm. During somitogenesis, MEF2D-containing cells are present on either side of the transverse myoseptum, and expression occurs in a rostral to caudal sequence (Ticho *et al.* 1996). MEF2A and MEF2C transcripts appear later, but follow the same pattern of expression within the somites as MEF2D (Ticho *et al.* 1996). Myogenin appears in the somites at approximately the same time as MEF2A (Ticho *et al.* 1996; Weinberg *et al.* 1996).

Although the zebrafish is an excellent system for studying early muscle commitment and differentiation, it does not provide a general model for post-larval muscle growth because of its modest ultimate body size (length 3–5 cm). Muscle growth in small species involves primarily the hypertrophy of the fibres formed in the embryo and during the early larval stages (Weatherley *et al.* 1988). In contrast, for species with indeterminate growth, including common carp, new muscle fibres continue to be recruited during the juvenile and adult stages from a stem cell population present under the basal lamina of muscle fibres (Stickland, 1983; Alami-Durante *et al.* 1997; Koumans *et al.* 1993; Johnston *et al.* 1998). In addition, carp fast muscle fibres change myosin heavy chain isoforms following temperature acclimation (Guo *et al.* 1994; Watabe *et al.* 1995*b*; Imai *et al.* 1997; Hirayama and Watabe, 1997), suggesting the participation of MRFs in muscle fibre recruitment for adult carp in association with environmental temperature fluctuation. However, almost nothing is known about the expression patterns and role of MRFs and MEF2 isoforms during post-larval growth in fish. The aim of the present study was to isolate cDNA clones of MyoD, myogenin, myf-5 and MEF2 isoforms from the common carp and to investigate their temporal expression patterns in relation to actin and myosin heavy chain gene expression in embryonic, larval and juvenile stages of the life cycle.

Materials and methods

Fish

Carp (*Cyprinus carpio* L.) were reared at the Yoshida Research and Training Station, Tokyo University of Fisheries, Japan. Eggs and milt were stripped from two male and two female fish to produce four families. Embryos and larvae were cultured in freshwater aquaria at approximately 20 °C. Somite formation was observed with a light microscope to assess the developmental stage of the embryos. Whole fish were quickly frozen in liquid N_2 for extraction of total RNA. Whole larvae at hatching were also used for polymerase chain reaction (PCR) amplification of myogenic regulatory factors after freezing in liquid N_2. Larvae aged 2 days were transported to the laboratory of the University of Tokyo and cultured for a further 7 months under a 14 h:10 h L:D photoperiod at approximately 20 °C. Fish were fed commercial pellets twice daily. Fast muscle was isolated from the trunk muscle of juveniles aged 1 and 7 months, frozen in liquid N_2, and stored frozen at –80 °C until used for northern blot analysis.

Total RNA extraction

Total RNA used for PCR amplification was isolated from larvae at hatching by the acidic guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987). Total RNAs used for northern blot analysis were extracted with Isogen solutions (Nippon Gene) from embryos at 30 h (three-somite stage), 42 h (15-somite stage), 61 h (heartbeat stage) and 77 h (eyed stage) post-fertilization and from larvae at hatching. Muscle samples were also taken from 1-month-old and 7-month-old juveniles. Approximately 300 μg of total RNA was extracted from 1 g of embryos, whereas 1 mg of total RNA was obtained from 1 g of larvae at hatching and from juvenile muscle. The

concentrations of RNA were determined from the absorbance at 260 nm using a Shimadzu UV-160 spectrophotometer.

cDNA cloning of the MyoD family: PCR amplification

First-strand cDNA was synthesized using a T-primed first-strand kit (Pharmacia Biotech). PCR primers were designed with reference to DNA nucleotide sequences of myogenin, MyoD and myf-5 from various vertebrate species (Table 1; Fig. 1). Primer 4 had the same sequence as an adapter region of the *Not*I $d(T)_{18}$ primer contained in the kit. PCR amplifications were carried out for 3 min at 94 °C, followed by 30 cycles of denaturation for 1 min at 94 °C, 1.5 min of annealing at 50 or 60 °C and 2 min of extension at 72 °C (the last extension step was extended for 5 min), using a DNA thermal cycler (model 2400; Perkin Elmer Applied Biosystems). The composition of the reaction mixture was as follows: each of the 100 µl reaction mixtures contained 40 pmol of forward and reverse primers, approximately 1 µg of first-strand cDNA from embryos at hatching, 20 nmol of dNTP mixture, 10 µl of 10× PCR buffer (100 mmol l^{-1} Tris/HCl, pH 8.3, 500 mmol l^{-1} KCl, 15 mmol l^{-1} $MgCl_2$, 0.01 %, w/v, gelatin) and 1 unit of *Taq* DNA polymerase (Perkin Elmer). Alternatively, DNA fragments were amplified by 5′-rapid amplification of cDNA ends (5′-RACE) PCR using a 5′-RACE system version 2.0 kit (Gibco BRL). Amplified DNA fragments were subcloned into plasmid pBluescript II (SK^-) using *Escherichia coli* strain MV1190 as a host bacterium.

DNA sequencing was performed using a dye-terminator cycle sequencing ready reaction kit with a DNA sequencer (model 373S; Perkin Elmer Applied Biosystems). The nucleotide sequence data for carp myogenin, MyoD and myf-5 will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB012881, AB012882 and AB012883, respectively.

cDNA cloning of the MEF2 family: screening of a cDNA library from adult carp

First, we tried to isolate cDNA clones encoding the MADS box and MEF2 domain by PCR from a cDNA library prepared from the fast myotomal muscle of carp acclimated to 10 °C for a minimum of 5 weeks (Imai *et al.* 1997). PCR primers 16 and 17 were designed with reference to DNA nucleotide sequences of MEF2C from various vertebrate species (Table 1). A PCR product of approximately 200 bp encoding the MADS box and MEF2 domain of carp MEF2C was randomly labelled with a digoxigenin DNA-labelling kit (Boehringer Mannheim) according to the manufacturer's instructions. Hybridization

Table 1. *Nucleotide sequences of primers used for polymerase chain reaction amplification of carp myogenin, MyoD, myf-5 and MEF2*

No.	Sequence	Nucleotide position*	Myogenic regulatory factor
1	5′-GTGTGTAAGCGCAAGTCTG-3′	418–436	Y
2	5′-GATGCT(C/G)TCCACGATGGA(C/G)G-3′	833–852	Y
3	5′-GGAGCAGTGCGTCTGAGC-3′	734–751	Y
4	5′-TTGACCTTCTTAAGCGCCG-3′	3′ end	Y, D and F
5	5′-TCAGGTTGGTTTGCTCT-3′	807–823	Y
6	5′-TCATGAGCGTGCTCCTCTTA-3′	516–535	Y
7	5′-TGCCTACTGTGGGCATGCAA-3′	392–411	D
8	5′-ACTCACTTCTGCTGATCTGC-3′	1214–1233	D
9	5′-ATCTCCACTTTGGGCAGCCT-3′	542–561	D
10	5′-TGCCTCATGTGGGCCTGCAA-3′	212–231	F
11	5′-AGGATCTCCACCTTGGGCAG-3′	365–384	F
12	5′-ACTGTGGACCGCCGGAGAGC-3′	254–273	F
13	5′-GCAGGCTGAAGAAGGTGAAC-3′	294–313	F
14	5′-GGCTTTCGATGTACTGGATG-3′	394–413	F
15	5′-GGATCTCCACCTTGGGAAGA-3′	364–383	F
AUAP	5′-GGCCACGCGTCGACTAGTAC-3′	5′ end	Y, D and F
16	5′-ATGGGGAG(A/G)AAAAAGAT(A/T/C)CAGAT-3′	424–448	E
17	5′-CTCCAC(A/G)ATGTCTGAGTT(A/T/G)GT-3′	663–683	E

*The positions of bases in myogenin, MyoD, myf-5 and MEF2C (DDBJ/EMBL/GenBank nucleotide sequence databases accession numbers AB012881, AB012882, AB012883 and AB012885, respectively).

Y, myogenin; D, MyoD; F, myf-5; E, MEF2.

was carried out as described previously (Imai *et al.* 1997). Positive clones were plaque-purified, and the inserts were excised in the form of pBluescript II (SK^-) plasmid vectors according to the manufacturer's protocol for sequencing. Sequence analysis was performed as described above. The nucleotide sequence data for carp MEF2A and MEF2C will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB012884 and AB012885, respectively.

Northern blot analysis

Samples for northern blot analysis were collected from various developmental stages of carp including embryos, larvae and juveniles. Two series of sample collection were carried out for northern blot analysis. Total RNAs were fractionated in 1.4 % (w/v) agarose gels containing 18 % (v/v) formamide and blotted onto nylon membranes (Amersham). The membranes were prehybridized at 65 °C for 1 h in 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. After hybridization, 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 for periods ranging from 2 days to 2 weeks at −80 °C using intensifying screens.

The DNA fragments used as probes encoding carp myogenin, MyoD, myf-5, MEF2A, MEF2C, fast skeletal myosin heavy chain and α-actin were amplified by PCR and labelled with [α-^{32}P]dCTP using a random primer DNA-labelling kit (Takara). The probes correspond to nucleotides 418–1264 of myogenin cDNA, 392–1233 of MyoD cDNA, 294–1242 of myf-5 cDNA, 648–1679 of MEF2A cDNA and 881–1927 of MEF2C cDNA, or were amplified from the DNA sequence encoding residues 534–717 from the N terminus for carp 10 °C-type fast skeletal myosin heavy chain (Hirayama and Watabe, 1997) or nucleotides 1–511 of carp α-actin (Watabe *et al.* 1995*b*).

It should be emphasized that the region selected for carp myosin heavy chain is thought to react with all known isoforms of this protein in carp (Hirayama and Watabe, 1997). Furthermore, it has been demonstrated that chicken embryonic (Gulick *et al.* 1985) and fast skeletal (Chao and Bandman, 1997) myosin heavy chains have 89.7 % homology for DNA nucleotide sequences in this region, suggesting that our probe should hybridize with mRNAs encoding any potential embryonic isoforms of myosin heavy chains in carp.

Results

cDNA cloning of carp myogenin

The cloning of cDNAs encoding carp myogenin was accomplished through several PCR amplifications (Fig. 1; Table 1). First, a 435 nucleotide cDNA fragment was successfully amplified using primers 1 and 2. Amplification of the 3′ non-coding region was carried out by nested PCR as follows. Primers 1 and 4 were used in the first PCR, then a second PCR was carried out with primers 3 and 4, resulting in amplification of a 722 nucleotide cDNA fragment. Subsequently, 5′-RACE PCR was carried out with primers 5 and 6 as well as AUAP in the kit, producing a 515 nucleotide cDNA fragment. The PCR products were subcloned into plasmid vectors and sequenced. Finally, we obtained the cDNA clone, which had a single major open reading frame (ORF) encoding putative 253 amino acid residues. The total DNA nucleotide sequence was confirmed by a single PCR. The first ATG at nucleotide 142 is probably the initiation codon because of its sequence similarity around the ATG (AACATGG) to a consensus sequence (A/CNNATGG) for eukaryotes (Kozak, 1981).

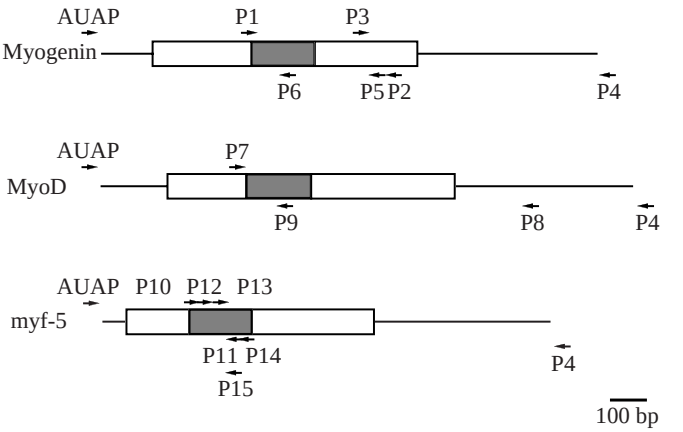

Fig. 1. Locations of primers (P) used for polymerase chain reaction amplification of carp myogenin, MyoD and myf-5 cDNAs. The boxes and solid bars indicate the coding and non-coding regions, respectively. The hatched areas in the coding regions represent the basic helix–loop–helix domains.

It has been proposed that the bHLH region in myogenic regulatory factors is required for DNA binding and heterodimerization with E proteins in higher vertebrates (Lassar *et al.* 1989; Davis *et al.* 1990). The corresponding region in carp myogenin (enclosed within a box in Fig. 2) was very conserved and showed almost the same sequence (93–98 % identity) as those of other vertebrates, including rainbow trout (*Oncorhynchus mykiss*) (Rescan *et al.* 1995). While myogenin was also well conserved at residues 81–94 and 156–164, the entire sequence of carp myogenin showed 69, 55 and 51 % sequence identity with those of rainbow trout (Rescan *et al.* 1995), chicken (Fujisawa-Sehara *et al.* 1990) and mouse (Edmondson and Olson, 1989), respectively. In addition, carp myogenin had additional sequences at residues 52–56 and 68–79 from the N terminus which were not observed in chicken and mouse.

cDNA cloning of carp MyoD

Cloning of carp MyoD was also carried out through sequential PCR amplifications. A 1194 nucleotide cDNA

fragment was first amplified with primers 4 and 7 (Table 1; Fig. 1). Subsequently, 5′-RACE PCR was carried out using primers 8 and 9 as well as AUAP. The resulting cDNA contained 1561 nucleotides with an ORF of 825 nucleotides that encoded 275 amino acid residues. The total DNA nucleotide sequence was confirmed by a single PCR. The first ATG in the ORF was considered as the initiation codon for the same reason as outlined for myogenin.

The bHLH region of carp MyoD was highly conserved with a sequence identity to other vertebrates, including zebrafish, in the range 93–98 %. In contrast to myogenin, however, MyoD from carp contained several other conserved regions at 27–53, 77–101, 189–211 and 246–261 amino acid residues from the N terminus, resulting in higher homology of the whole molecule in carp with that of higher vertebrates. MyoD in carp showed 93, 81, 73 and 71 % identities with MyoD from zebrafish (Weinberg *et al*. 1996), rainbow trout (Rescan *et al.* 1994), *Xenopus laevis* (Hopwood *et al.* 1989) and chicken (Lin *et al.* 1989), respectively (Fig. 3). Carp MyoD was relatively similar to other fish MyoDs sequenced, containing deletions at 9–21, 66–76, 171–175 and 306–312 amino acid residues from the N terminus that are not observed in higher vertebrates.

cDNA cloning of carp myf-5

The cloning of cDNAs encoding carp myf-5 was accomplished through several PCR amplifications (Table 1; Fig. 1). First, a 173 nucleotide cDNA fragment was successfully amplified using primers 10 and 11. Amplification of the 3′ non-coding region was carried out by nested PCR as follows. Primers 12 and 4 were used in the first PCR, then a second PCR was carried out with primers 13 and 4, resulting in amplification of a 1089 nucleotide cDNA fragment. Subsequently, 5′-RACE PCR was carried out with primers 14 and 15 as well as AUAP in the kit, producing a 385 nucleotide cDNA fragment. The PCR products were subcloned into plasmid vectors and sequenced. Finally, we obtained the cDNA clone, which had a single major ORF encoding putative 240 amino acid residues. The total 1288-nucleotide sequence was confirmed by a single PCR. The first ATG in the ORF was considered to be the initiation codon, for the same reason as outlined for myogenin.

The bHLH region of carp myf-5 was highly conserved, showing an identity with other vertebrate myf-5s of 82–84 %. Carp myf-5 contained several other conserved region at residues 64–77, 139–151 and 205–216, and the whole molecule showed 56, 57 and 56 % identities with myf-5 from *Xenopus laevis* (Hopwood *et al.* 1991), chicken (Saitoh *et al.* 1993) and mouse (Buonanno *et al.* 1992), respectively. Carp myf-5 contained deletions at postions 4–8, 28–30 and 37–45 from the N terminus relative to mouse and chicken (Fig. 4).

cDNA cloning of the carp MEF2 family

The cloning of cDNA encoding the carp MEF2 family was accomplished through screening of a cDNA library from adult carp acclimated to 10 °C with a PCR product encoding the MADS box and MEF2 domain of carp MEF2C. We isolated cDNA clones containing the complete coding sequence for carp MEF2A and MEF2C. Carp MEF2A and MEF2C were 2746 and 2031 nucleotides in length, respectively, and had ORFs that coded for proteins of 472 and 474 amino acids, respectively. The first ATG in the ORF was considered to be the initiation codon, for the same reason as outlined for myogenin. The region of the MEF2A and MEF2C cDNAs encoding the MADS box and MEF2 domains showed

```
Carp     MELLETNPYF FADQRFYEGG DNFFQSRLTG GFDQTGYQDR SSMMGLCGDG   50
Trout
Chicken  ...F...... .PE....D.- E..LG...Q. -YEAAAFPE. PEV-T..PES
Mouse    ...Y..S... YQEPH..D.- E.YLPVH.Q. -.EPP..-E. TEL-S.SPEA

Carp     RLLSNGVGLE DKPSPSSSLG LSLSPHQEQQ HCPGQCLPWA CKVCKRKSVT  100
Trout
Chicken  .-----GA.. E.D.TLP--- ---------E .......... ..I....T.S
Mouse    .-----GP.. E.GLGTP--- ---------E .......... ....._____S

Carp     MDRRKAATLR EKRRLKKVNE AFEALKRSTL MNPNQRLPKV EILRSAIQYI  150
Trout       .....M.. .......... .......... .......... ..........
Chicken  I...R..... .......... .......... L......... ..........
Mouse    V...R..... .......... .......... L......... ..........

Carp     ERLQALVSSL NQQEHEQGN- -LHYRSTAPQ A-VSSSSDQG SGSTCCSSPE  200
Trout    .....|..... ....ND..TQ G.Q..TGPA. PR.....E.. ..........
Chicken  ....S.L... ....R..-R E.R.PA..-- PQPAAP.EC. ...SS..-..
Mouse    .....|L... ..E.R----- D.R..GGGG- PQPMVP.ECN .H.AS..-..

Carp     WSSASEQCAP AYSSTHEDLL NDDSSEQTNL RSLTSIVDSI TGTEVTPVPY  250
Trout    ..NT.DH..Q S..--N.... SA..P..... .......... .AA.GA.LA.
Chicken  ..TQL.FGTN P----ADH.. S..QA.DR.. H..S...E.. AVED.AV---
Mouse    .GN.L.FGPN P----GDH.. AA.PTDAH.. H......... .VEDMSV---

Carp     SVDISK                                                  256
Trout    P.PVGTFPNK PRAADRHVCC
Chicken  -----TFPEE RVQN
Mouse    -----AFPDE TMPN
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Fig. 2. Comparison of the deduced amino acid sequence of carp myogenin with those from other vertebrates. Data were taken from rainbow trout (Rescan *et al.* 1995), chicken (Fujisawa-Sehara *et al.* 1990) and mouse (Edmondson and Olson, 1989). Amino acid residues identical to those of carp are shown by periods, and gaps are represented by dashed lines. The box indicates the basic helix–loop–helix domain. Note that the N-terminal sequence has not yet been determined for rainbow trout.

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Carp        MELSDIPF-- ---------- -PIPSADDFY DDPCFNTNDM HFFEDLDPRL VHV-SLLKPD   60
Zebrafish   ........-- ---------- -......... .......... .......... ...-......
Trout       ......S.-- ---------- -.VT...... .......S.. .......... ...-G.....
Xenopus     ...LPP.LRD MEVT--EGSL CAF.TP.... .......S.. S......... ...T-....E
Chicken     .D.LGP---- MEMT--EGSL CSFTA..... .......S.. .......... ...GG....E
Mouse       ...LSP.LRD IDLTGPDGSL CSFET..... .....DSP.L R......... ...GA....E

Carp        EHHHL----- ------EDEH VRAPSGHHQA GRCLLWACKA CKRKTTNADR RKAATMRERR  120
Zebrafish   ....I----- ------.... .......... .......... .......... ..........
Trout       D..YN----- ------.... I......... .......... .......S.. ..........
Xenopus     .P..N----- ------.... .......... .......... .......... ..........
Chicken     ..P.TRA--- PPREPT.E.. .......... .......... .......... ..........
Mouse       ..A.FSTAVH PGPGAR.... .......... .......... .......... ..........

Carp        RLSKVNDAFE TLKRCTSNNP NQRLPKVEIL RNAISYIESL QALLRGQE-E -----NYYPV  180
Zebrafish   .......... .......T.. .......... .......... ....S..-D -----.....
Trout       ..G....... N......... .......... .......... .S.....DG. -----.....
Xenopus     ......E... ....Y..T.. .......... ....R..... ....HD.D-. -----AF...
Chicken     ......E... .......T.. .......... ....R..... ....E..-D -----A....
Mouse       ......E... .......S.. .......... ....R...G. ....D.DAA PPGAAAF.AP

Carp        --------LE HYSGDSDASS PRSNCSDGMM DFMGPTCQSR RRNSYDSSYF NDTPNADARN  240
Zebrafish   --------M. .......... .......... ........T. .......... ..A.......
Trout       --------.. .......... .Q........ .YNA...T.A ..SN...... AE.....S.S
Xenopus     --------.. .......... .......... .YNS.P.G.. ........FY S.S..-.S.L
Chicken     --------.. ....E..... .......... EYS..P.S.. .........Y TES..-.PKH
Mouse       GPLPPGRGS. .......... .......... .YS..PSGP. .Q.G..TA.Y SEAVR-ES.P

Carp        TKSSVVSSLD CLSSIVERIS TETPACPVLS VPEG-HEGSP CSPQEGSVLS ETGAPAPSPT  300
Zebrafish   N.N....... .......... .......... ...A-..... ...H...... D..TT.....
Trout       N.NAA..... ...N...... .D.S..T... GQ..-S.... .......I.. RN.GTV....
Xenopus     G....I.... .......... .QS.S...PT AVDSGS.... ...LQ.ET.. .RVITI...S
Chicken     G......... .......... .DNST..I.P PA.AVA.... ......GN.. DS..QI....
Mouse       G..AA..... .......... .DS..APA.L LADAPP.SP. -G.P..AS.. D--TEQ-GTQ

Carp        -TCPQ----- --QQARDPIY QVL                                          323
Zebrafish   -S..------ --...QET.. ...
Trout       -N..------ --P-SH.... ...
Xenopus     N..T.LSQDP SST-----.. H..
Chicken     N-.TPLPQES SSSSSSN... ...
Mouse       TPS.DAAPQC PAGSNPNA.. ...
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Fig. 3. Comparison of the deduced amino acid sequence of carp MyoD with those from other vertebrates. Data were obtained from zebrafish (Weinberg *et al.* 1996), rainbow trout (Rescan *et al.* 1994), *Xenopus laevis* (Hopwood *et al.* 1989), chicken (Lin *et al.* 1989) and mouse (GenBank accession number M84918). Amino acid residues identical to those of carp are shown by periods, and gaps are represented by dashed lines. The box indicates the basic helix–loop–helix domain.

```
Carp        MDV-----FS TSQIFYDSTC ASSPEAL--- EFGPGG---- -----ELAG-  50
Xenopus     .EMVDSCH.. P.EF....S. IP...EGYTE DYEH.-MSIY GAHKKD.EE-
Chicken     .E.MDSCQ.. P.EL....S. L....GEFPE D.E.RELPPF GAPAPTEPAC
Mouse       ..MTDGCQ.. P.EY..EGS. IP...DEFGD Q.E.R-VAAF GAHKA..Q.-

Carp        SEEDEHVRVP GAPHQPGHCL QWACKACKRK ASTVDRRRAA TMRERRRLKK 100
Xenopus     .D......A. IGH..A.N.. M.......... S..T...K.. ..........
Chicken     P..E....A. SGH..A.... M.......... ST.M...K.. ..........
Mouse       .DDE....A. TGH..A.... M.......... ST.M...K.. ..........

Carp        VNHAFEALRR CTSANPSQRL PKVEILRNAI QYIESLQELL REQVENYYSL 150
Xenopus     ..Q...T.K. ..TT..N... .......... .......D.. ..........
Chicken     ..Q...T.K. ..T...N... .......... R.........D .........H.
Mouse       ..Q...T.K. ..TT..N... .......... R.........D ..........

Carp        PMESSSEPAS PSSSCSESMV DCNSPVWPQM NPNFGNNYNF EAQNASAVDR 200
Xenopus     .GQ.CT..G. .M....DG.S ..S..Q.SGR .SS.D.V.CS DL.TSFSSTK
Chicken     .GQ.C...T. ......DV.A .SR.....AR GSS.EAG.CR .MPHGY.TEQ
Mouse       .GQ.C...T. .T.N..DG.P E......SRK .SS.DSI.CP DVS..C.A.K

Carp        TPGASSLQCL SSIVDRLSSV DTGVAMGMRN MVALSPTGS- DSQCSSADSP 250
Xenopus     LT-L...D.. ......I..P QQC-SLPIPD SITP...S.T ..LPR.P.AH
Chicken     SGAL...D.. ........PA EEP-GLPL.H AGS...GA.I ..GPGTPG..
Mouse       SS-V...D.. ......IT.T EPS-ELALQD TAS...AT.A N..PATPGPS

Carp        SNRPVYHVL                                                  259
Xenopus     DC..I....
Chicken     PP.RT.QA.
Mouse       .S.LI....
```

Fig. 4. Comparison of the deduced amino acid sequence of carp myf-5 with those from other vertebrates. Data were obtained from *Xenopus leavis* (Hopwood *et al.* 1991), chicken (Saitoh *et al.* 1993) and mouse (Buonanno *et al.* 1992). Amino acid residues identical to those of carp are shown by periods, and gaps are represented by dashed lines. The box indicates the basic helix–loop–helix domain.

94–100 % sequence identity with other vertebrate MEF2 isoforms. Carp MEF2A contained deletions at 211–229 and 424–437 amino acid residues from the N terminus compared with sequences for higher vertebrates, the entire sequence showing 91, 72 and 70 % sequence identity with those of zebrafish (Ticho *et al.* 1996), mouse (Lin *et al.* 1995; GenBank database accession number U30823) and human (Yu *et al.* 1992), respectively (Fig. 5). Carp MEF2C contained a deletion at 345–348 amino acid residues from the N terminus compared with sequences for higher vertebrates. As shown in Fig. 6, the amino acid sequences of zebrafish (Ticho *et al.* 1996), mouse (Martin *et al.* 1993) and human (McDermott *et al.* 1993) MEF2C are 85, 80 and 80 % identical to the carp MEF2C sequence, respectively.

Northern blot analyses of carp myogenin, MyoD, myf-5, MEF2A and MEF2C in comparison with expression patterns of carp skeletal myosin heavy chain and α-actin

Total RNAs were extracted from carp at various developmental stages and subjected to northern blot analysis for myogenin, MyoD, myf-5, MEF2A and MEF2C mRNAs as well as for muscle-specific genes encoding skeletal myosin heavy chain and α-actin. Three determinations were carried out from two series of sample collections during ontogeny. Since good reproducibility was obtained for each determination, only typical results are shown in Fig. 7.

A single mRNA band of approximately 1.5 kb encoding carp myogenin was first detected in embryos 42 h post-fertilization at the 15-somite stage (Fig. 7). Strong signals were detected in embryos at 61 h post-fertilization, at approximately the time that the heartbeat was first observed, as well as in 1-month-old juveniles. In contrast, relatively weak signals were obtained for myogenin transcripts in eyed-stage embryos (77 h post-fertilization), in larvae at hatching and in 7-month-old juveniles (Fig. 7).

Carp MyoD mRNA was detected in embryos 30 h post-fertilization at the three-somite stage, appearing earlier than myogenin mRNA (Fig. 7). The developmental stages that

```
Carp       MGRKKIQITR IMDERNRQVT FTKRKFGLMK KAYELSVLCD CEIALIIFNS SNKLFQYAST   60
Zebrafish  ..........  ..........  ..........  ..........  ..........  ..........
Mouse      ..........  ..........  ..........  ..........  ..........  ..........
Human      ..........  ..........  ..........  ..........  ..........  ..........

Carp       DMDKVLLKYT EYNEPHESRT NSDIVEKLRN KGHNDCASPD PDDCFGHSPL MDDRFSKLNE  120
Zebrafish  ..........  ..........  ..........  ......P...  ..........  .....G....
Mouse      ..........  ..........  ......T..K  ..L.G.EN..  A..Y.E....  SE...I....
Human      ..........  ..........  ......A.NK  .E.RG.D...  ..TSYVLT.H  TEEKYK.I..

Carp       ESDLMYK-RC GPTALPPQNFS MHVAVPVTN  PNAMS-YNHG  ASLSSQSLSA ATA-SLSDGA  180
Zebrafish  .......-..  ...........  .........  .....-..P.  ..........  .A.A......
Mouse      D..FIF.--R ..PGF......  .S.T....S  ..PL.DT.P.  S..V.P..--  .ASST.AETS
Human      .F.N.MRNHK IAPG.......  .S.T....S  ...L......  S..V.P..--  .ASST.T..S

Carp       MLSPPQGSLH RNLVSAGPPQ RPPSTGSAAG ---------- ---------N GFVNSRGSPG  240
Zebrafish  ........M.  .SV.---...  .......T-. ----------  ---------. ..........
Mouse      .....PAT..  ..-..P.A..  ........S. MLSTTDLTVP NGAGNSPVG. ......A..N
Human      S.....TT..  ..-..P.A..  ......N.G. MLSTTDLTVP NGAGSSPVG. ......A..N

Carp       LLGTPSGNGL GKVMPAKSPP PPGG-NMGIG SRKPDLRVVI PPSSKGMMPP LQSEEEEMEL  300
Zebrafish  ..........  ..........  ....-.....  ..........  ..........  .---------
Mouse      .I.NTGA.S.  .....T....  ....GSL.MN  ........A.  ..........  .-.....L..
Human      .I.ATGA.S.  .....T....  ....G.L.MN  ..........  ..........  .-........

Carp       NTHRISSSQS TQPLATPVVS ITTPSLPPQG LVYSGMPTAY NSEYSLSSAE LSSLQGFGSP  360
Zebrafish  ..Q.......  ..........  V.....A...  ..........  ..........  ..........
Mouse      .AQ......A  ..........  V.........  ....A.....  .TD...T..D  ..A....T..
Human      ..Q......A  ..........  V.........  ....A.....  .TD...T..D  ..A....N..

Carp       G-LSIGSMSA WQQHQLGQAA LNSLVGGGHL PQGSNLSINT SQNVNIKSEP ISPPRERITP  420
Zebrafish  .-.....V..  ..........  .S........  ..........  ..........  .......V..
Mouse      .M..L.QA..  ..E.H...TT  .S...A..Q.  S.........  N..I......  .....D.M..
Human      .M..L.QV..  ....H.....  .S...A..Q.  S.........  N..IS.....  .....D.M..

Carp       SGF------- -------PPQ QQQQPPSSRP --DMGRSPVD SLSSSCSSYD GSDREDHRHD  480
Zebrafish  ...-------- --------... ...-...G.. --........  ..........  ........P.
Mouse      ...QHHHHHP QQQPPP---- ---...QPQ.  RQE.......  .....S....  ......P.G.
Human      ...QQQQQQQ QQQQPPP...  P.P...QPQ.  RQE.......  .....S....  ......P.G.

Carp       FHSPLGLGRP RPPGQREGD- PTVKRMRMDT WVT                                513
Zebrafish  .......... PAG.AD.RES  .S........ ...
Mouse      ....IV..--  ..ANTEDRES  .S........ ...
Human      ....IV..--  ...NTEDRES  .S.......A ...
```

Fig. 5. Comparison of the deduced amino acid sequence of carp MEF2A with those from other vertebrates. Data were obtained from zebrafish (Ticho *et al.* 1996), mouse (GenBank accession number U30823) and human (Yu *et al.* 1992). Amino acid residues identical to those of carp are shown by periods, and gaps are represented by dashed lines. Open and shaded boxes indicate the MADS box and MEF2 domain, respectively.

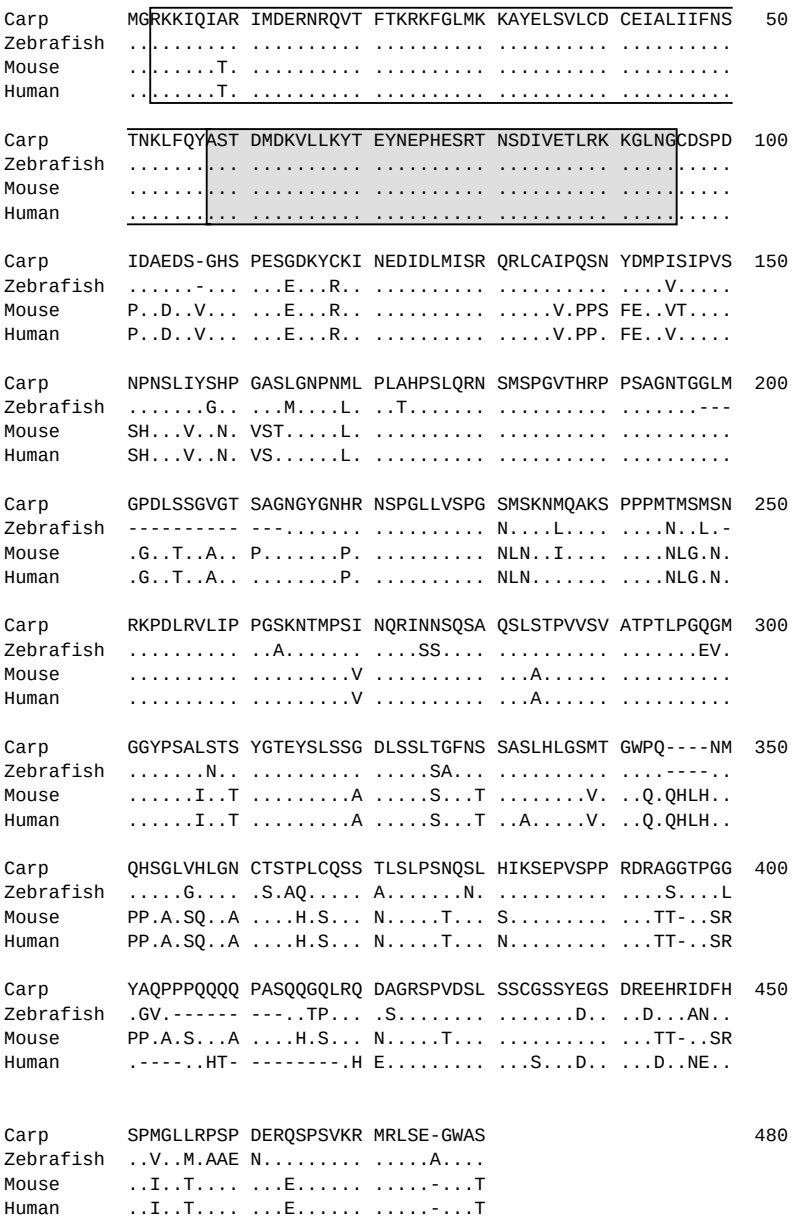

Fig. 6. Comparison of the deduced amino acid sequence of carp MEF2C with those from other vertebrates. Data were obtained from zebrafish (Ticho *et al.* 1996), mouse (Martin *et al.* 1993) and human (McDermott *et al.* 1993). Amino acid residues identical to those of carp are shown by periods, and gaps are represented by dashed lines. Open and shaded boxes indicate the MADS box and MEF2 domain, respectively.

showed maximal expression levels were different for myogenin and MyoD. While the accumulated levels of myogenin transcripts showed two peaks, those of MyoD transcripts had only one. The abundance of the MyoD transcripts increased during development until hatching and thereafter declined, although transcripts were still present in the fast myotomal muscle of 7-month-old juveniles.

The carp myf-5 probe detected one size of transcript, which was present in three-somite embryos (30 h post-fertilization) and was still weakly detectable in 7-month-old juveniles. The strongest signal for myf-5 mRNA was in early embryonic samples, at 30 and 42 h post-fertilization.

Transcripts corresponding to MEF2A were first detected, though faint, in 15-somite embryos (42 h post-fertilization) coincident with the appearance of myogenin mRNA. The expression pattern of MEF2A during development was similar to that of myogenin in terms of having two peaks, with the maximal level of MEF2A mRNA being detected in 1-month-old juveniles. MEF2C was first detected in three-somite embryos, and the level increased gradually during development, although the signal obtained from eyed-stage embryos 77 h post-fertilization was weak. Levels of carp MEF2A and MEF2C transcripts increased after hatching. In contrast to other MRFs, a strong signal for MEF2C was detected in the fast muscles of 7-month-old juveniles.

The mRNA levels of skeletal myosin heavy chain and α-actin were also examined to determine their temporal relationship with those of myogenin, MyoD, myf-5, MEF2A and MEF2C (Fig. 7). The signals of the two muscle-specific mRNAs were first detected in embryos 61 h post-fertilization at approximately the time that the heartbeat was first observed, although the abundance of α-actin transcripts was very low at this time.

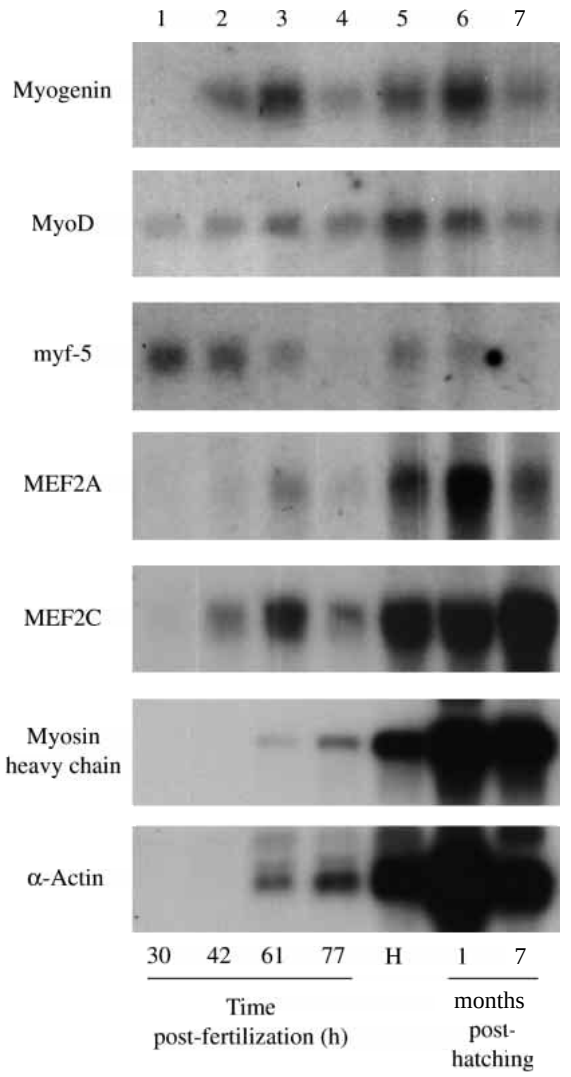

Fig. 7. Northern blot analyses of mRNAs encoding carp myogenin, MyoD, myf-5, MEF2A and MEF2C compared with those for carp skeletal myosin heavy chain and α-actin. Total RNAs (20 μg for MyoD, myf-5, MEF2A, MEF2C, skeletal myosin heavy chain and α-actin and 30 μg for myogenin) were electrophoresed in 1.4 % agarose gels and transferred onto nylon membranes. The membranes were then hybridized with ^{32}P-labelled polymerase chain reaction products of MyoD, myogenin, myf-5, MEF2A, MEF2C, skeletal myosin heavy chain and α-actin. Lanes 1–7 contain total RNAs from whole embryos at 30 h, 42 h, 61 h and 77 h post-fertilization, from whole larvae at hatching (H) and from muscle of juveniles 1 and 7 months post-hatching, respectively.

Discussion

The MyoD family contains four myogenic regulatory factors, all of which contain the bHLH DNA-binding domain. They act at multiple points in the skeletal muscle lineage to establish the skeletal muscle phenotype. In addition to the MyoD family, members of the MEF2 family have been shown to play important roles in the control of muscle-specific gene expression (Molkentin and Olson, 1996). In all known skeletal muscle lineages, myf5/MyoD expression is followed by upregulation of myogenin and of MEF2 family factors, which enhance expression of differentiation genes (Yun and Wold, 1996). The last myogenic regulatory factor to be activated in most muscle types is MRF4, which is expressed until adulthood (Rhodes and Konieczny, 1989).

We have now determined the coding and non-coding regions of the cDNAs from the MyoD and MEF2 families of transcription factors in the common carp. Comparison of the deduced amino acid sequences of carp MRFs with those from other vertebrates revealed that MyoD was more highly conserved than myogenin and myf-5, including in the bHLH DNA-binding domain (see Figs 2–4). It is thought that myogenic activity maps onto two amino acid residues (alanine and threonine) in the centre of the MyoD basic domain and one amino acid residue in the junction region of the first helix of MyoD (Davis and Weintraub, 1992). Black *et al.* (1998) found that the myogenic residues alanine and threonine were required for MyoD to activate transcription synergistically with MEF2, but were not required for interaction with MEF2. Marked differences were observed between the amino acid sequences of carp myogenin, MyoD and myf-5, showing only 73–87 % identity in the bHLH region and 31–47 % identity in the whole coding region. Similar sequence divergence has been reported between myogenin, MyoD and myf-5 in higher vertebrates (Lin *et al.* 1989; Fujisawa-Sehara *et al.* 1990; Edmondson and Olson, 1989; Pinney *et al.* 1988; Saitoh *et al.* 1993; Buonanno *et al.* 1992). A comparison of the predicted amino acid sequences of MEF2A and MEF2C from carp showed 93 % identity in the MADS box and MEF2 domain and 58 % identity in the whole coding region.

The present study has shown differences in the relative timing of expression of MyoD and MEF2 transcription factors during embryogenesis in the carp. It should be noted that, although expression of the MyoD family is specific to skeletal muscle, MEF2 transcripts are also present in nervous and cardiac tissue of zebrafish (Ticho *et al.* 1996). In mammals, genes for MEF2 are expressed in a broad range of cell types, including brain and neural crest cell derivatives as well as skeletal, cardiac and visceral muscle (Edmondson *et al.* 1994). The first MRF to be expressed during somitogenesis in the carp is probably myf-5, since mRNAs for this factor are already present at high levels in three-somite embryos (30 h post-fertilization) (Fig. 8). mRNAs for MEF2C and MyoD can be detected at much lower levels in three-somite embryos, whereas mRNAs for myogenin and MEF2A were not detected until the 15-somite stage (42 h post-fertilization). In contrast, mRNAs for skeletal myosin heavy chain and α-actin were not detected in northern blot analysis until the heartbeat stage (61 h post-fertilization), which is consistent with their expression being a downstream consequence of MRF expression (Fig. 8). The interpretation of our results is complicated by the fact that total RNA was extracted from the whole body of embryos and larvae, whereas myotomal muscle samples were used from juveniles. A comparison with other expression data reveals differences in the transcriptional regulation of the MyoD and MEF2

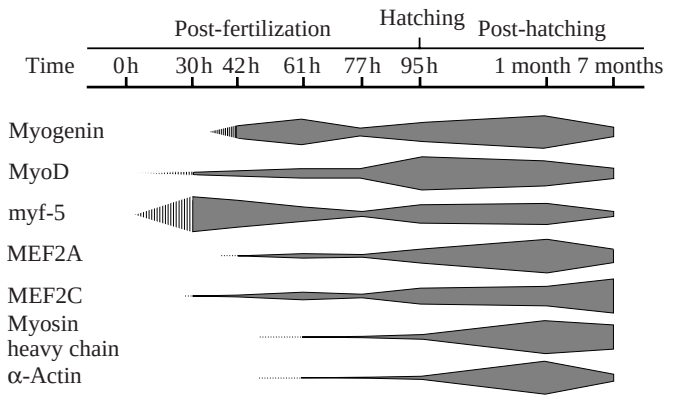

Fig. 8. Expression patterns of mRNAs encoding carp myogenin, MyoD, myf-5, MEF2A and MEF2C compared with those for carp skeletal myosin heavy chain and α-actin.

families between muscle types and species. For example, myf-5 is the first MRF to be expressed in the precursor cells for myotomal muscle in mouse (Ott *et al.* 1991), zebrafish (Weinberg *et al.* 1996) and carp (the present study), but MyoD is the first to be expressed in the quail (Pownal and Emerson, 1992). MyoD expression occurs earlier than myogenin expression in quail and zebrafish (Pownal and Emerson, 1992; Weinberg *et al.* 1996). In contrast, MyoD transcripts are first detected approximately 2 days after myogenin transcripts begin to accumulate in the precursor cells for the myotomal muscle of the mouse (Sassoon *et al.* 1989). In zebrafish, MEF2D is first activated in the presomitic mesoderm, followed by MEF2A and then MEF2C (Ticho *et al.* 1996). MyoD expression occurs at the six-somite stage, and MEF2D and myogenin expression occur at the 10-somite stage in zebrafish (Ticho *et al.* 1996). In contrast, MEF2C transcripts appear first, followed by MEF2A and MEF2D in the mouse (Edmondson *et al.* 1994). In carp, the pattern of accumulation of mRNA for the MyoD family is therefore similar to that described in quail and zebrafish (Ticho *et al.* 1996; Pownall and Emerson, 1992), whereas expression patterns for the MEF2 family are similar to those in the mouse (Molkentin and Olson, 1996). Transcripts of the MEF2 family were expressed in both somatic and cardiac cells in zebrafish (Ticho *et al.* 1996). Since the whole embryonic body and larvae at hatching were used in the northern blot analysis in the present study with carp, it is necessary to determine which cells, somatic or cardiac, express the MEF2 family by *in situ* hybridization.

Andres and Walsh (1996) claimed that myogenin-positive C2C12 myoblasts remained capable of replicating DNA. In contrast, subsequent expression of the cell cycle inhibitor p21 in differentiating myoblasts correlated with the establishment of the postmitotic state (Andres and Walsh, 1996). Later during myogenesis, postmitotic mononucleated myoblasts activated expression of muscle structural proteins such as myosin heavy chain and then fused to form a multinucleated myotube. It seems from these results that muscle-specific genes are upregulated by myogenin. However, myogenin(–/–) mice maintained certain, although decreased, levels of many muscle-specific transcripts (Hasty *et al.* 1993). Forced expression of myogenin or MyoD in 10T1/2 fibroblasts induced MEF2 DNA-binding activity, and it has been shown that MEF2 proteins lack myogenic activity on their own, but are able to act synergistically with myogenic bHLH proteins during myogenic conversion of 10T1/2 fibroblasts in culture. It is thought that the synergy is mediated by direct protein–protein interactions between MEF2 factors and heterodimers formed between myogenic bHLH factors and E proteins (Ludolph and Konieczny, 1995; Molkentin and Olson, 1996). Thus, the overall cascade for expression of myogenic regulatory factors and induction of muscle-specific genes during myogenesis remains unclear.

In mammals, the number of muscle fibres is fixed at birth, and post-natal muscle growth involves the hypertrophy of muscle fibres (Rowe and Goldspink, 1969). The expanding muscle fibres require a source of additional nuclei that is provided by a pool of undifferentiated myogenic stem cells located beneath the basal lamina (Moss and LeBlond, 1971). These myogenic stem cells (satellite cells) are also activated following muscle injury. Following activation, the division products of the myogenic stem cell population express myogenic bHLH proteins (Yablonka-Reuveni and Rivera, 1994; Megeney *et al.* 1996). Muscle growth in the common carp and other fish involves the production of new muscle fibres in addition to fibre hypertrophy (Stickland, 1983; Koumans *et al.* 1993; Johnston *et al.* 1998). The continued expression of members of the MyoD family and MEF2 isoforms in the carp (Fig. 8) presumably reflects activated myogenic cells. Such cells constitute a declining proportion of the total number of muscle nuclei during the larval and juvenile stages as growth proceeds (Koumans *et al.* 1993; Johnston *et al.* 1998). Cornelison and Wold (1997) used a multiplex single-cell reverse-transcription PCR assay to monitor the expression of myogenic regulatory factors in mice following injury and found that activated satellite cells began to express either MyoD or myf-5 first, followed by myogenin and MRF4. MRF4 is one of myogenic regulatory factors in mammals that has been claimed to play an important role in muscle maturation and to maintain the adult muscle phenotype (Hinterberger *et al.* 1991). Although we have been trying to clone carp MRF4, we have not yet obtained any homologous clone. MRF4 has not yet been cloned from any fish species.

The skeletal muscle phenotype can be modified by temperature acclimation in cyprinid fish, including *Cyprinus carpio*. Cold-acclimation results in an increase in myofibrillar ATPase activity in fast muscle fibres (Johnston *et al.* 1975; Sidell, 1980). A minimum of three isoforms of fast skeletal muscle myosin heavy chain have been identified in common carp which are expressed in an acclimation-temperature-dependent fashion, each with different Mg^{2+}-ATPase activities and thermal stabilities (Guo *et al.* 1994; Nakaya *et al.* 1995; Watabe *et al.* 1995*a*; Imai *et al.* 1997; Hirayama and Watabe 1997). We now propose to use *in situ* hybridization to

investigate the expression of MyoD family and MEF2 family transcription factors of carp in relation to both developmental stage and acclimation temperature.

We thank Dr K. Sakai and Dr G. Yoshizaki, Tokyo University of Fisheries, for helping with the sampling of carp embryos. Thanks are also due to Dr T. Kurisaki, The Tokyo Metropolitan Institute of Medical Science, for his invaluable advice on cDNA cloning. S.W. was supported by Grant-in-Aids for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan and from the Asahi Glass Foundation, Japan. Y.H. and K.K. were supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists. I.A.J. was supported by a Royal Society–JSPS exchange programme.

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