*The Journal of Experimental Biology* **198**, 1603–1611 (1995)
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# SMALL-DIAMETER WHITE MYOTOMAL MUSCLE FIBRES ASSOCIATED WITH GROWTH HYPERPLASIA IN THE CARP (*CYPRINUS CARPIO*) EXPRESS A DISTINCT MYOSIN HEAVY CHAIN GENE

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*Accepted 17 March 1995*

## Summary

**A carp myosin heavy chain gene isoform was isolated from a genomic clone, restriction mapped and partially sequenced to reveal the location of various exons. The clone contains a complete gene of approximately 12.0 kb which is half the size of the corresponding mammalian and avian myosin heavy chain genes. The mRNA transcript of this gene, however, is the same size as mammalian and avian striated muscle myosin heavy chain genes (about 6000 nucleotides), illustrating that the difference in size at the genomic level is due to shorter introns. A 169 bp *Nsi*I restriction fragment containing only the 3′ untranslated region of this gene was subcloned and used as an isoform-specific probe to study the expression of this particular isoform. Hybridisation analysis could only detect expression of this myosin heavy chain gene in the white muscle of adult carp that had been subjected to an increased environmental temperature. No expression of this gene was detected in carp under 1 year of age. *In situ* hybridisation demonstrated that expression of this gene is limited to small-diameter white muscle fibres of adult carp, which are thought to be responsible for muscle growth by fibre hyperplasia.**

Key words: myosin heavy chain, muscle, carp, *Cyprinus carpio*, growth.

## Introduction

Growth of skeletal muscle in fish differs from that of mammals and birds in a number of ways. Fish myotomal muscle grows by both fibre hyperplasia and hypertrophy (Greer-Walker, 1983, Stickland, 1983; Weatherley and Gill, 1984) whereas in mammals and birds, increases in the number of fibres stop shortly after embryonic development (Goldspink, 1972). Also, muscle growth rates in fish can vary significantly throughout life depending on factors such as the availability of food or environmental temperature (Loughna and Goldspink, 1985). Furthermore, the muscle of some cyprinid species shows a remarkable adaptability to seasonal temperature changes, with the myofibrillar ATPase activity being 2.8 times greater in cold-acclimated than in warm-acclimated fish (Johnston *et al.* 1975).

Myosatellite cells are thought to be involved in both hypertrophic and hyperplasic growth of muscle. In hypertrophic growth, they fuse with the existing muscle fibres, supplying additional nuclei, whilst in the hyperplasic growth of carp (*Cyprinus carpio*), they have been shown to be the source of the new muscle fibres (Koumans *et al.* 1993*a*,*b*). However, there may be species-dependent mechanisms of fibre hyperplasia since studies in the sonic muscle fibres of the toadfish have shown that increases in fibre number occur by fibre splitting (Fine *et al.* 1993).

Small-diameter fibres (less than 25 $\mu$m in diameter) present in the white myotomal muscle of carp (Akster, 1983; Rowlerson *et al.* 1985) are generally assumed to have arisen from myosatellite cells and hence to be involved in the process of fibre hyperplasia. Such fibres have been shown to differ from the larger-diameter fibres both histochemically and immunohistochemically, suggesting that their myosin heavy chain content (MyoHC) is also different (Rowlerson *et al.* 1985).

Like many other contractile proteins, the MyoHCs are encoded by a highly conserved multigene family (Nguyen *et al.* 1982). There are thought to exist at least eight separate striated muscle *MyoHC* genes in mammals and as many as 31 in chicken (Robbins *et al.* 1986). Gerlach *et al.* (1990) reported that there

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are up to 28 *MyoHC* genes present in the carp. Expression of the various isoforms of the *MyoHC* gene in mammals has been shown to be under both developmental and tissue-specific control, with specific isoforms expressed in the heart, extraocular muscles, different fibre types and at different stages in development (for a review, see Pette and Staron, 1990).

During the regeneration of damaged mammalian skeletal muscle fibres, myosatellite cells proliferate and fuse either into multi-nucleated myotubes or with the ends of damaged muscle fibres (Hinterberger and Barald, 1990). Recruitment of myosatellite cells in muscle regeneration is known to involve the expression of the embryonic and neonatal isoforms of the myosin heavy chain (Sartore *et al.* 1982). Whilst histochemical and immunohistochemical studies suggest that the newly formed small-diameter fibres in carp white myotomal muscle contain a different isoform of the myosin heavy chain from that in the larger fibres (Rowlerson *et al.* 1985), it is not known whether this corresponds to the re-expression of the embryonic/larval isoforms. Isolation and characterisation of the predominant MyoHC isoform expressed in the new small-diameter muscle fibres would provide a valuable tool with which to investigate the mechanisms of fibre hyperplasia in fish growth.

Work in our laboratory has focused on the genes encoding the myosin heavy chain protein in carp. We have previously constructed a carp genomic library and isolated 28 different lambda clones, containing *MyoHC* gene sequences (Gerlach *et al.* 1990). Here we describe the characterisation of one of the carp *MyoHC* isogenes isolated. Using the 3′ untranslated region as a probe in hybridisation studies, the expression of this gene is localised to the small muscle fibres of adult growing carp, which are believed to be involved in post-embryonic fibre hyperplasia.

## Materials and methods

### *Isolation of an isoform-specific carp MyoHC probe*

The isolation of genomic clones containing carp *MyoHC* gene sequences has previously been described (Gerlach *et al.* 1990). DNA sequencing of various restriction fragments from one of these genomic clones, λFG2, allowed the size of the complete gene to be estimated and various exons to be localised (Fig. 1). Nucleotide sequence data from the 3′ end of this *MyoHC* isogene are shown in Fig. 2. It is known that the sarcomeric *MyoHC*s isolated from mammalian and avian species show a high degree of sequence homology in their coding region both between and within species (see, for example, Moore *et al.* 1992). Hence, nucleotide probes for the study of gene expression patterns of individual *MyoHC* isoforms within the same species have to be chosen with great care. Large probes which cover the coding region of the gene have been shown to cross-hybridise to a number of *MyoHC* isogenes within the same species (Eller *et al.* 1989) and are therefore of limited use in determining the expression patterns of an individual *MyoHC* isogene. However, the 3′ untranslated region (3′ UTR) of the gene does show considerable divergence between isogenes, and the 3′ UTRs of *MyoHC* genes from mammals have been used extensively in hybridisation experiments to characterise the expression of the individual isogenes (Loughna *et al.* 1990; Sutherland *et al.* 1991; DeNardi *et al.* 1993).

For the carp FG2 *MyoHC* isogene, a 169 bp *Nsi*I restriction digest product, containing only the 3′ untranslated region sequence (Fig. 2), was subcloned into pBS$^{(+)}$ vector (Stratagene), named FG2UTR and subsequently used as an isoform-specific probe in Northern and *in situ* hybridisation to characterise the expression pattern of this particular isoform.

### *Experimental animals*

Common carp (*Cyprinus carpio* L.), were obtained from a commercial supplier and kept in 110 l tanks of circulating aerated tap water under a 12 h:12 h light:dark photoperiod. Twenty-five adult carp of standard body length (18±2 cm) and mass (120±15 g) were acclimated to 10 °C for a period of 8 weeks. Five fish were killed at this stage and the water temperature was then raised to 28 °C over a period of 7 days and at a rate of 3 °C per day. Once the water temperature had reached 28 °C, four more fish were killed. The water temperature was then maintained at 28 °C and four fish were killed each week for a further 4 weeks. The fish at 10 °C were fed to their maximal intake and this rate of feeding was kept

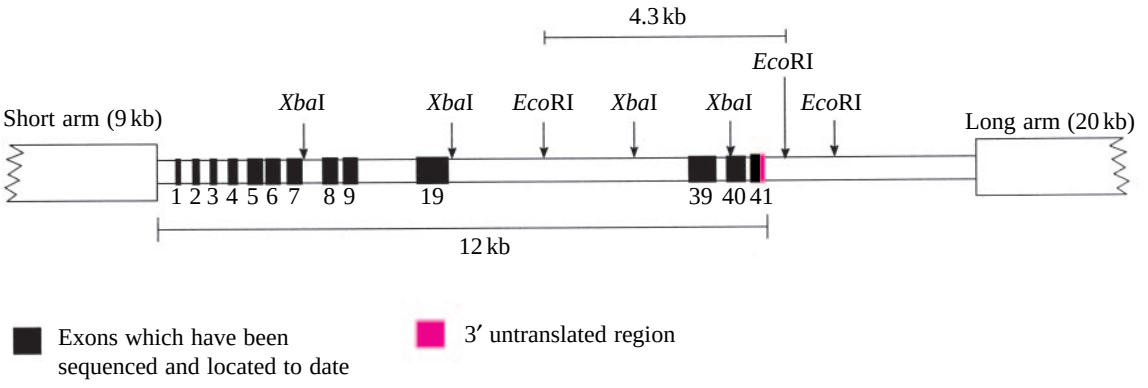


Fig. 1. Restriction map of the genomic clone λFG2 indicating the positions (black) of exons that have been sequenced and the sites of action of endonucleases.


Fig. 2. Nucleotide sequence of the 3′ end of the FG2 carp *MyoHC* gene. The deduced protein sequence of the equivalent to the rat embryonic MyoHC gene (Strehler *et al.* 1986) exons 40 and 41 is given in the one-letter IUPAC code beneath the nucleotide sequence. A putative polyadenylation signal (AATAAA) and the stop codon are highlighted in pink. The blue text corresponds to the probe FG2UTR used in hybridisation studies. (EMBL accession number Z37108.)

constant throughout the whole experiment. Samples of red and white muscle for RNA extractions and muscle blocks for *in situ* hybridisation were taken from each fish following killing by decapitation. Muscle samples were taken from the same region of each fish, the region directly below the end of the dorsal fin on the flank of the fish. Carp fry of varying ages and juvenile carp 12 months of age and 5.5–6 cm in length were also used for RNA extractions.

### *Hybridisation analysis*

Total RNA was extracted by the method described by Chomczynski and Sacchi (1987). In the case of juvenile and adult carp, 0.25–0.75 g of tissue was dissected from individual fish and pooled. For carp fry, five whole fish were used for each sample. Electrophoresis of RNA (30 $\mu$g) was performed in 1.5 % agarose gels made in 3-(*N*-morpholino)-propanesulphonic acid (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× SSC (saline sodium citrate, 1× concentration; 150 mmol $l^{-1}$ NaCl, 15 mmol $l^{-1}$ sodium citrate, pH 7.0) and fixed by baking at 80 °C for 2 h. For slot blotting, total RNA (30 $\mu$g) was applied to 'Zeta Probe' nylon membrane according to the manufacturer's instructions. DNA probes used for hybridisations were labelled by primer extension with $\alpha$-[$^{32}$P]dATP (3000 Ci mmol $^{-1}$, Amersham International). The FG2UTR probe was specifically primed to give a full-length antisense labelled probe and the FGA101 probe was labelled by random priming (Feinberg and Vogelstein, 1984). Hybridisation and subsequent washes were carried out at 20 °C below the calculated melting temperature of the probe duplex (64 °C) according to the method of Church and Gilbert (1984). 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 *hybridisations*

Muscle tissue blocks were fixed in 4 % paraformaldehyde at 4 °C for 4 h and then partially dehydrated by 20 min incubations in three changes of 50 % ethanol and three changes of 70 % ethanol before being processed in an automated wax-embedding machine (Tissue TEK III) as in standard histological procedures. Sections at a thickness of 5 $\mu$m were cut on a sledge microtome, mounted on aminopropyltrimethoxy silane (APTES)-treated slides and dried overnight at 37 °C before storage at room temperature. Prior to hybridisation, sections were dewaxed by heating to 65 °C for 10 min followed by two 10 min incubations in xylene at room temperature. Sections were then rehydrated through a series of graded alcohols and subjected to pre-hybridisation treatment according to the protocol of Zeller and Rogers (1992). Sense and antisense cRNA probes of FG2UTR were labelled with digoxigenin-11-UTP according to the manufacturer's instructions (Boehringer Mannheim). Probes were heated to 85 °C for 2 min, rapidly cooled on ice and then diluted 1:10 in hybridisation buffer (50 % formamide, 0.3 mol $l^{-1}$ NaCl, 20 mmol $l^{-1}$ Tris–HCl, pH 8.0, 10 mmol $l^{-1}$ sodium phosphate, pH 8.0, 10 % dextran sulphate, 1× Denhardt's, 0.5 mg $ml^{-1}$ yeast tRNA) (Denhardt's consists of 0.02 % bovine serum albumin, 0.02 % polyvinylpyrrolodone, 0.2 % Ficoll) to give a final probe concentration of 5 ng $\mu l^{-1}$. The probe mixture (30 $\mu$l) was then added to each section and a siliconised coverslip applied. Sections were incubated for 12 h in a humid chamber at 42 °C. After hybridisation, slides were dipped into 4× SSC in order to remove coverslips and the majority of unbound probe. The slides were then washed twice in 2× SSC (15 min each time at room temperature) followed by RNAase A treatment at a concentration of 100 $\mu$g $ml^{-1}$ in 2× SSC for 30 min at 37 °C. Slides were washed three times in 0.1× SSC (twice for 20 min at 42 °C and once for 10 min at room temperature). For detection of hybridised probe, alkaline-phosphatase-conjugated anti-digoxygenin antibody (1:500 dilution in Boehringer Mannheim buffer 2) with NBT/X-phosphate precipitation was used according to the manufacturer's instructions (Boehringer Mannheim). Unbound antibody was removed with three 5 min washes in 100 mmol $l^{-1}$ Tris–HCl (pH 7.4) with 150 mmol $l^{-1}$ NaCl at room temperature. The colour precipitation reaction was left for 16 h and the sections were dehydrated through graded alcohols, cleared in xylene and mounted with DPX.

## Results

### *The carp MyoHC gene FG2 is approximately half the size of mammalian MyoHC genes*

The genomic clone λFG2 was shown to contain a full *MyoHC* gene from 900 bp 5′ of the translational start site through to the stop codon and 3′ untranslated region (Fig. 1). Characterisation of the 5′ regulatory region of this gene is currently in progress and will be described elsewhere. The nucleotide sequence of exons 1–7 of the gene is deposited in the EMBL sequence data base under the accession number Z37999 whilst the 3′ end nucleotide sequence (Fig. 2) is under the accession number Z37108.

The size of the complete gene is 12 kb and this is approximately half the size of the complete mammalian and chicken striated muscle *MyoHC* genomic sequences published to date, which range from 22.8 to 24.6 kb (Jaenicke *et al.* 1990; Strehler *et al.* 1986; Molina *et al.* 1987; Matsuoka *et al.* 1991). This difference in size at the genomic level is due to the size of the introns. The 13 exons of the gene that have been sequenced to date are all of similar size and show high sequence identity to the equivalent exons in the mammalian and chicken *MyoHC* genes. The introns, however, are consistently shorter. For further evidence supporting this theory of shorter introns, see Fig. 5, where Northern blot analysis shows that the corresponding mRNA transcript is approximately 6 kb in length, the same size as the mammalian and chicken striated muscle *MyoHC* mRNAs.

Of the four complete striated muscle *MyoHC* genomic sequences published to date, only one, the rat embryonic *MyoHC* (Strehler *et al.* 1986), has a final exon coding for five amino acids. In the other three complete sequences, the human β cardiac (Jaenicke *et al.* 1990), the chicken embryonic (Molina *et al.* 1987) and the human α cardiac (Matsuoka *et al.* 1991), these five amino acids are at the end of the previous exon. The carp FG2 *MyoHC* gene is similar to the rat embryonic *MyoHC* in that these five amino acids are also located on a separate exon. It is difficult to postulate the significance of the location of this final exon without additional information about the 3′ exon structure of *MyoHC* genes from a divergent range of species. However, the location of this exon may reflect the evolution of the different MyoHC isoforms from common ancestral genes.

### *Specificity of the carp FG2 MyoHC 3′ UTR probe*

In order to verify that the FG2UTR probe does not cross-hybridise with other members of the carp *MyoHC* gene family, it was necessary to perform hybridisation analysis of carp genomic DNA digested with restriction endonuclease (Fig. 3). As would be expected from the restriction map presented in Fig. 1, the FG2UTR probe hybridises to a single 4.3 kb *Eco*RI fragment. Single genomic restriction fragments of 8.5 kb (*Xba*I) and 3.7 kb (*Pst*I) also hybridised with the FG2UTR probe, further demonstrating that the FG2UTR probe is indeed isogene-specific.

Sequence comparison of the 3′ coding region of the FG2 *MyoHC* isogene with the equivalent regions in other published

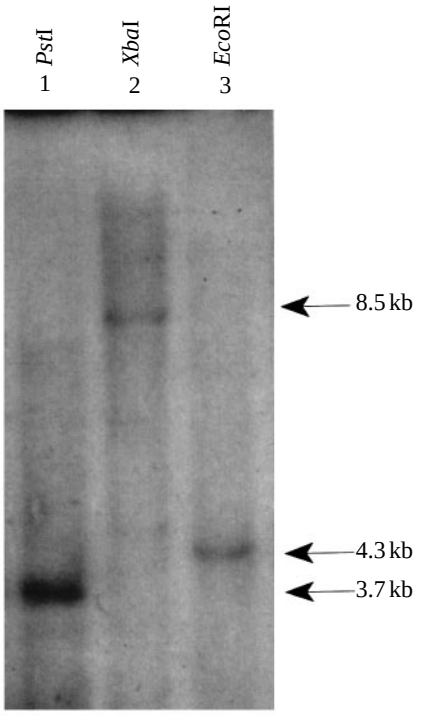


Fig. 3. Genomic Southern hybridisation. Genomic DNA was extracted from adult carp liver as previously described (Gerlach *et al.* 1990). The restriction endonucleases *Pst*I, *Xba*I and *Eco*RI (Boehringer Mannheim) were then used to digest 10 μg samples of genomic DNA to completion. The digested DNA was separated by agarose gel electrophoresis (1% gel in TBE buffer), transferred to 'Zeta Probe' nylon membrane and hybridised with the FG2UTR probe which had been labelled by primer extension with α-[$^{32}$P]dATP. Hybridisation and washing conditions were according to the method of Church and Gilbert (1984) at a temperature of 60 °C. With each restriction enzyme, a single hybridising band was observed, confirming the specificity of the FG2UTR probe.

*MyoHC* genes shows a high degree of sequence homology (Fig. 4). The 3′ UTR of the FG2 *MyoHC* isogene, however, does not show sequence homology with any published *MyoHC* sequence. With the mammalian *MyoHC* genes, it has become apparent that sequence homology exists within the 3′ UTRs of equivalent isoforms in different species. The 3′ UTR of the rat fast 2A *MyoHC* gene (DeNardi *et al.* 1993) is highly homologous to the 3′ UTR of the human fast 2A *MyoHC* gene (Ennion *et al.* 1995). Since the 3′ UTR of the carp FG2 *MyoHC* isogene does not show any homology with any other published *MyoHC* isogene, it is not possible to consider it in the context of the nomenclature of myosin heavy chain genes adopted for mammals.

### *The expression of the FG2 MyoHC gene is restricted to the white muscle of adult carp acclimated to a warm temperature*

The use of the isogene-specific FG2 3′ UTR probe in Northern hybridisation analysis demonstrated the dynamic nature of *MyoHC* isogene expression in carp subjected to changes in environmental temperature. Expression of the FG2

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FG2              EEQANTHLSRYRKVQHELEEAQERADIAESQVNKLRAKSRD_AGK____SKDEE
1.HUMANEMB       DEQANAHLTKFRKAQHELEEAEERADIAESQVNKLRAKTRDFTSSRMVVHESEE
2.RATEMB         DEQANVHLTKFRKAQHELEEAEERADIAESQVNKLRAKTRDFTSSRMVVHESEE
3.HUMANβ         EEQANTNLSKFRKVQHELDEAEERADIAESQVNKLRAKSRDIGTK____GLNEE
4.BABOONβ        EEQANTNLSKFRKVQHELDEAEERADIAESQVNKLRAKSRDIGTK____GLNEE
5.RATβ           EEQANTNLSKFRKVQHELDEAEERADIAESQVNKLRVKSRDIGAK____GLNEE
6.HUMANα         EERANTNLSKFRKVQHELDEAEERADIAESQVNKLRAKSRDIGAK____KMDEE
7.RATα           EEQANTNLSKFRKVQHELDEAEERADIAESQVNKLRAKSRDIGAK__QKMHDEE
8.RABBITα        EEQANTNLSKFRKVQHELDEAEERADIAESQVNKLRAKSRDIGAK__QKMHDEE
9.RATEOM         EEQANTQLSKFRKVQHELEEAEERADIAESQVNKLRFKSRD_________KMEE
                  ***** *   ** **** ** ************** * *          **
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Fig. 4. Comparison of the 3′ coding region of FG2 with other *MyoHC* genes. Amino acid sequences are given in the one-letter IUPAC code. *MyoHC* genes with which FG2 was compared and associated accession numbers are as follows: (1) human embryonic (X13988); (2) rat embryonic (X04267); (3) human $\beta$ cardiac (M30605); (4) baboon $\beta$ cardiac (M19932); (5) rat $\beta$ cardiac (X15939); (6) human $\alpha$ cardiac (D00943); (7) rat $\alpha$ cardiac (X15938); (8) rabbit $\alpha$ cardiac (K011867); (9) rat extrocular isoform (M28654). An asterisk indicates amino acid residues conserved in all the sequences.

*MyoHC* isogene was only detected in adult white muscle of carp that had been exposed to a warm (28 ˚C) environmental temperature. No expression was observed in white muscle from carp at 10 ˚C (Fig. 5, lanes 3 and 4), or up to 1 week at 28 ˚C (lane 5). Strong expression of the FG2 MyoHC isoform was detected through weeks 2, 3 and 4 at 28 ˚C and a slightly weaker expression by week 5. To correlate the expression of the FG2 *MyoHC* isogene with levels of muscle tissue mRNA,

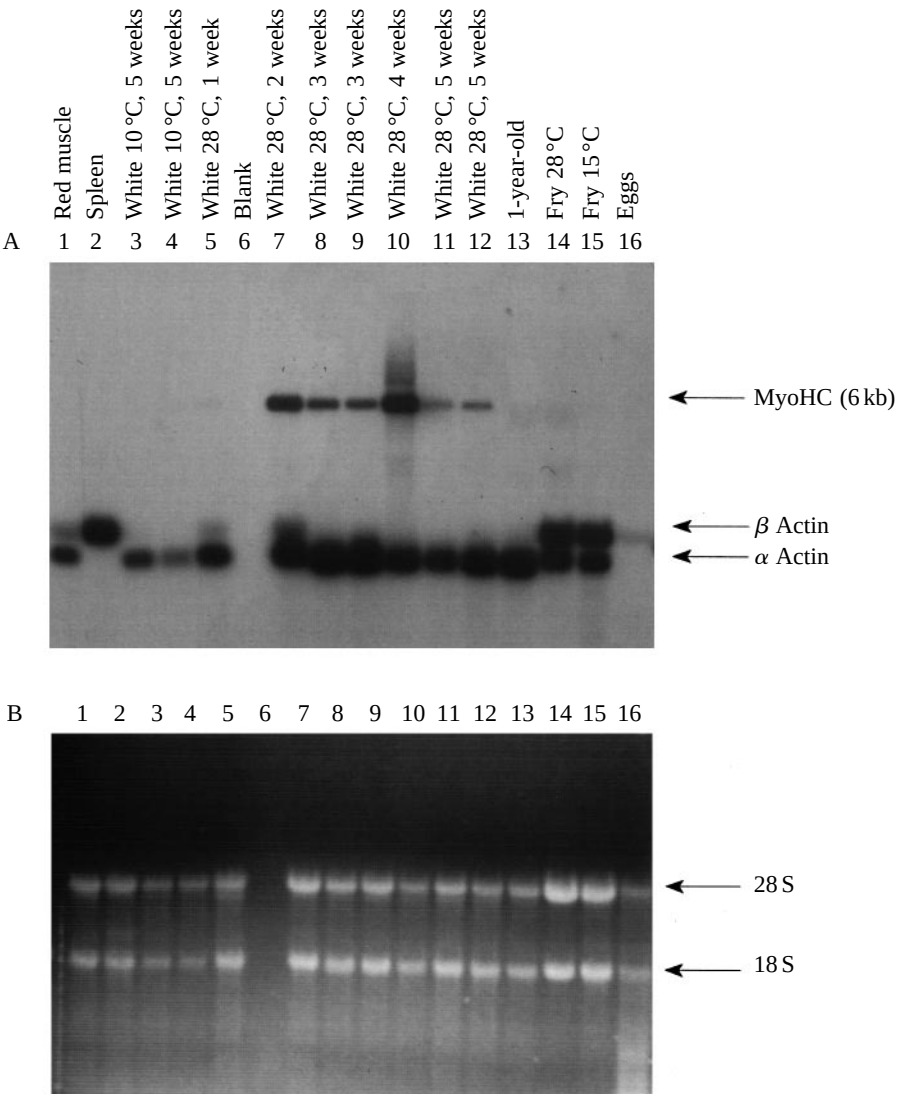


Fig. 5. Northern blot analysis. Total RNA (30 $\mu$g) from carp was separated by electrophoresis, transferred to nylon membrane and hybridised sequentially with the carp *MyoHC* gene probe FG2UTR and the carp actin probe FGA101 (Gerlach *et al.* 1990). Lanes contain RNA from the pooled muscle samples of a number of fish as indicated below. (A) The membrane was hybridised under high-stringency conditions with the probe FG2UTR and the blot exposed to X-ray film at −70 ˚C for 1 week. Subsequently, the membrane was stripped and reprobed under high-stringency conditions with the carp actin probe FGA101. The blot was exposed to X-ray film at −70 ˚C for 72 h and superimposed on the autoradiograph of the FG2UTR hybridisation. (B) Ethidium bromide staining of the original RNA agarose gel. Lanes are as follows. (1) Red muscle from four carp acclimated to 28 ˚C for 5 weeks. (2) Carp spleen. (3) White muscle from three carp acclimated to 10 ˚C for 5 weeks. (4) White muscle from two carp acclimated to 10 ˚C for 5 weeks. (5) White muscle from four carp acclimated to 28 ˚C for 1 week. (6) Blank lane, no RNA. (7) White muscle from four carp acclimated to 28 ˚C for 2 weeks. (8) White muscle from two carp acclimated to 28 ˚C for 3 weeks. (9) White muscle from two carp acclimated to 28 ˚C for 3 weeks. (10) White muscle from four carp acclimated to 28 ˚C for 4 weeks. (11) White muscle from two carp acclimated to 28 ˚C for 5 weeks. (12) White muscle from two carp acclimated to 28 ˚C for 5 weeks. (13) White muscle from three juvenile (1-year-old) carp. (14) Five whole 60-day-old carp fry, 1.2 cm long, acclimated to 28 ˚C for 2 weeks. (15) Five whole 60-day-old carp fry, 1.2 cm long, acclimated to 15 ˚C for 2 weeks. (16) Unhatched carp eggs 24 h after fertilisation.

the membranes were stripped and rehybridised with FGA101 (Gerlach *et al.* 1990), a carp actin probe that hybridises with both isoforms of actin ($\alpha$ and $\beta$). When compared with the level of actin expression in carp which had been maintained at 28 °C for more than 1 week, the level of actin mRNA expression in carp sampled at 10 °C and in the carp maintained at 28 °C for 1 week was lower. However, the level of actin expression did not correlate with the amount of FG2 *MyoHC* mRNA expression in carp that had been at 28 °C for more than 1 week (Fig. 5, lanes 7–12). Expression of the FG2 MyoHC isoform was not detected in carp red muscle, 1-year-old carp, carp fry of various ages or unhatched carp eggs (Figs 5A, 6). Hybridisation of the carp actin probe FGA101 and ethidium bromide staining of the RNA gel (Fig. 5B) confirm the integrity of the RNA samples.

### *Expression of the FG2 MyoHC isoform is localised to small-diameter white muscle fibres*

*In situ* hybridisation experiments using sense and antisense labelled FG2UTR probes revealed that the FG2 MyoHC isoform is expressed in a specific population of white muscle fibres in fish that had been acclimated to a warm temperature (Fig. 7). The fibres shown to be expressing the FG2 MyoHC isoform in the warm-acclimated carp were less than 25 $\mu$m in diameter (Fig. 7B,D). These small-diameter fibres were also present in the cold-acclimated fish (Fig. 7E), but in these fish they were far fewer in number and no expression of the FG2 MyoHC isoform could be detected by either *in situ* hybridisation or Northern blot analysis. This suggests that the FG2 MyoHC isoform is only expressed in adult carp when they are in a growth phase.

## Discussion

The results obtained in this study by *in situ* hybridisation with the FG2UTR probe (Fig. 7) show that the small white muscle fibres express a *MyoHC* gene (FG2) that is not expressed in the red muscle fibres or in the large fibres of the white muscle. This and the fact that a clear boundary was always observed between the fibres expressing the FG2 isoform and surrounding fibres is supportive to the theory that, in carp, the myosatellite cells are the source of the new small-diameter muscle fibres. Recently, Rowlerson *et al.* (1995) have shown that the small-diameter white muscle fibres of the gilthead seabream *Sparus aurata* have a high mitotic activity, which also indicates that their origin is from myosatellite cells. The carp FG2 *MyoHC* gene is apparently expressed transiently when the small fibres are induced to grow. However, it cannot be considered as an embryonic or neonatal MyoHC equivalent isoform since hybridisation analysis (Figs 5, 6) showed that it is not expressed in juvenile carp, carp fry and unhatched carp eggs.

Mosaic white muscle containing both large- and small-diameter fibres has also been described in the sea bass *Dicentrarchus labrax* (Scapolo *et al.* 1988). In this species, the small-diameter fibres in the mosaic white muscle of the larvae have different histochemical and immunohistochemical properties from the small-diameter fibres present in adult fish. Scapolo and coworkers interpreted these results as an indication of different mechanisms of fibre hyperplasia in adult and young fish. Therefore, small fibres present in adult carp arising from myosatellite cells would not necessarily show expression of embryonic or neonatal MyoHC equivalents.

As it is likely that the source of the new muscle fibres expressing the FG2 gene is the myosatellite cells (Koumans *et al.* 1993*a*,*b*), then the pattern of MyoHC isoform expression during myosatellite cell recruitment in carp muscle hyperplasic growth would appear to differ from that in myosatellite cell

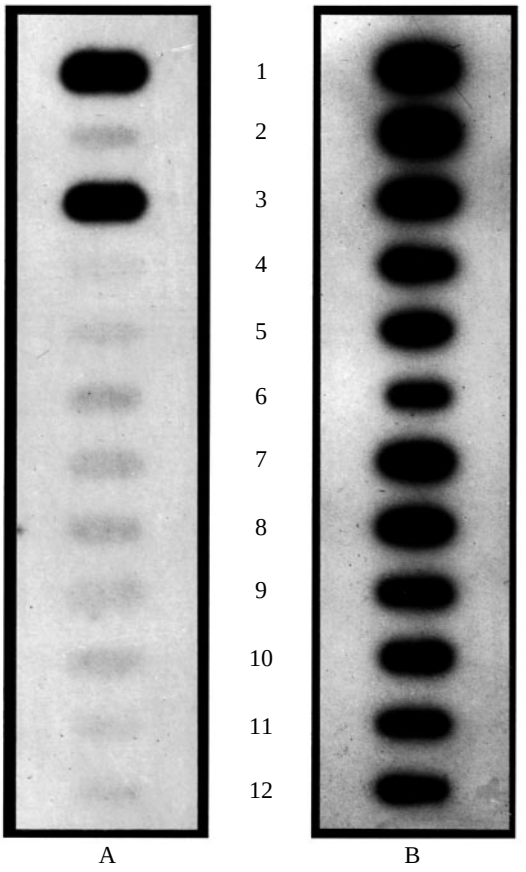


Fig. 6. Slot blot hybridisation of carp total RNA to the probes FG2UTR and FGA101. The RNA loaded on each slot (30 $\mu$g) was extracted from the pooled muscle samples of three fish. The membrane was hybridised under high-stringency conditions with FG2UTR and exposed to X-ray film for 24 h (A). Subsequently, the membrane was stripped of bound probe and rehybridised with the carp actin probe FGA101 (B, exposure 24 h). Lanes are as follows. (1) Deep white muscle from adult carp (3 years old, 16.2–17.5 cm in length) acclimated to 28 °C for 5 weeks. (2) Red muscle from adult carp (same fish as in slot 1). (3) Superficial white muscle from adult carp (same fish as in slot 1). (4) Adult carp spleen. (5) Whole heart from adult carp. (6) 20-day-old carp fry (five whole fish). (7) 12-day-old carp fry (five whole fish). (8) 8-day-old carp fry (five whole fish). (9) 4-day-old carp fry (five whole fish). (10) 2-day-old carp fry (five whole fish). (11) Red muscle from adult carp (3 years old, 16.0–18.0 cm in length) acclimated to 10 °C for 5 weeks. (12) White muscle from adult carp (same fish as in slot 11).

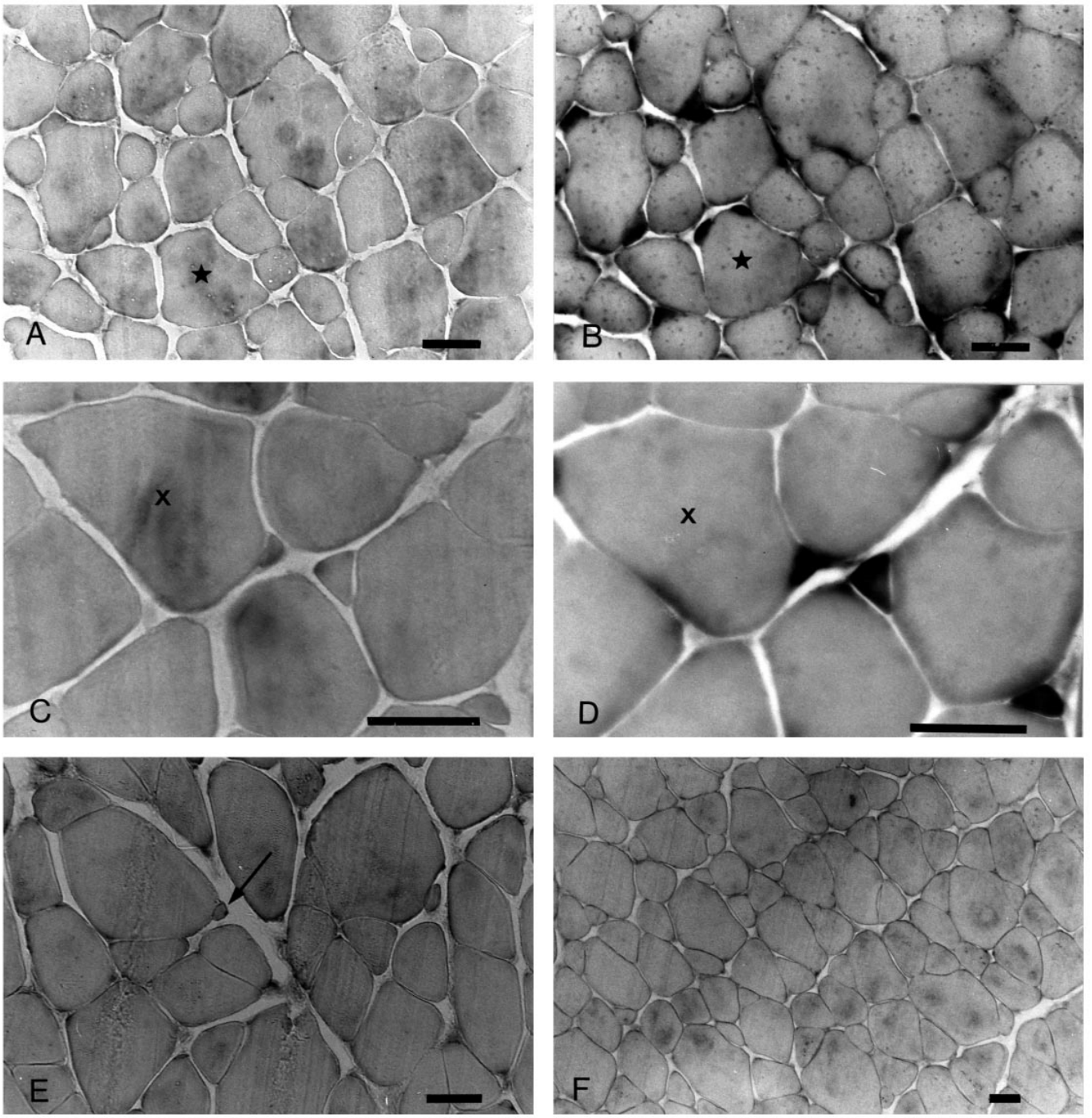


Fig. 7. *In situ* hybridisation using the FG2UTR probe on muscle sections from warm- and cold-acclimated carp. A and B show transverse sections of white muscle from adult carp exposed to 28 °C for 2 weeks. The star (★) indicates the same muscle fibre in serial sections. C and D show transverse sections of white muscle from adult carp exposed to 28 °C for 5 weeks. The cross (×) indicates the same muscle fibre in serial sections. A and C correspond to the sense labelled FG2UTR probe. B and D correspond to the antisense labelled FG2UTR probe. E and F show transverse sections of white muscle fibres from adult carp kept at 10 °C for 5 weeks. Both E and F correspond to the antisense labelled FG2UTR probe. Scale bars, 50 μm.

recruitment during muscle regeneration in mammals, since in mammals the embryonic and neonatal MyoHC isoforms are re-expressed (Sartore *et al.* 1982). Thus, the carp FG2 *MyoHC* gene is best regarded as a 'growth isoform gene' for which a mammalian equivalent has not been described.

That there is a *MyoHC* gene which is expressed during the growth phase of the small-diameter white muscle fibres in carp and that this gene does not appear to be expressed at any other time are interesting observations highlighting the differences between muscle growth in fish and in other species. The

requirement for the expression of a growth-related isoform in fish, or indeed for the expression of embryonic/neonatal MyoHC isoforms during early muscle development, is unresolved. According to the so-called 'functional' hypothesis (Gros and Buckingham, 1987), the differential expression of various MyoHC isoforms throughout development and in the different fibre types of the adult are consequences of various functional demands on the molecule dictating the evolution of a multigene family by selective pressure. Such functional demands may possibly reside in the S1 region of the molecule, where differences in primary structure could affect its actin binding properties and ATPase activity. Alternatively, the functional demands that dictate evolutionary pressure for multiple MyoHC isoforms may reside in the light meromyosin (LMM) region, where differences in structure may be required for the correct construction of the thick filament in particular cellular environments (Taylor and Bandman, 1989). Thus, if the expression of the carp FG2 MyoHC isoform in the small-diameter newly developing white muscle fibres is driven by functional demands, then one would hypothesise that properties in the LMM that facilitate the correct formation of thick filaments could be important since it is difficult to envisage changes in ATPase activity in such a population of fibres affecting the locomotory ability of the fish.

An alternative to the 'functional' hypothesis is that isoform polymorphism confers regulatory advantages at the level of gene expression rather than controlling changes in protein function. That is to say, although the MyoHC isoforms themselves may be very similar in their functional properties, they differ significantly in the regulatory regions of their genes. The cellular environment is probably sufficiently different in the newly developing or growing muscle fibres to necessitate the existence of isogenes that differ in their regulatory regions. Thus, it may be necessary to build the initial myofibrillar infrastructure using a MyoHC isoform that is transiently but strongly expressed. Thereafter, this MyoHC isoform can be readily exchanged for the molecules of the adult type genes. A study of the promoter region of the different *MyoHC* isogenes should shed light on the developmental expression of such isoforms of the *MyoHC* gene family.

Fundamental biological processes such as growth and adaptation can often be studied better in non-mammalian species such as fish. Certainly the use of gene probes to determine the switches in gene expression that initiate the phenotypic changes will enable the cellular mechanisms to be elucidated at a fundamental level.

We are grateful to Mr T. Gysbers for his preparation of photographic material. This work was supported by grants to Professors Goldspink and Butterworth from the SERC (S.E.) and the NERC (L.G.).

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