A STUDY OF THE MYOSIN HEAVY CHAIN GENE FAMILY IN THE CARP

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ABSTRACT

Skeletal muscle has a striking potential for plasticity with an inherent ability to adapt to altered functional demands. The expression of various isoforms of the myosin heavy chain (MyoHC) plays a central role in facilitating these adaptive changes by conferring changes in contractile characteristics. In contrast to mammals, very little is known about the MyoHC isoforms and their genes in fish. This study aimed to characterise the family of MyoHC isogenes in the carp (*Cyprinus carpio*).

Using genomic clone analysis and 3' RACE-PCR, the 3' untranslated regions of seven separate carp MyoHC isogenes (arbitrarily named types 1 to 7) were isolated and used to characterise the expression patterns of individual MyoHC isoforms. Northern blot analysis demonstrated that the carp MyoHC gene family is developmentally regulated and that their expression is also determined by environmental temperature. Two isoforms, types 1 and 5, are expressed in both adult and immature carp and three, types 2, 3 and 4, are expressed exclusively in immature carp. *In situ* hybridisation localised the expression of the type 2 MyoHC isoform to the developing pink muscle fibre layer in fry and 12 month old carp and demonstrated that distinct isoforms of the MyoHC are expressed in the red and white muscle fibres types. Types 6 and 7 MyoHC isoforms were shown to be expressed exclusively in the white muscle fibres of adult carp which had been acclimated to a warm (28°C) temperature. The expression of the type 7 MyoHC gene was localised, by *in situ* hybridisation, to the small diameter (10-25μm) white muscle fibres which are thought to be involved in fibre hyperplasia. The size of this gene at the genomic level was shown to be half the size of mammalian MyoHC isoforms and this difference in size was attributed to shorter introns.
I would like to express my gratitude to Professor Geoffrey Goldspink for providing me with the opportunity to carry out the research described in this thesis and for his continuing advice and encouragement. I am also grateful to Dr Thomas Jaenicke who provided valuable guidance during the initial stages of my work and continued to be an expert source of information. Thanks must also go to Dr Tania Webb for her advice on RACE PCR. Finally, special thanks must go to my parents for their constant support, understanding and encouragement during my time as a student.

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<table>
<thead>
<tr>
<th>CONTENTS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title Page</td>
<td>1</td>
</tr>
<tr>
<td>Abstract</td>
<td>2</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>3</td>
</tr>
<tr>
<td>Contents</td>
<td>4</td>
</tr>
<tr>
<td>List of Figures and Tables</td>
<td>8</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>12</td>
</tr>
<tr>
<td><strong>Chapter 1: General Introduction</strong></td>
<td>13</td>
</tr>
<tr>
<td>1.1 Introduction</td>
<td>14</td>
</tr>
<tr>
<td>1.2 Muscle fibre structure</td>
<td>14</td>
</tr>
<tr>
<td>1.3 Muscle fibre typing</td>
<td>16</td>
</tr>
<tr>
<td>1.4 Polymorphism of contractile proteins</td>
<td>18</td>
</tr>
<tr>
<td>1.4.1 Myosin</td>
<td>19</td>
</tr>
<tr>
<td>1.4.2 Isoforms of the myosin light chain.</td>
<td>21</td>
</tr>
<tr>
<td>1.4.3 Isoforms or the myosin heavy chain.</td>
<td>22</td>
</tr>
<tr>
<td>1.4.3.1 Structure of the MyoHC gene</td>
<td>23</td>
</tr>
<tr>
<td>1.4.3.2 Adult skeletal myosin heavy chain isoforms.</td>
<td>26</td>
</tr>
<tr>
<td>1.4.3.3 Developmental myosin heavy chain isoforms</td>
<td>29</td>
</tr>
<tr>
<td>1.4.3.4 Cardiac myosin heavy chain isoforms.</td>
<td>32</td>
</tr>
<tr>
<td>1.5 Factors modulating myosin heavy chain isoform expression.</td>
<td>33</td>
</tr>
<tr>
<td>1.5.1 Innervation</td>
<td>33</td>
</tr>
<tr>
<td>1.5.2 Chronic electrical stimulation.</td>
<td>35</td>
</tr>
<tr>
<td>1.5.3 Mechanical stimuli.</td>
<td>37</td>
</tr>
</tbody>
</table>
1.5.3.1 Work overload. 37
1.5.3.2 Stretch and disuse. 38
1.5.4 Thyroid hormone. 39
1.5.5 Transcription factors. 41

1.6 Aims 42

Chapter 2: Isolation of specific probes for individual isoforms of the carp myosin heavy chain gene. 43

2.1 Introduction. 44

2.2 Materials and methods. 46

2.2.1 Genomic bacteriophage λ clones. 46
2.2.2 Preparation of bacteriophage λ DNA. 46
2.2.3 Restriction endonuclease mapping of λ clones. 47
2.2.4 Transfer of DNA to nylon membranes. 47
2.2.5 Radionucleotide labelling of DNA probes. 48
2.2.6 Southern hybridisations. 48
2.2.7 Subcloning of DNA fragments derived from λ clones. 49
2.2.8 Preparation of plasmid DNA. 49
2.2.9 Sequencing of plasmid DNA. 49

2.3 Results 50

2.3.1 Isolation of the 3' end of the myosin heavy chain gene cloned into λFG2. 50

2.3.2 The use of λFG2 derived subclones to isolate 3' end containing subclones from other myosin heavy chain isoforms. 54

2.3.3 Sequence data analysis 59

2.3.4 Generation of plasmid constructs 62
2.4 Discussion.

Chapter 3: Amplification of myosin heavy chain 3' untranslated regions by RACE PCR

3.1 Introduction

3.2 Materials and methods.
  3.2.1 Oligonucleotides.
  3.2.2 cDNA synthesis.
  3.2.3 PCR amplification.
  3.2.4 Cloning of PCR products.

3.3 Results
  3.3.1 Optimisation of PCR conditions.
  3.3.2 Preliminary PCR amplifications.
  3.3.3 PCR amplifications of cDNA from various carp muscle samples.
  3.3.4 Analysis of sequence data.
  3.3.5 Summary of MyoHC isoforms isolated.

3.4 Discussion.

Chapter 4: Characterisation of the expression patterns of carp MyoHC genes during post hatching development.

4.1 Introduction.

4.2 Materials and methods.
  4.2.1 Experimental animals.
  4.2.2 Extraction of RNA.
  4.2.3 Electrophoresis of RNA.
  4.2.4 Northern blotting of RNA.
4.2.5 Slot blotting of RNA.

4.2.6 Labelling of DNA probes for northern hybridisations.

4.2.7 Hybridisation conditions for Northern blots.

4.2.8 In situ hybridisations.

4.2.9 Synthesis of labelled cRNA.

4.2.10 Hybridisation and washing conditions.

4.2.11 Detection of bound probe on in situ hybridisation sections.

4.3 Results

4.3.1 Northern hybridisation analysis of the expression patterns of seven MyoHC genes during post hatching development.

4.3.2 In situ hybridisation

4.3.2.1 Expression pattern of the type 2 MyoHC isoform.

4.3.2.2 In situ hybridisation analysis with the human β cardiac MyoHC derived probe HIIATP.

4.4 Discussion.

Chapter 5: An investigation of the temperature dependent expression of carp MyoHC genes.

5.1 Introduction.

5.2 Materials and methods.

5.2.1 Experimental animals.

5.2.2 Temperature acclimation experiment 1.

5.2.3 Temperature acclimation experiment 2.

5.2.4 RNA extractions and Northern hybridisations.

5.2.5 In situ hybridisations.

5.2.6 Analysis of the clone FG1706.
5.3 Results.

5.3.1 Analysis of the clone FG1706. 138

5.3.2 Temperature acclimation experiment 1. 142

5.3.3 Temperature acclimation experiment 2. 147

5.4 Discussion. 157

Chapter 6: General discussion. 163

6.1 Summary of results 164

6.2 Do the seven 3' MyoHC sequences isolated correspond to seven separate genes? 165

6.3 Possible mechanisms involved in creating a MyoHC multigene family 167

6.4 Sequence comparisons between the 3'UTR sequences of MyoHC isoforms from a variety of species 168

6.5 The biological relevance of multiple MyoHC isoforms. 173

6.6 Future studies. 174

Appendix 1: Plasmid constructs for the generation of DNA and cRNA probes. 176

Appendix 2: Publications 186

References 187
LIST OF FIGURES AND TABLES.

Chapter 1:

Fig 1.1 Schematic representation of the sarcomere 15
Fig 1.2 Schematic representation of the myosin molecule. 19
Fig 1.3 Structure of the rat embryonic MyoHC gene. 24
Fig 1.4 Electrophoretic mobilities of MyoHC protein isoforms in rat. 27
Table 1.1 Mammalian striated MyoHC isoforms characterised to date. 30

Chapter 2:

Fig 2.1 Restriction maps of λFG2 and associated subclones. 51
Fig 2.2 Restriction digests of λFG2. 52
Fig 2.3 Nucleotide sequence of the 3' end of λFG2. 53
Fig 2.4 Nucleotide sequence obtained from the 3' end of λFG19. 56
Fig 2.5 Nucleotide sequence obtained from the 3' end of λFG17. 57
Fig 2.6 Nucleotide sequence obtained from the 3' end of λFG16. 58
Fig 2.7 Multiple alignment of carp MyoHC gene sequences. 59
Fig 2.8 Comparison of carp MyoHC amino acid sequences with other species. 61
Table 2.1 Fragments of λ genomic clones which hybridise to probes containing exon 40 from λFG2. 54

Chapter 3:

Fig 3.1 Schematic representation of the RACE PCR method used. 71
Fig 3.2 Optimisation of Mg²⁺ concentration for RACE PCR. 72
Fig 3.3 PCR reactions on total RNA and mRNA (PolyA⁺). 74
Fig 3.4 PCR products of cDNA samples from various carp muscle samples.

Fig 3.5 Dendrogram of PCR product sequence alignments.

Fig 3.6 Nucleotide sequence alignment of MyoHC PCR products.

Fig 3.7 Deduced amino acid sequence alignment of PCR products.

Table 3.1 Tissue types used for cDNA preparation.

Table 3.2 Sequence identity between the 3' ends of seven carp MyoHC isoforms.

Chapter 4:

Fig 4.1 Muscle types of the carp.

Fig 4.2 Northern hybridisation of carp RNA to the probes, WHITEC1, FG17UTR and HIIATP.

Fig 4.3 Northern hybridisation of carp RNA to the probe FRY2811.

Fig 4.4 Northern hybridisation of carp RNA to the probe EGGS22.

Fig 4.5 Northern hybridisation of carp RNA to the probe FG19UTR.

Fig 4.6 Slot blot hybridisation of carp RNA to the probes FG2UTR.

Fig 4.7 In situ hybridisation on 14 month old carp using 35S labelled FG17UTR probe.

Fig 4.8 In situ hybridisation on carp fry using 35S labelled FG17UTR probe.

Fig 4.9 In situ hybridisation on 14 month old carp using digoxigenin labelled FG17UTR probe.

Fig 4.10 In situ hybridisation on adult carp using 35S labelled HIIATP probe.

Fig 4.11 In situ hybridisation on adult carp using digoxigenin labelled HIIATP probe.

Table 4.1 Northern hybridisation temperatures.
Table 4.2  Expression patterns of seven carp MyoHC isoforms.

Chapter 5:

Fig 5.1  Restriction map and partial sequence of the subclone FG1706.

Fig 5.2  Northern hybridisation of carp total RNA with the probe FG1706.

Fig 5.3  Northern hybridisation of RNA extracted from carp subjected to increasing temperature.

Fig 5.4  Northern hybridisation of RNA extracted from carp subjected to decreasing temperature.

Fig 5.5  Hybridisation of the probes FG2UTR and EGGS24 with carp RNA from temperature acclimation experiment 2.

Fig 5.6  Hybridisation of the FG19UTR probe with RNA from carp maintained at warm and cold temperatures.

Fig 5.7  In situ hybridisation on warm and cold acclimated carp using digoxigenin labelled FG2UTR probe.

Table 5.1  Water temperatures for groups A and B in temperature acclimation experiment 1.

Chapter 6:

Figure 6.1  Dendrogram alignment of MyoHC 3'UTR sequences from a variety of species.

Table 6.1  Conserved sequence motifs in MyoHC 3'UTRs
**ABBREVIATIONS.**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>APTES</td>
<td>3-aminopropyltriethoxysilane</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>C</td>
<td>cytidine</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine 5' triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytidine 5' triphosphate</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>G</td>
<td>guanine</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobasepairs</td>
</tr>
<tr>
<td>MyoHC</td>
<td>myosin heavy chain</td>
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<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque forming unit</td>
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<tr>
<td>RACE</td>
<td>rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>T</td>
<td>thymidine</td>
</tr>
<tr>
<td>Tris HCl</td>
<td>tris (hydroxymethyl) aminomethane hydrochloride</td>
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</tbody>
</table>
Chapter 1
General Introduction.
1.1 Introduction

Three main classes of muscle have emerged during the evolution of vertebrates: smooth, cardiac and skeletal. These main classes are adapted for specific contractile functions, the extremes of which are exemplified by skeletal muscle, which is under voluntary neural control and cardiac muscle, which is an electrically and mechanically coupled tissue with an inherent genesis of contractile activity. In addition to this gross tissue adaptation of muscle, each class of muscle also has an intrinsic adaptability and is capable of changing its contractile characteristics in response to various functional demands imposed during development and also during adult life (Goldspink 1985). This introduction will deal only with aspects of myosin isoform expression in the context of skeletal and cardiac muscle adaptability.

1.2 Muscle fibre structure

Skeletal muscle is composed of bundles of relatively enormous multinucleated cells, known as muscle fibres, which can be up to 100μm in diameter and many centimetres in length. The sarcoplasm (cytoplasm) of the muscle fibre is densely packed with threadlike contractile structures, about 1μm in diameter, known as myofibrils. Although the myofibrils extend lengthwise within the muscle fibre, their prominent alternating dark and light bands give the whole muscle fibre a striped or striated appearance. These striations arise from a highly organised arrangement of subcellular structures within the myofibrils known as sarcomeres which are the molecular structures responsible for muscle contraction (reviewed by Craig, 1986).

Each sarcomere is bordered by dark narrow (0.1μm) structures known as the Z lines, which mainly consist of the protein α-actinin (Schollmeyer et al., 1973). The Z line bisects a lighter band of about 1μm long named the I band because it is isotropic in polarised light. Each I band is shared by two sarcomeres (Figure 1.1). At the centre of the sarcomere is a dark 1.6 μm long A band, so called because it appears anisotropic in polarized light. The A band itself is bisected by a less dense region called the H zone, the centre of which
contains a still lighter region called the pseudo H zone or the bare zone and this itself shows a region of higher density called the M line.

Electronmicroscopy on ultra thin muscle sections (Huxley, 1957) allowed the banding pattern of the muscle fibre to be explained in terms of a precisely ordered arrangement of contractile filaments within the myofibrils (Figure 1.1). Each sarcomere consists of two sets of parallel and partly overlapping filaments known as the thick and thin filaments which are both polymers of non covalently associated protein molecules. The thick filaments (about 15 nm in diameter) consist of approximately 300 myosin molecules each (Lamvik, 1978; Reedy et al., 1981) and extend across the full length the A band. Each half I band contains an array of thin filaments (about 8 nm in diameter), which are composed mainly of F actin with tropomyosin and the troponin complex associated. The thin filaments extend from anchoring points in the Z lines to interdigitate with the thick filaments within the A band. The H zone is less dense than the rest of the A band owing to the absence of overlapping thin filaments in this area.

Contraction of muscle is brought about as a result of the sliding of the thick and thin filaments past each other, producing greater overlap of the filaments without a change in the length of the filaments themselves. (Huxley, H.E. and Hanson, 1954; Huxley, A.F. and
Niedergerke, 1954). This sliding of filaments across each other is facilitated by projections from the myosin molecules within the thick filaments, known as cross-bridges (Huxley, 1957), which make contact with the thin filaments. The crossbridges themselves act in a cyclic manner, attaching to actin, hydrolysing ATP, changing conformation (and thus pulling on actin), detaching, then attaching further along the filament before repeating the cycle (Huxley, 1969).

1.3 Muscle fibre typing

The locomotor muscles of all animals have to provide a wide range of forces and contractile speeds ranging from sustained postural contraction to rapid bursts of maximum speed. The solution adopted universally for these conflicting requirements has been to divide the locomotor system into populations of different fibre types which have characteristic contractile properties. In mammals these fibre types are arranged typically in a mosaic like fashion throughout different muscles. In fish however, the different fibre types are arranged in discrete anatomical locations (described in more detail in the introduction of Chapter 4).

A muscle fibre type can be defined as a group of muscle fibres which have the same functional properties. Therefore any categorisation of muscle fibre types should ideally be based on purely functional criteria (Dahl and Roald, 1991). However, since it is impractical to measure the force-velocity relationships of a great number of muscle fibres (for example Bottinelli, et al., 1991), it has been the tradition in the past to use various histochemical methods to try and estimate the functional characteristics of individual fibres. Two different histochemical approaches are presently used to distinguish between different fibre types. One method is based upon the myofibrillar actomyosin adenosine triphosphatase (ATPase) activity whilst the other method is based upon reference enzymes of oxidative and glycolytic metabolism.

An important milestone in the histochemical method based on myofibrillar ATPase is the paper by Bárány, (1967) which demonstrated that actomyosin ATPase activity can be used as an indicator of contractile speed. Subsequently, it was observed that fast and slow myosins have different alkaline and acid liabilities with the myosin of fast fibre types being
alkali stable, acid labile and the myosin of slow fibres being alkali labile, acid stable with respect to ATPase activity. Several modifications of the basic ATPase assay exist and a number of different designations of fibre types and subtypes have been introduced for mammalian muscle, only some of which are used to any extent (for review see Pette and Staron, 1990).

One crucial problem with the histochemical methods used in muscle studies at present is that the classifications produced by one method are not completely compatible with the classifications produced by another. In addition to this problem it must also be noted that the fibre type classification schemes have been developed using relatively few muscles from a small number of species. Therefore, a particular classification scheme may not apply to every muscle within the same animal or during cases of altered phenotypic expression (Pette and Staron, 1990). Moreover, the fibre classification used within one class of animal may be inappropriate in another class, as has been demonstrated between the fibre types of fish and mammals and also between different species of fish (Sänger, 1991).

The most widely accepted classification of mammalian muscle fibres is that proposed by Brooke and Kaiser, (1970). This method distinguishes between one slow fibre type (type I) and three types of fast (type II) fibres (type IIA, type IIB and type IIC) based on their ATPase activity after preincubations at three different values of pH. Subsequently an additional subtype of type II fibres has also been described in specific muscles of some species (Schiaffino et al., 1989). This additional fibre type, type IIX, is widely distributed in rat skeletal muscles and can be distinguished from IIA and IIB fibres by histochemical ATPase activity and by the unique staining pattern of seven anti-MyoHC monoclonal antibodies. It is possible that in earlier fibre classification studies the type IIX fibres were erroneously classified as type IIB fibres and this may be one of the reasons why different fibre classification systems are incompatible (Pette and Staron, 1990). Furthermore, it should also be noted that rather than all muscle fibres lying in three or four distinct types, it is more likely that a continuum of fibre type hybrids exists.

The contraction rate of a muscle fibre is largely determined by the ATPase activity of the myosin heavy chain (MyoHC) isoform or isoforms it expresses (Bottinelli et al., 1991). Therefore, to a first approximation, the MyoHC isoform content of the fibre is the molecular determinant of fibre type. Different types of muscle fibre contain different isoforms of the
MyoHC (Reiser et al., 1988; Klitgaard et al., 1990). Slow type I muscle fibres contain isoforms of the MyoHC which have a low ATPase activity, whilst fast type II fibres contain MyoHC isoforms with a high ATPase activity (Bárány, 1967; Reiser et al., 1988). However, the MyoHC content of a fibre does not exclusively govern contractile properties, a number of other elements such as the troponin isoforms present, the properties of the T tubule system and the myosin light chain composition may also make a contribution (Pette and Staron, 1990).

1.4 Polymorphism of contractile proteins

Many of the proteins present in eukaryotic organisms exist in multiple forms. These closely related forms of the same protein are known as isoforms or isoenzymes and are very similar in overall structure and function. However, subtle differences in structure between members of the same isoform family give certain characteristics which optimise each isoform for a particular application. The regulated expression of tissue specific and developmentally regulated isoforms is essential for cell differentiation, ontogenic development and physiological adaptation.

The diversification of protein isoforms can be brought about at several different levels. Many protein coding genes are members of multigene families which code for closely related isoforms such that each isoform at the protein level also has an isogene at the genomic level. In some cases the same gene can give rise to several different protein products by the process of alternative splicing of exons as is seen in alkali myosin light chain and troponin gene families (Gros and Buckingham, 1987). Posttranslational modifications can also play a role in creating protein diversification.

The contractile proteins present in muscle exist as multiple isoforms encoded by multigene families (Syrový, 1987; Gros and Buckingham, 1987). The existence of these multiple isoforms not only allows a wide variation in the types of muscle present in any one species but also facilitates a wide degree of functional plasticity within a particular muscle allowing for adaptation to various functional needs.
1.4.1 Myosin

Myosin is the major contractile protein found in all muscle cells and also some non-muscle cells (Warrick and Spudich, 1987). The family of myosin proteins can be split into two main classes, myosin I and myosin II. Both are mechanochemical proteins with myosin I involved in cytokinesis, and organelle motility whilst myosin II proteins are involved in muscle contraction. In skeletal muscle the myosin molecule is located in the thick filament of the myofibril and together with actin takes part in the mechanism by which the chemical energy of ATP is converted into mechanical work. It is thought that both myosin I and myosin II are universally present in eukaryotes (Goodson and Spudich., 1993). This introduction will only deal with the isoforms of myosin II heavy chains present in vertebrate striated muscle.

In its native state, striated muscle myosin is a hexameric protein consisting of two "heavy" polypeptide chains of approximately 220 KDa each and four "light" chains approximately 17-20 KDa each (Weeds and Lowey, 1971). Both the heavy and light chains of myosin exist as multiple isoforms which produce distinct isoforms of myosin all with similar, but not identical, structure and function (Gros and Buckingham, 1987).

The two MyoHCs intertwine at their carboxyl termini forming an α-helical coiled-coil, about 150nm long, termed the rod region of the molecule. During myofibrillogenesis the rod regions of MyoHC molecules aggregate to form the myosin filaments and for this reason the length and charge of the rod region is critical (Lowey, 1986). The NH₂ terminus of each individual MyoHC molecule consists of a globular head about 19nm long and 5nm wide (at the widest point) such that the myosin molecule as a whole contains two head regions (Figure 1.2). The head region of the MyoHC forms the "crossbridge" between the thick and thin filaments of the
sarcomere. It contains an actin binding domain, the sites where the myosin light chains interact and the region where the ATPase activity of the molecule is localised (Craig, 1987). The myosin I proteins contain a region homologous to the myosin II head but lack the coiled-coil tail.

Early attempts to elucidate structure-function relationships of the myosin II molecule involved the characterisation of subdomains of the molecule based on the actions of various proteolytic enzymes (reviewed by Lowey, 1986). Chymotrypsin cleaves the protein between the globular head and fibrous rod domains resulting in an 800 amino acid long globular fragment termed S1 which can be further digested into three subdomains of 23, 50 and 24 KDa respectively (see Figure 1.3). The S1 fragment alone has been shown to contain ATPase activity and has the ability to bind actin (Balint et al., 1978). Spudich and coworkers (Toyoshima et al., 1987) have also demonstrated that the S1 fragment is sufficient to generate movement of actin in an in vitro motility assay.

The three dimensional structure of the S1 fragment from myosin of chicken pectoralis muscle has been recently elucidated by X-ray diffraction (Rayment et al., 1993a;b). This three dimensional crystal structure of the S1 fragment provides the first structural framework for understanding the molecular basis of muscle contraction and has allowed a more detailed hypothesis of how the individual myosin, actin and ATP molecules interact during the cross-bridge cycle.

The domain connecting the globular S1 head and the helical rod region is termed as the "hinge" region or S2 fragment and is thought to permit and amplify the movement of the head along actin relative to the thick filament. The fibrous rod region of the molecule can also be isolated by proteolytic digestion and is termed light meromyosin (LMM).

In order to more closely investigate structure/function relationships within the MyoHC molecule, much work has been done in terms of elucidating the actual amino acid and nucleotide sequences of myosin heavy and light chains from various species. The information generated from such sequencing endeavours revealed the complexities of diversification within the myosin family and has also allowed some interesting phylogenetic deductions.
1.4.2 Isoforms of the myosin light chain

Bound to each MyoHC head are two proteins, making a total of four per myosin molecule, known as the myosin light chains. Two classes of myosin light chain have been characterised. The phosphorylatable, or regulatory, light chains (LC2) and the alkali or essential light chains (LC1 and LC3). The regulatory light chains (LC2) can be dissociated from the myosin head by treatment with 5,5-dithio-bis-2-nitrobenzoate (DTNB) whilst the alkali (LC1 and LC3) light chains are dissociated from the myosin head by treatment with high pH.

One of each of these two types of myosin light chain are bound to each MyoHC head. The regulatory light chain is wrapped around the head/rod junction and the alkali light chain is positioned more distal to this (Katoh and Lowey, 1989). The function of the myosin light chains however remains unclear.

The regulatory light chain LC2 has a calcium binding site which can be phosphorylated by a calcium dependent kinase and has been postulated to modulate the contractile response (Perrie et al., 1973). The role of the alkali light chains has been suggested to be involved with the modulation of myosin and thin filament interactions and is therefore associated with the different contractile properties of different muscle types (Barton et al., 1985). Using an in vitro motility assay, Lowey et al., (1993) demonstrated that removal of the light chains from myosin dramatically reduces the velocity at which actin filaments are moved by the S1 molecule. It was also shown that neither of the light chains are essential for the ATPase activity of myosin. From these data Lowey and coworkers suggest that the globular head region of myosin alone can function as an actin activated ATPase and a minimal motor, but that the presence of the light chains is necessary for the transduction of the energy of hydrolysis into rapid movement.

Different muscle types have different isoforms of both the alkali and regulatory light chains. Fast adult mammalian skeletal muscle contains two distinct alkali light chains, LC1f and LC3f and one regulatory light chain, LC2f. The LC1f and LC3f are encoded by the same gene which is differentially spliced to give specific N-termini (Barton and Buckingham 1985). Variants of the LC1f and LC2f isoforms have been described in the superfast fibres.
present in the jaw closing muscles of the cat (Rowlerson et al., 1981) and the dog (Shelton et al., 1988). Pernelle et al., (1986) have described four variants of the LC2f isoform and three variants of the LC3f isoform in rabbit. Avian muscle is thought to contain at least three variants of the LC1f isoform (Rushbrook et al., 1988).

Slow mammalian skeletal muscle contains the alkali light chain LC1s and the regulatory light chain LC2s which appear to be identical with the cardiac ventricular myosin light chains LC1v and LC2v respectively. Indeed in the mouse and rat it has been shown that LC1s and LC1v are encoded by the same gene (Barton et al., 1985; Periasamy et al., 1989a). Variants of the slow LC1 and LC2 isoforms (LC1sa, LC1sb, LC2s and LC2') have also been described in some species (Pinter et al., 1981; Biral et al., 1982; Houston et al., 1985). The rabbit is thought to have four variants of LC1s and three variants of LC2s (Pernelle et al., 1986).

Embryonic isoforms of the LC1 and LC2 light chains have also been described in human and rat (Whalen et al., 1982; Biral et al., 1984; Pons et al., 1987).

The myosin light chains present in fish have been shown to be broadly similar to those of mammalian muscles. The fast white fibres of fish species generally contain three types of myosin light chain, LC1f, LC2f and LC3f whereas the slow red muscle contains two types, LC1s and LC2s (Rowlerson et al., 1985; Martinez et al., 1989; Martinez et al., 1990 a; Martinez et al., 1990 b; s Martinez et al., 1991). The white muscle of trout however has been shown to be exceptional in that it also contains LC1s (Rowlerson et al., 1985).

As protein electrophoretic techniques gain higher resolution and more DNA probes for specific myosin light chains become available, it is likely that this already complex list of myosin light chain isoforms will become even larger in the future, revealing the full complexities of this isoform family.

1.4.3 Isoforms of the myosin heavy chain

Although extensive polymorphisms of the myosin light chains have been shown in a variety of species, the predominant contributor to the functional diversity of muscle fibres is thought to be the MyoHC. 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 (Table 1.1) and as many as 31 in chicken (Robbins et al., 1986). Gerlach et al., (1990) reported the number of MyoHC genes present in the carp to be as many as 28. No pseudogenes have yet been identified in any of the species studied, although the total number of MyoHC genes which have been cloned and characterised is fewer than nine for any one species.

To date the process of alternative exon splicing has not been observed in the MyoHC genes of vertebrate striated muscle and it is thought that in such species each individual MyoHC isoform at the protein level is encoded by a separate gene (Wydro et al., 1983; Leinwand et al., 1983). In Drosophila there are two MyoHC genes, one of which codes for a non muscle isoform and one a muscle specific isoform which can be alternatively spliced to give different transcripts (Rozek and Davidson, 1983; Bernstein et al., 1983; Kiehart et al., 1989; Kronert et al., 1991).

1.4.3.1 Structure of the MyoHC gene

In human and mouse, the family of sarcomeric MyoHC genes has been shown to be split into two subfamilies located on separate chromosomes. Six MyoHC genes including the embryonic, neonatal, fast and a putative extraocular gene have been shown to be located within a 500Kb segment on chromosome 17p13.1 in the human (Yoon et al., 1992). The order of the genes in this cluster however does not correspond to the developmental pattern of expression of the individual members. Similarly, the developmental and adult fast skeletal MyoHC have been shown to be clustered on chromosome 11 in the mouse (Weydert et al., 1985). The cardiac MyoHC isoforms (α and β) are also tandemly linked on chromosome 14 in both mouse and human where they are separated by only 4 to 5 Kb (Weydert et al., 1985; Saez et al., 1987; Yamauchi-Takahara et al., 1989; Matsuoka et al., 1989; Qin et al., 1990; Gulik et al., 1991). Chicken embryonic and neonatal fast MyoHC genes have also been shown to be linked by less than 7.5Kb (Gulick et al., 1987).

Stedman and coworkers (1990a) presented sequence comparison data which suggests that the mammalian slow type I (β Cardiac) MyoHC isoform diverged from a common ancestor of the mammalian embryonic, neonatal and adult fast genes more than 50
million years ago. They also hypothesise that the physical segregation of the cardiac/slow isoform and the skeletal/fast isoforms to different chromosomes occurred approximately 400-600 million years ago.

Due to their large size, relatively few vertebrate MyoHC genes have been sequenced completely (Table 1.1). Those vertebrate MyoHC genes which have been entirely sequenced are very similar in size at both the genomic and RNA levels, where they are approximately 24kb and 6000 nucleotides respectively, and show a high degree of organisational and sequence homology. Both the human β cardiac (Jaenicke et al., 1990) and the chicken embryonic (Molina et al., 1986) MyoHC genes have a total of 40 exons whilst the rat embryonic MyoHC gene (Strehler et al., 1986) has 41 exons. However, the exon positions in all three genes differs slightly in a minority of cases. The equivalent of exon 37 in the

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**Figure 1.3 Structure of the rat embryonic MyoHC gene**

The 41 exons of the gene are numbered and represented as black boxes. Positions of the TATA box, ATG start site and polyadenylation signal (AATAAAA) are shown as arrows. The positions of the two reactive thiol groups (SH1 and SH2), the hinge region (S2 hinge) and the ATP binding site (ATP) are also shown. A schematic of the protein subdomains above the gene structure indicates the positions of various tryptic fragments. (Re-drawn from Strehler et al., 1986).
human \( \beta \) cardiac gene is split into two exons in the chicken and rat embryonic genes such that the position of the additional intron is identical in both of the latter two genes. Also, the intron separating the final two exons of the human \( \beta \) cardiac (exons 39 and 40) and the rat embryonic (exons 40 and 41) is absent in the chicken embryonic gene. The determination of the deduced amino acid sequences of complete MyoHC proteins from genomic and cDNA sequences has allowed the various subdomains of the protein (reviewed by Lowey 1986) to be assigned to specific regions within the protein sequence (Figure 1.3). The globular S1 domain, which accounts for approximately 40% of the protein, begins at the first amino acid and ends at the proline residue at position 838 in the rat embryonic MyoHC gene (Strehler et al., 1986). The tryptic fragments of the S1 head of the rat embryonic MyoHC were also assigned to amino acid residues 1 to 206 (23 KDa fragment), 207 to 633 (50 KDa fragment) and 634 to 838 (24KDa fragment). The S2 region is encoded by approximately seven exons and covers positions 839 to 1279 and the rod (LMM) region covers positions 1280 to 1939.

The binding and cleavage of ATP is assigned to regions within the 23 KDa of S1 with an active centre around the tryptophan residue at position 130 (Okamoto and Yount, 1985). Additional nucleotide binding sites are localised on the 50 KDa subfragment (Jaenicke et al., 1990). The binding of actin is thought to occur in two regions on the 50 KDa and also on the 24 K Da fragment (Jaenicke et al., 1990). The 24 KDa fragment of the S1 head also contains two active thiol groups, termed SH1 and SH2 which correspond to cysteines at positions 700 and 710. These groups are also thought to be important in the ATPase activity of the molecule (Molina et al., 1987).

Extensive sequence homology exists between MyoHC genes. This homology is present between species as diverse as nematode and rat and is especially noticeable between different isoforms within the same species. The sequence of the globular head region (S1) of the molecule is more highly conserved between species than the rod region (Karn et al.1983; Strehler et al.1986). Highly conserved sequences within the S1 region include the ATP binding site, the region of the two active thiols and sequences in the 50 kD domain thought to be involved in actin binding. Divergent regions also exist in the S1 head which include the hinge region and the 80 amino acid N-terminal sequence (Strehler et al.1986).

The rod regions of both muscle and non muscle MyoHCs have a repetitive sequence
organisation characteristic of a helical coiled-coil proteins. All vertebrate MyoHC rod
regions sequenced to date have two common features. They all contain forty 28 residue
repeats, the first 39 of which are complete (termination of the rod region occurs within
repeat 40) (Stedman et al. 1990), and they all contain an extra "skip" residue, the function
of which is unknown, at the end of repeats 13, 20, 27, and 35 (McLaughlin & Karn, 1983).

1.4.3.2 Adult skeletal muscle MyoHC isoforms

Investigations characterising the expression patterns of different MyoHC isoforms
in different muscle types and during muscle development have been conducted at three
levels: i) protein electrophoresis ii) immunological studies and iii) molecular biological
studies. In general, the nomenclature of the isoforms characterised by such studies in adult
mammalian skeletal muscle has followed the fibre typing nomenclature of Brooke and Kaiser
(1970). Thus the MyoHC isoform present in type IIA fibres is named HClia, the isoform
in type IIB fibres is called HClIb and the isoform in type I fibres HCl. Type IIC fibres are
thought to contain a mixture of MyoHC isoforms. The percentage of such fibres which
coequipresent different MyoHC isoforms varies between species and is seen to increase under
conditions of induced fibre transformation, for example in response to chronic electrical
stimulation as described later in this introduction (Maier et al., 1988).

All MyoHC protein isoforms have a similar molecular weight and charge. Therefore,
early protein electrophoretic studies tended to have limited resolution due to comigrations
of the different isoforms. However, technical advances in protein electrophoretic techniques
have allowed several MyoHC isoforms to be distinguished. In rat adult skeletal muscle,
Termin and coworkers (1989) were able to separate four MyoHC isoforms by gradient
polyacrylamide gel electrophoresis of extracts from single fibres and whole muscle
homogenates, the electrophoretic mobilities of which are depicted in Figure 1.4. The HClia,
HClIb and HCl isoforms were shown to be uniquely present in histochemically defined fibre
types IIA, IIB and I, respectively and the isoforms HCl and HClia were shown to coexist,
in variable ratios, in type IC and IIC fibres. An additional fast MyoHC isoform with an
electrophoretic mobility between HClia and HClIb was designated as HClId because of its
abundance in fast large diameter fibres of the diaphragm. Circumstantial evidence suggests
that the type IIId MyoHC isoform described by Termin et al., (1989) is identical with the MyoHC isoform found in the 2X fibres described by Schiaffino et al., (1989).

\[
\begin{align*}
\text{HCIIa} & \rightarrow \quad \text{Embryonic} \\
\text{HCIIId/IX} & \rightarrow \quad \text{Neonatal} \\
\text{HCIIb} & \rightarrow \quad \text{Migration} \\
\text{HCI/β cardiac} & \rightarrow \\
\end{align*}
\]

**Figure 1.4** Electrophoretic mobilities of MyoHC protein isoforms in rat (re-drawn from Termin et al., (1989)).

Immunohistochemical techniques have also played a valuable role in the characterisation of MyoHC isoforms and all of the previously mentioned MyoHC proteins can now be distinguished in the rat by means of monoclonal antibodies, (Schiaffino et al., 1989; Gorza, 1990). A further two fast MyoHC isoforms have also been characterised immunohistochemically in specialised muscles. The HCIIm isoform has been described in the tensor tympani and masseter muscles of the cat and dog (Rowlerson et al., 1981; Mascarello et al., 1982; Mascarello et al., 1983; Hoh et al., 1988), whilst the HCEom isoform has been described in the fast contracting fibres of the extraocular muscles (Wieczorek et al., 1985; Sartore et al., 1987). The gene coding for the extraocular MyoHC isoform of the rat has been isolated and characterised (Wieczorek et al., 1985) however, no gene has been isolated to date which corresponds to the HCIIm isoform. The α cardiac MyoHC isoform, which can be considered as a fast isoform (Swynghedauw, 1986), was once thought to be exclusively expressed in cardiac muscle. However expression of this isoform has recently been reported in the masseter (Bredman et al., 1991; d'Albis et al., 1993) and extraocular (Domellöf et al., 1992) muscles.

Slow (type I) skeletal muscle fibres contain the MyoHC isoform HCI, which is thought to be identical to the β cardiac isoform (Lompre et al., 1984). In humans, it has been reported that a second β cardiac like isoform is also expressed in slow type I fibres (Jandreski et al., 1987). The cDNA sequence of this skeletal muscle specific β cardiac like
isoform was shown to have a 3' untranslated region and 482 nucleotides specifying the
carboxyl coding region which were 100% homologous to the β cardiac MyoHC isoform. However, the skeletal clone sequence diverged from the β cardiac isoform at the 5' end. Whether this skeletal muscle specific clone isolated by Jandreski et al., (1987) represents a true human MyoHC isoform remains to be determined unequivocally, since there is a possibility that it could have arisen through a cloning artefact. It has also been postulated that a second slow type MyoHC gene is expressed in the rabbit tibialis anterior muscle (Goldspink et al., 1992). Stretch and stimulation, a condition known to transform fast fibre types into slow fibre types (see later in this introduction), of the rabbit tibialis anterior muscle showed an increase in the mRNA for slow type I MyoHC when Northern blots were probed with a cDNA containing coding region of the rabbit β cardiac MyoHC gene. However, when the same Northern blots were hybridised with a probe containing only the 3' untranslated region of the rabbit β cardiac MyoHC gene, no increase in message was observed, suggesting that the slow MyoHC gene induced by stretch and stimulation is different than the β cardiac MyoHC gene. Therefore, the nature of this second slow MyoHC gene in rabbit appears to be different than the one proposed by Jandreski et al., (1987) in human.

The slow tonic muscle fibres of the extraocular muscle, the tensor tympani muscle and muscle spindles contain a slow MyoHC isoform termed HClton (Pierobon-Bormiloi et al., 1980; Mascarelo et al., 1982; Mascarelo et al., 1983; Sartore et al., 1987) which is different from the β cardiac MyoHC isoform. However, no gene sequences corresponding to this isoform have been cloned to date.

Molecular biological techniques have confirmed many of the results obtained by protein electrophoresis and immunohistochemistry by demonstrating the existence of separate genes for the individual MyoHC isoforms. The best characterised mammalian species in terms of MyoHC gene sequences is the rat and to date a total of eight separate striated muscle MyoHC genes have been described for this species (Table 1.1).

Protein electrophoretic studies in fish species have tended to be less informative regarding MyoHC diversity. It has long been known that fish skeletal muscle myosin is similar in overall size and sub-unit structure to mammalian myosins, however like the myosins present in other cold blooded vertebrates it is more unstable than mammalian
myosin and rapidly aggregates upon storage, losing its ATPase activity (Connel, 1960). Huriaux and coworkers (1991), using SDS-polyacrylamide gel electrophoresis, were able to separate four distinct MyoHC proteins in adult barbel striated muscle, specific to white muscle, the red muscle, the ventricles and the head muscles. Martinez and coworkers, (Martinez et al., 1989; Martinez et al., 1990a; Martinez et al., 1990b) electrophoretically separated one MyoHC protein band in red muscle and one or two bands in the white muscle of a variety of marine fish. However the authors also point out that these protein "bands" do not necessarily represent a single protein isoform and that comigrations of multiple isoforms are likely.

The genes coding for MyoHC isoforms in fish species have not been studied in any depth to date. Gerlach et al., (1990) isolated 28 different genomic clones which contained MyoHC gene sequences from the carp. Partial sequencing of some of these clones (Turay, 1991) revealed conserved intron/exon structure with mammalian MyoHC genes in the regions studied. This work, to the authors knowledge, is the only published description of the cloning of MyoHC gene sequences from a species of fish.

1.4.3.3 Developmental myosin heavy chain isoforms

The precursors of muscle fibres are mono-nucleated embryonic mesenchymal cells which do not themselves fuse or synthesize any of the muscle specific myofibrillar proteins. During muscle formation these cells proliferate and differentiate into mono-nucleated myoblasts which fuse together forming multinucleated myotubes. In mammals a biphasic process of fibre formation is observed. The first myotubes to form are known as primary myotubes and these provide a framework along which remaining myotubes longitudinally orientate themselves and subsequently fuse to form the secondary myotubes. This process leads to a "rosette" arrangement of muscle fibres with the larger primary myotubes surrounded by smaller secondary myotubes. The difference in size between primary and secondary myotubes gradually diminishes until the two populations are indistinguishable in the adult. In fish this biphasic development of primary and secondary myotubes is not observed. However, newly formed small muscle fibres in the later stages of development have been shown to be immunohistochemically different from more mature fibres (Akster,
<table>
<thead>
<tr>
<th>Myosin Isoform</th>
<th>Rat</th>
<th>G</th>
<th>C</th>
<th>Human</th>
<th>G</th>
<th>C</th>
<th>Mouse</th>
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<tbody>
<tr>
<td>HClIa</td>
<td>DeNardi et al., (1993)</td>
<td>P</td>
<td>Ennion ¹ Z32858</td>
<td>P</td>
<td>N/A</td>
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<td>Extraocular</td>
<td>Wieczorek et al., (1985)</td>
<td>P</td>
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1. Nucleotide sequence not published. EMBL accession number for sequence is as shown.
2. The sequence was not attributed to a specific fibre type at the time of publication. However, later comparison with the Rat IIX sequence (DeNardi et al., (1993) showed a high homology within the 3' untranslated regions of these genes leading the authors to propose that the human and mouse sequences are homologous to the rat IIX gene.
3. Sequence not designated to a particular fibre type in original publication. However, the 3' untranslated region of this sequence shows very high homology to the rat IIB gene.

Key: G= Genomic sequence. C= cDNA sequence. F= Full gene sequence in Database. P= Only a partial sequence in Database. N/A= Gene not yet isolated.

Note: More than one publication may record the same gene. The references given in this table represent the largest continuous stretch of sequence present in the EMBL database at the time of writing or the reference where the gene has been assigned to an actual isoform.
During the development of muscle fibres a complex pattern of MyoHC isoforms are expressed. In the rat and human, the primary muscle fibres initially express the embryonic (Molina et al., 1987; Eller et al., 1989; Karsch-Mizrachi et al., 1989; Strehler et al., 1986), neonatal (termed perinatal in human) (Periasamy et al., 1984; Feghali and Leinwand, 1989; Karsch-Mizrachi et al., 1989; Weydert et al., 1987) and the slow β cardiac (Barbet et al., 1991; Narusawa et al., 1987) MyoHC isoforms, but none of the fast MyoHC isoforms. The secondary fibre population of myotubes express embryonic, neonatal, and fast MyoHC isoforms in a very heterogeneous manner, but never the slow twitch MyoHC isoform (at least in humans) (Barbet et al., 1991). Later in embryonic development (at about 35 weeks gestation in the human) the expression of embryonic isoform decreases and shortly after birth the expression of the neonatal isoform also disappears. Concomitantly with the elimination of the embryonic and neonatal isoforms, the adult slow and fast MyoHC isoforms begin to be expressed predominantly as the muscle begins to take on its adult phenotype. Also at this stage, certain fibres cease to express the slow β cardiac isoform and begin to express the adult fast isoforms (Barbet et al., 1991).

Immunological studies, (Hughes et al., 1993a) suggest that there at least three different slow MyoHC isoforms expressed during development in humans and rats, thus the above account of MyoHC switching during development is probably oversimplified. A second embryonic MyoHC isoform has also been described in chicken skeletal muscle (Hofmann et al., 1988), suggesting that MyoHC isoform switching during development is more complex in this species.

The specific factors controlling the transition from embryonic/neonatal isoforms to adult isoforms are not known. Russell et al., (1993) found that developing fibres of the rat hind limb muscle consisted of two populations which exhibited different responses after denervation at birth regarding the disappearance of neonatal MyoHC and the accumulation of adult fast MyoHC isoforms. From these results, the authors postulate that fibres containing neonatal MyoHC in rat hind limb muscles at birth are already preprogrammed to accumulate either the adult fast IIa or IIb MyoHC isoforms and that the neonatal to adult MyoHC transitions occurring in these two fibre populations are controlled by different mechanisms.
Expression of the embryonic and neonatal isoforms is not restricted to stages of
development. Adult muscle has also been shown to express these isoforms in certain
circumstances, for example in regenerating muscle, denervated muscle, dystrophic muscle
and in rhabdomyosarcomas (Sartore et al., 1982; Sartore et al., 1987; Cerny and Bandman,
1987; Harris et al., 1989; Rushbrook et al., 1987). Some normal adult muscles also express
the developmental isoforms, for example extraocular muscles, intrafusal fibres and the
masseter muscle (Wieczorek et al., 1985; d’Albis et al., 1986; Rowlerson et al., 1985).

The fact that the so called embryonic and neonatal MyoHC isoforms are also, in
some cases, expressed in adult skeletal muscle and that both cardiac MyoHC genes are also
expressed in skeletal muscle suggests that a future revision of the nomenclature of MyoHC
isoforms is necessary.

1.4.3.4 Cardiac myosin heavy chain isoforms

Two distinct isoforms of the MyoHC are expressed in mammalian heart: the α and
β MyoHCs. These two isoforms can exist as dimers within the same myosin molecule, two
α MyoHCs form a homodimer called V1, one α and one β MyoHC form a heterodimer
called V2 and two β MyoHCs form a homodimer called V3 (Morkin, 1993).

Expression of the two cardiac MyoHC genes varies during development with the β
isoform predominating in the myocardium of embryonic mammals (Lompre et al., 1984).
After birth, rodents rapidly switch to a predominance of α MyoHC expression in the
ventricles and atria (Lompre et al., 1984) and this expression pattern continues through adult
life. However, in senescent rats the β MyoHC isoform has been reported to reaccumulate
to as much as 80% of the total MyoHC (Schwartz et al., 1992). In large mammals the β
isoform predominates through all stages of development and is expressed mainly in the
ventricles with minor amounts in the atria whereas the α MyoHC is expressed mainly in the
atria with minor amounts in the ventricles (Gorza et al., 1984). The rat α cardiac MyoHC
gene has only a small number of nonidentical amino acids with the rat β cardiac gene (131
of 1938), yet these differences account for the characteristic differences in ATPase activity
and other biochemical properties observed between these two isoforms (McNally et
al., 1989).
The expression of both α and β cardiac MyoHC genes is regulated by thyroxine (Sinha et al., 1982; Everett et al., 1983) and increased haemodynamic load (Gorza et al., 1984). Treatment with thyroxine increases the predominance of the α MyoHC isoform in the ventricles, whereas hypothyroidism reverses the relationship causing a reduction in the α isoform mRNA (Everett et al., 1984).

The rat α cardiac MyoHC gene has been shown to have three different polyadenylation sites downstream from a single polyadenylation signal (Sindhwani et al., 1994). Hypothyroid rats show a significant increase in the proportion of the longest and a decrease in the shortest mRNA transcripts however, the functional significance of this phenomenon remains unclear. Sindhwani et al., (1994) also demonstrated the existence of an alternative splice site at position 1931 of the rat α MyoHC gene resulting in either the inclusion or exclusion of a glutamine amino acid, the significance of which is also unknown.

With regards to myosin isoforms present in fish cardiac muscle, studies in the literature are comparatively few. Using protein electrophoresis, Huriaux et al., (1991) demonstrated the existence of a distinct MyoHC protein in the ventricles of barbel. Martinez et al., (1991) found that the ventricles and atria of Arctic char each contain a distinct myosin protein. These two isoforms did not appear to differ in their light chain content but were shown to have distinct MyoHC proteins by electrophoresis and V8 protease mapping.

1.5 Factors modulating MyoHC isoform expression

Fully differentiated muscle fibres have a potential for plasticity and can respond to altered functional demands with specific adaptive changes. The expression of varying proportions of the MyoHC isoforms plays a major role in facilitating the plasticity of skeletal muscle and a variety of stimuli, including altered patterns of innervation, mechanical overload and hormonal signals have been shown to affect their expression.

1.5.1 Innervation

Each different muscle fibre type of vertebrate skeletal muscle is innervated by an appropriate type of motor neuron. Hence, fast muscle fibres are innervated by motor
neurons that discharge in high frequency bursts interspersed with long quiescent periods, whereas slow fibres are innervated by motor neurons that are activated at a relatively constant low frequency associated with prolonged postural movements. The fundamental questions of how muscle fibre types form and become innervated by the appropriate motor neurons during development and how altered patterns of innervation can affect fibre phenotype have been the focus of much research over recent years.

With regard to the formation of fibre type diversity during development, it was initially thought that the activity pattern of the innervating motor neuron dictated phenotype (Gambke et al., 1983; Rubinstein and Kelly, 1981). However, innervation alone is now known to be insufficient to account for fibre type diversity since it has been shown that different fibre types form during early embryonic development in the absence of interactions with motor neurons (Condon et al., 1990; Harris et al., 1989). Hence, the information required for the formation of specific motor units must be carried, at least in part, by the muscle cells themselves. Indeed, it has been shown that there are multiple types of intrinsically different myoblasts which give rise to different muscle fibre types in the absence of innervation (reviewed by Miller, 1991). Whilst the role of myoblast lineage is likely to be important in establishing the initial fiber type pattern, subsequent postnatal development is independent of cell lineage and extrinsic signals override any pre-programmed patterns of gene expression (Hughes and Blau, 1992).

In the later stages of development some aspects of fibre diversification and muscle formation are profoundly affected by a lack of innervation. Therefore, whilst the early stages of myogenesis are largely independent of innervation, continued growth and refinements of fibre diversification is dependent on the appropriate motor innervation.

Skeletal muscles of the adult respond to denervation in a complex and tissue specific manner with regard to MyoHC isoform expression. While proportions of slow type I and fast IIB MyoHC proteins decrease in the rat soleus and extensor digitorum longus muscles after denervation, the proportion of the fast IIA MyoHC isoform increases (Jakubiec-Puka et al., 1990). Thus it would seem that the expression of the IIB MyoHC is more susceptible to lack of innervation than the IIA MyoHC.

Cross-innervation experiments where the nerves of fast muscles have been surgically transferred to innervate the slow soleus muscle have also been used to investigate the role
of innervation in determining fiber types in the rat (Thomas and Ranatunga, 1993). In such experiments the complement of slow type I fibres decrease whilst the complement of fast type II fibres increase in the re-innervated soleus. Furthermore, it was shown that the type of fast muscle nerve used to cross-innervate the soleus is important in determining the type of transformation. The Extensor digitorum longus nerve, unlike the peronius longus nerve produces a significant increase in the type IIA MyoHC content of the re-innervated soleus.

1.5.2 Chronic electrical stimulation

In recent years the effects of chronic electrical stimulation have been studied extensively as a method of inducing specific changes in muscle properties (for review see Pette and Vrbova, 1992). Chronic artificial stimulation bypasses the central nervous system activating all motor units equally, thus unlike the case of exercise induced stimulation where the motor units of many muscles are activated in a graded hierarchial manner, chronic stimulation allows the study of separate muscles which are less likely to be influenced by other changes in the body which are caused by training. Indeed chronic stimulation of skeletal muscle, especially when combined with passive stretch, is capable of evoking changes which exceed those induced by other forms of increased contractile activity and has thus provided information of the maximal extent of stimulation induced muscle plasticity (Goldspink et al., 1992).

Chronic electrical stimulation at a frequency of 10Hz converts the predominantly fast twitch extensor digitorum longus (EDL) and tibialis anterior (TA) muscles of the rabbit into slower contracting ones (Salmons and Vrbova, 1969). These changes manifest themselves as an increase in the time to peak twitch tension and half relaxation times, a decrease in the maximum rate of tetanic tension development and an increased resistance to fatigue. Many factors are involved in changing these contractile characteristics including the capillary supply to the muscle, which is increased upon chronic stimulation, the Ca²⁺- regulatory system, energy metabolism, changes in numbers of mitochondria and changes in the myofibrillar apparatus. This brief overview will only deal with stimulation induced changes in the MyoHC content of the myofibrillar apparatus.

Myofibrillar ATPase histochemistry and immunocytochemistry with monoclonal anti-
MyoHC antibodies has revealed stimulation induced increases in the number of slow type I fibres in fast muscles of a variety of species including rabbit, cat, sheep, goat, and human (Pette and Vrbová, 1992 and references therein). Moreover the conversion of a muscle containing predominantly fast IIB fibres to one which contains predominantly slow type I fibres appears, at least in the rabbit, to be facilitated by a sequential change in fibre types from type IIB → type IID → type IIA → type I with transient intermediate fibres occurring between each main type (Pette and Vrbová, 1992). However, it should be noted that such stimulation induced changes of fast to slow phenotypes are species dependent. The rat is thought not to show an increase in slow type I fibres in the EDL and TA muscles even after prolonged chronic stimulation (Termin et al., 1989). In this species, stimulation induced changes in fibre populations of fast muscle types are restricted to a rearrangement of the fast fibre subtypes such that a type IIB → type IID → type IIA transition occurs. During this transition an increase in the numbers of hybrid fibres is observed (Termin et al., 1989a; Termin et al., 1989b). In contrast to these findings, Mayne et al. (1993) did show an increase of the slow type I MyoHC isoform in chronically stimulated rat hind limb muscle however, the fast MyoHC isoforms were also still expressed. In the mouse TA muscle, no such dramatic changes in fibre type populations are observed after chronic stimulation (Pette and Vrbová, 1992).

The changes in fibre types induced by electrical stimulation are facilitated by corresponding changes in the expression of MyoHC isoforms. Peptide cleavage studies (Hoffman et al., 1985) and electrophoresis of MyoHC isoforms (Staron et al., 1987) have demonstrated changes from fast to slow MyoHC protein isoforms in chronically stimulated fast muscles. These changes in MyoHC protein isoforms are facilitated by alterations in the levels of expression of the respective MyoHC isogenes. The use of specific MyoHC probes in S1 nuclease mapping assays, (Kirschbaum et al., 1989; Kirschbaum et al., 1990) and in situ hybridisation studies (Aigner and Pette, 1990) have revealed changes in mRNA levels for the various MyoHC isoforms in response to chronic stimulation. In the stimulated rat EDL and TA muscles a rapid decrease in type Iib MyoHC mRNA is observed after two days of stimulation which decreases to almost undetectable levels after seven days. The opposite change occurs with the type Iia mRNA which rapidly increases over the same period (Kirschbaum et al., 1989). At the time these experiments were carried out, no sequence
information of the rat IIX MyoHC gene (Denardi et al., 1993) was available.

In addition to these fast to slow changes of skeletal muscle induced by electrical stimulation, the soleus, a predominantly slow muscle, can be induced to change to a predominantly fast muscle by direct phasic high frequency pattern of stimulation (Gorza et al., 1988). In this study, the transition from fibres containing predominantly the slow type I MyoHC isoform to fibres containing predominantly fast isoforms was demonstrated immunocytochemically. Subsequently the fast MyoHC containing transformed soleus muscle fibres were shown to contain the type 2X MyoHC (Schiaffino et al., 1988; Schiaffino et al., 1989).

It has been hypothesised that the motor impulse pattern, i.e. low frequency repetitive stimulation, is responsible for slow muscle type phenotypic expression (Pette and Vrbová, 1992). However, it has been shown that higher frequency stimulation with 30Hz (Ferguson et al., 1989) or 60Hz (Sreter et al., 1982) stimulation was more effective in transforming fast muscles into slow muscles than low frequency (10Hz) stimulation. An alternative hypothesis proposed by Goldspink and coworkers (Goldspink et al., 1991; Goldspink et al., 1992) is that rather than impulse frequency per se governing the slow fibre type phenotype, it is those stimulation regimes that cause maximal force generation, particularly when combined with stretch, that are responsible for the slow phenotype expression. Hence, muscle fibres stay phenotypically fast unless they are subjected to stretch and isometric force development. When the muscle is not subjected to stretch or force generation, the fast MyoHC genes, particularly the fast IId MyoHC gene is expressed by default.

1.5.3 Mechanical stimuli

1.5.3.1 Work overload

Muscle is a tissue in which mechanical signals play an important role in regulating gene expression. The removal or tetotomy of synergistic muscles has been used as an experimental model to study work-overload induced changes in skeletal muscle (Morgan and Loughna, 1989; Periasamy et al., 1989; Gregory et al., 1990; Leferovich et al., 1991). In such experiments the intact muscle, which is subjected to work overload, undergoes a rapid
hypertrophy which is associated with alterations in the proportions of the MyoHC genes expressed. The expression of the fast IIa MyoHC gene is increased in the fast-twitch plantaris and decreased in the slow twitch soleus during work overload hypertrophy in the rat (Periasamy et al., 1989; Morgan and Loughna, 1989). Expression of the fast IIb MyoHC isoform however, decreases markedly in both muscles. Results regarding the expression of the slow type I isoform are contradictory. Periasamy et al., (1989) showed an increase in slow type I MyoHC mRNA in both fast (plantaris) and slow (soleus) skeletal muscles of the rat during work overload induced hypertrophy with the increases being more pronounced in the plantaris (+310% after 11 weeks) than the soleus (+27% after 11 weeks). In contrast to this, Morgan and Loughna (1989), in an almost identical experiment, found no significant increases in the level of slow type I mRNA in either muscle. However, the time course of 20 days used by Morgan and Loughna may have been too short to observe changes in the levels of slow type I mRNA which were shown to peak after 11 weeks by Periasamy et al., (1989).

Experiments investigating the effects of work overload on neonatal rat muscle, (Leferovich et al., 1991) have also shown increases in the expression of slow type I MyoHC in the soleus and plantaris muscles. However this response is tissue specific since work overload of the extensor digitorum longus (EDL) muscle did not alter the normally occurring developmental decline of slow type I MyoHC in this muscle. Also, whilst mechanical overload in the rat neonate was shown to modulate the rate of progression of maturational adjustments in MyoHC expression it did not change the overall course of development. Hence, in the work-overloaded soleus muscle of the rat neonate, slow type I MyoHC expression is accelerated so that expression of this isoform is significantly above controls at 30 days. By 60 days however, the proportion of slow type I in control rat neonate soleus was the same as in the overloaded muscles.

1.5.3.2 Stretch and disuse

Immobilisation of limbs in both lengthened and shortened positions produces muscle specific responses with regards to MyoHC isoform expression. Immobilisation in the shortened position causes a rapid induction in the expression of the fast IIb MyoHC gene
in the rat soleus muscle but not in the plantaris or gastrocnemius muscles (Loughna et al., 1990). When immobilisation is in the lengthened position, a significant reduction in the expression of the IIb MyoHC gene is observed in fast muscles and no rapid induction of the IIb MyoHC in the soleus is observed. Expression of the slow type I MyoHC gene in the rat soleus is not affected by immobilisation in the shortened position but expression is much reduced after immobilisation in the lengthened position (Loughna et al., 1990; Goldspink et al., 1992). In contrast to this, expression of the slow type I MyoHC in the plantaris and gastrocnemius muscles, is reduced to undetectable levels by disuse in the shortened position. Passive stretch however, prevents this reduction in the plantaris muscle and causes an accumulation of slow type I MyoHC mRNA in the gastrocnemius. The effects of passive stretch on the fast IIA MyoHC gene are very similar to those of the slow type I in that when compared to control levels, the amount of IIA MyoHC mRNA is increased in the gastrocnemius and plantaris and reduced in the soleus. Similarly, immobilisation in the shortened position causes a reduction in the level of IIA MyoHC expression in the plantaris and gastrocnemius muscles and causes no detectable change in the soleus.

When passive stretch is combined with electrical stimulation, these two stimuli have a synergistic effect (Goldspink et al., 1992). Increases of up to 30% wet weight were recorded in the TA muscle of rabbits subjected to passive stretch and electrical stimulation for four days. This dramatic hypertrophy was accompanied by a concurrent increase in the expression of the slow type I mRNA and a decrease in the fast MyoHC mRNAs. The increase in type I MyoHC gene expression observed with stretch and stimulation is far more rapid than those changes observed with cross-innervation, chronic electrical stimulation or work overload.

1.5.4 Thyroid hormone

The multigene family of sarcomeric MyoHCs respond to thyroid hormone in a highly tissue specific manner. Izumo et al., (1986) investigated the effects of thyroid hormone on the expression of six separate MyoHC genes in seven different muscles of the rat. It was shown that all six MyoHC genes investigated are responsive to thyroid level and that the same MyoHC gene can be regulated by thyroid hormone in highly different modes, even in
opposite directions, depending on the tissue in which it is expressed. Furthermore, the embryonic and neonatal MyoHC isoforms can be reinduced by hypothyroidism in specific adult rat muscles. In general, hyperthyroidism either increases the mRNA level of the fast MyoHC isoforms or decreases the mRNA level of the slow β cardiac MyoHC isoform whilst hypothyroidism brings about the opposite changes.

In euthyroid rats there is little or no expression of the β cardiac MyoHC gene in the heart. Hypothyroidism however, causes a dramatic induction of this gene in the ventricles and a less pronounced induction in the atria. Hypothyroidism does not cause a significant induction of the β cardiac MyoHC gene in the soleus muscle and diaphragm, where expression is already high. Hyperthyroidism however causes a decrease in the expression of this gene in these muscles, indicating that normal physiological levels of thyroid hormone are capable of suppressing the expression of the β cardiac MyoHC gene in rat heart but not in the soleus or diaphragm. The expression of the α cardiac MyoHC gene is independent of thyroid hormone in the atrium but highly dependent of thyroid hormone in the ventricle where it is significantly reduced by hypothyroidism.

The fast IIA MyoHC gene exhibits a particularly striking response to changes in thyroid hormone in the rat. Generally the fast IIA MyoHC gene is up-regulated by thyroid hormone in slow twitch muscles and down regulated, with different degrees of sensitivity, in fast twitch muscles. The fast IIB MyoHC gene is responsive to thyroid hormone in some muscles, for example the soleus, masseter and diaphragm where hypothyroidism decreases its expression and hyperthyroidism slightly increases its expression, however, in other muscles the IIB MyoHC gene does not respond to thyroid hormone level.

Given this complex regulation of MyoHC gene expression in different muscle tissues, the question of how thyroid hormone exerts its effects on gene expression arises. Whilst the complex signal transduction pathways affecting MyoHC gene expression are largely unknown, recent studies have shed some light on how thyroid hormone effects transcription of the α cardiac MyoHC gene in heart (Morkin, 1993). The known nuclear effects of thyroid hormone are mediated by DNA binding proteins which belong to the c-erbA / retinoic acid receptor family of transcription factors (Yu et al., 1991; Glass et al., 1989). Moreover, thyroid responsive elements have been identified within the 5' flanking region of the α cardiac MyoHC gene (Flink and Morkin, 1990; Markham et al., 1987; Tsika et al., 1990) and
have been shown to be necessary for transcriptional activation by thyroid hormone using deletion and mutagenesis studies in cultured cardiac cells (Ojamaa and Klein, 1993). There is also evidence for the existence of a negatively acting thyroid responsive element in the β cardiac MyoHC gene 5' flanking region (Morkin, 1993). Additional interactions between thyroid hormone and steroid hormones at both cellular and molecular levels are likely to play an important role in the overall pattern of MyoHC gene regulation in the heart and skeletal muscles (Morkin, 1993).

1.5.5 Transcription factors

The members of the MyoD family of transcription factors (MyoD1, myogenin, myf5 and MRF4) contain a structural motif termed the helix-loop-helix (HLH) and following dimerisation with other HLH proteins bind to specific DNA sequences in the promoter region of muscle specific genes, regulating their expression. This family of transcription factors play an important, but as yet not fully understood, role in muscle fibre differentiation (Weintraub et al., 1991) and it has been suggested that each member of the family regulates a different subset of muscle specific genes and contributes to the formation of different types of fast and slow muscle fibres (Miller, 1991). Myogenin, MyoD and myf5 have also been shown to be present in a species of electric fish (*Torpedo californica*) where they are expressed in both skeletal muscle and the electric organ (a skeletal muscle homolog) (Neville and Schmidt, 1992).

Whether the MyoD family of helix-loop-helix proteins play a role in maintaining the distinct patterns of gene expression in the fibre types of the adult remains to be established. Hughes et al. (1993b) have shown that MyoD and myogenin mRNAs selectively accumulate in hind limb muscles of the adult rat such that MyoD is prevalent in fast twitch muscles and myogenin in slow twitch muscle. Moreover, the distribution of MyoD and myogenin transcripts within a single muscle were also shown to correlate with areas of fast glycolytic and slow oxidative muscle fibres respectively. This, together with the fact that alterations of the fast/slow fibre type distribution by thyroid hormone treatment or by cross-innervation resulted in a corresponding alteration in the MyoD/myogenin mRNA distribution, led Hughes and coworkers to hypothesise that MyoD and myogenin may be regulatory
molecules involved in the mechanisms whereby the stimuli described previously in this chapter are integrated to allow functional adaptations in adult muscle fibres.

1.6 Aims

A major determinant of the contractile characteristics of a skeletal muscle fibre is the MyoHC isoform content. Hence, polymorphisms within the MyoHC family play a major role in determining the locomotor ability of an organism. Whilst much work has been carried out characterising the MyoHC isoform family in mammals and chicken, comparatively little work has been done to characterise the MyoHC isoforms present in fish species. The functional demands placed on the locomotor musculature of fish species differ in many ways from those of terrestrial animals, not least, the temperature range over which the muscle has to function. Therefore, as well as allowing interesting cross species comparisons, further information regarding MyoHC polymorphism in fish would also provide a valuable insight into the molecular mechanisms employed by fish in muscle adaptation during development and in adult life.

The carp (*cyprinus carpio*) was chosen as an experimental animal since this species, like many other cyprinids, has a remarkable ability to adapt the contractile characteristics of skeletal muscle in response to seasonal changes in environmental temperature (described in more detail in Chapter 5). Earlier attempts to elucidate MyoHC polymorphisms in fish species at the protein electrophoretic level have had minimal success due to an inherent instability of fish myosin and similar overall charge and mass of the isoforms leading to comigrations. It was therefore decided to conduct this investigation of MyoHC polymorphism in carp at the level of MyoHC gene expression. The initial aim of this work was to obtain specific gene probes for individual isoforms of the MyoHC in carp. Once isolated these probes were used to investigate tissue specific, developmental and adaptational expression patterns of MyoHC isoforms in carp.
Chapter 2

Isolation of specific probes for individual isoforms of the carp myosin heavy chain gene.
2.1 Introduction

The first MyoHC gene to be sequenced completely was the unc-54 gene of nematode (*Caenorhabditis elegans*) (Karn et al., 1983). cDNA clones encoding this gene were prepared from mRNA enriched for MyoHC transcripts by size selection (MacLeod et al., 1981). Putative MyoHC cDNA clones were then selected by hybridisation to mRNA from nematodes with a mutant unc-54 gene which produces shortened unc-54 transcripts. Deduced amino acid sequence from the cDNA clones isolated confirmed that they encoded a protein homologous to existing published MyoHC protein sequences. A nematode genomic library was then screened with a cDNA clone containing the unc-54 transcript allowing sequencing of this gene at the genomic level.

Rat skeletal muscle MyoHC gene sequences were first obtained from L6E9 cells, a subclone of the L6 rat myogenic cell line, by Medford et al., 1980. In the differentiated myotubes from this cell line 50% of the RNA transcripts present are from MyoHC genes. Medford et al., (1980) isolated polysomal RNA from such differentiated cells and further enriched for MyoHC transcripts by size fractionation with sucrose-gradient density centrifugation. The 26S region of this gradient was shown to be highly enriched for MyoHC transcripts by cell free translation experiments and was subsequently used for cDNA synthesis and cloning. A recombinant DNA plasmid, designated pMHC25, isolated by such cloning was subsequently shown to contain a rat skeletal muscle MyoHC gene. The identity of the MyoHC sequence insert in pMHC25 was determined by muscle tissue specificity, inhibition of MyoHC protein synthesis *in vitro* by hybrid-arrested translation, and hybridization to a 33S cytoplasmic mRNA found only in differentiated muscle cells.

A third strategy which has been successfully used to obtain MyoHC sequences is to raise polyclonal antibodies against the MyoHC protein and then screen cDNA expression libraries for positive clones, for example, DeLozanne et al., 1985; Eller et al., 1989; Stedman et al., 1990; Sun & Chantler, 1991; Nyitray et al., 1991 and Moore et al., 1992.

Once regions of nucleotide sequence for MyoHC genes had been isolated, these gene fragments could be used to probe cDNA and genomic libraries from a variety of species and most of the sequences in the literature to date have been isolated in this way (Robbins et al., 1986; Feghali & Leinwand, 1989; Yamauchi-Takahara et al., 1989; Bober et
As previously described in Chapter 1, the sarcomeric MyoHCs show a high degree of sequence homology both between and within species. Comparisons of the light meromyosin nucleotide sequences of five members of the chicken fast MyoHC family showed that extremely high homology exists within this family (Moore et al., 1992). All of the chicken sequences studied showed greater than 90% homology to each other and two of the most similar isoforms shared complete nucleotide identity in two regions of over 250 base pairs. The high sequence homology between MyoHC isoforms means that nucleotide probes to be used for the characterisation of the expression patterns of different isoforms within the same species have to be chosen with great care. Large probes which cover coding region of the gene have been shown to cross hybridise to a number of MyoHC isoforms within the same species (Eller et al., 1989; Stedman et al., 1990) and are therefore of limited use in determining the expression patterns of individual isoforms. The 3' untranslated region (3'UTR) of the gene however shows considerable divergence between isoforms since in this region the evolutionary pressure to maintain protein sequence is removed. The 3' UTRs of MyoHC genes from a variety of species have been used extensively in hybridisation experiments to characterise the expression of individual isoforms (Morgan and Loughna, 1989; Sutherland et al., 1991; DeNardi et al., 1993).

The aim of the work presented in this chapter was to isolate 3'UTRs from carp MyoHC isoforms. Once subcloned these regions could be used as gene specific probes to facilitate the characterisation of the expression patterns of different MyoHC isoforms in carp. Gerlach et al., (1990) isolated 28 MyoHC λ clones from a carp genomic library by hybridisation with two rabbit cDNA clones; pMHCβ174 which is a slow MyoHC specific probe and pMHC20-40 which is a fast MyoHC specific probe. Turay (1991) sequenced various randomly chosen fragments derived from these carp genomic λ clones and generated partial sequence information for coding regions of carp MyoHC genes. One of these sequenced fragments, a 1.1 Kb Hind III fragment from the clone λFG2, yielded sequence data from intron 39 and part of exon 40, the penultimate exon of the gene. This fragment was therefore a good starting point for the current study as it was likely to contain the 3' untranslated region of that particular isoform and the exon 40 sequence isolated from this clone could possibly be used to locate 3' sequences from other myosin λ clones by Southern
hybridisation.

2.2 Materials and methods

2.2.1 Genomic Bacteriophage λ clones

Lysates from twenty eight different bacteriophage λ clones (λ 2001) which contained carp MyoHC gene sequences were a kind gift from Gerlach et al., (1990).

2.2.2 Preparation of bacteriophage λ DNA

Titration of bacteriophage λ lysate stocks and small scale bacteriophage λ DNA preparations were performed according to Maniatis et al.,(1989). Large scale, high purity, λ DNA preparations were made by a modified version of the method described by Reddy et al.,(1987) as follows:

A single colony of the E. coli strain NM539 was grown overnight in 100ml of LB medium supplemented with 0.2% (w/v) maltose and 10mM MgSO₄. Lysates of the λ phage clones were diluted in SM buffer (10mM Tris HCl pH 7.4, 10mM MgSO₄ and 0.01% (w/v) gelatin) to give 0.5 x 10⁸ PFU /ml and 1 ml of this dilution was added to 5 ml of the overnight culture and incubated at 37°C for 20 minutes to allow the phage particles to adsorb to the E. coli cells. Prewarmed LB medium (250 ml) supplemented with 10mM MgSO₄ in a 2 litre Erlenmeyer flask was then inoculated with the phage/E.coli mixture and grown at 37°C. Growth of the culture was monitored by measuring the optical density at 600nm. After lysis had occurred, about 4-5 hours later, 1.25 ml of chloroform and sodium chloride crystals, to a final concentration of 0.5 M, were added and the culture incubated for a further 10 minutes. Cell debris were then removed from the lysate by centrifugation at 6000g (4°C) for 30 minutes. Polyethylene glycol (PEG) M.W 8000, was added to a concentration of 10% (w/v) and the solution stirred at room temperature until the PEG had dissolved. The lysate was then incubated on ice for 1 hour followed by centrifugation at 6000g (4°C) in order to precipitate the phage particles. The pellet of phage particles was then gently resuspended in 5 ml of TM buffer (50 mM Tris HCl pH 7.5, 10 mM MgSO₄) and
extracted with one volume of chloroform by centrifugation at 2000g for 10 minutes. The PEG solution was back extracted with a further 2.5 ml of TM buffer and the two aqueous phases combined. The phage suspension was then applied to a 1.5 x 30 cm DEAE-cellulose column (prepared according to the manufacturers instructions) and the phage particles eluted with 35 ml of TM buffer. Aliquots of 1ml fractions collected from the column were then screened by agarose gel electrophoresis in order to determine which fractions contained the phage particles (5μl of fraction, 2μl of 0.1M EDTA, 2μl of 2% (w/v) SDS and 1μl 10x DNA gel loading buffer were mixed and run on a 0.7 % agarose gel in TBE buffer). The purest fractions were combined and NaCl added to a final concentration of 400mM. Isopropanol was added (40% of initial volume) and the solution was incubated at -20°C for 15 minutes. After centrifugation at 6000 x g (4°C) for 10 minutes, the pellet of phage particles was resuspended in 1ml of TE buffer, phenol chloroform extracted, ethanol precipitated and the resulting λ phage DNA dissolved in 500μl TE buffer (10mM Tris-HCl, 1mM EDTA, pH8.0)

2.2.3 Restriction endonuclease mapping of λ clones

Restriction endonuclease enzymes were obtained from the following commercial sources: Amersham International, Boehringer Mannheim, Northumbria Biologicals, Promega and Pharmacia. Restriction digests were carried out to completion according to the manufacturers recommendations and products analysed by both agarose (0.6% in TBE buffer) and polyacrylamide (8% 19:1 acrylamide : bis-acrylamide in TBE buffer) gel electrophoresis.

2.2.4 Transfer of DNA to Nylon membranes

Restriction endonuclease digested λ and plasmid DNA were transferred from agarose gels to positively charged nylon membranes (Zeta-probe GT) by alkaline capillary transfer according to the manufacturers instructions (BIO-RAD). DNA was fixed to the nylon membrane by baking at 80°C for 2 hours.
2.2.5 Radionucleotide labelling of DNA probes

DNA probes larger than 300 base pairs were labelled with $\alpha^{32}$P dCTP (3000 Ci/mmol) (Amersham International) by the random priming method described by Feinberg and Volgstein, (1983).

For DNA probes smaller than 300 base pairs a "specific priming" method was used to incorporate $\alpha^{32}$P dATP (3000 Ci/mmol) (Amersham International). The DNA to be labelled was excised from the vector DNA with restriction endonucleases in such a way that a portion of polylinker was left attached to the 3' end. This area of polylinker contained one of the standard vector primer sequences, for example T7, T3, M13 forward or M13 reverse. The same reaction conditions were then followed as described by Feinberg and Volgstein, (1983) except the random hexanucleotide primer was replaced with the appropriate sequencing primer at a concentration of 0.3µM.

2.2.6 Southern hybridisations

Southern hybridisations, in rotating glass bottles, were performed in a purpose designed hybridisation oven (Hybaid). Pre-hybridisation, hybridisation and washing solutions were those of the standard protocol recommended by BioRad, the manufacturers of the nylon membrane. Hybridisations, and subsequent washes, were carried out at either 20°C (for high stringency) or 35°C (for low stringency) below the calculated melting temperature of the probe duplex with a sodium ion concentration of 0.5M and no formamide present. Where the sequence of the probe was unknown, hybridisation and washing temperatures of 65 °C, for high stringency and 50°C for low stringency were used.

Hybridised probe was detected by exposure of the washed membrane to X-Ray film (Du-Pont) at -70 °C using cassettes with intensifying screens.
2.2.7 Subcloning of DNA fragments derived from carp genomic λ clones

Restriction endonuclease fragments of the carp genomic λ clones were subcloned into various versions of the pBluescript phagemid (Stratagene) by standard DNA cloning techniques as described by Ausubel et al., (1992). The *E. coli* strain XL1-Blue (Bullock et al., 1987) was used for transformations. Both insert and vector DNA were electrophoretically purified before ligations were performed.

2.2.8 Preparation of plasmid DNA

Crude, small scale preparations of plasmid DNA were performed by the method described by Zhou et al., (1990). The quality of DNA isolated by this method was sufficient for endonuclease reactions but not good enough for DNA sequencing, due to contaminating bacterial nucleic acids. For high quality preparations of plasmid DNA commercially available kits (Promega Magic minipreps and Qiagen columns from Hybaid) were used according to the manufacturers instructions.

2.2.9 Sequencing of plasmid DNA

Plasmid DNA (5μg) was made single stranded by the alkaline denaturation method described by Wang, (1988). Dideoxy-chain termination sequencing (Sanger et al., 1977) was then carried out with Sequenase (Version 2.0) enzyme according to the manufacturers instructions (Amersham). Gel electrophoresis was carried out using either the Macrophor sequencing system from Pharmacia or the S2 system from BRL. DNA sequence analysis was performed with the PC-GENE computer program from Intelligenetics.
2.3 Results

2.3.1 Isolation of the 3' end of the myosin heavy chain gene cloned into λFG2

The genomic clone λFG2 was restriction mapped fully with *Xba*I and *EcoRI* restriction enzymes and partially with the enzyme *Hind III* (Figure 2.1). A 1.1Kb *HindIII* fragment of this clone was subcloned into Bluescript KS + at the *HindIII* site and named pFG2H1.1. Partial sequencing of pFG2H1.1 showed that this subclone terminated 10 base pairs after the end of exon 40 (Figure 2.1) and so did not contain the 3' untranslated region as had been postulated from analysis of the partial sequence data of Turay (1991).

Hybridisation studies confirmed the restriction map in Figure 2.1 and showed that pFG2H1.1 is located on a 4.3 Kb *EcoRI* fragment of λFG2 (Figure 2.2). This 4.3 Kb *EcoRI* fragment was subcloned into the *EcoRI* site of Bluescript KS+ (Stratagene), named pFG2E4.3 and restriction mapped. The subclone, pFG2Exbl was constructed by digesting the plasmid pFG2E4.3 with *XbaI* followed by re-ligation of the plasmid and remaining insert.

After further subcloning and sequencing of pFG2Exb1, the 3' end of the λFG2 MyoHC isoform was located. A plasmid containing only 3' untranslated region sequence (named FG2UTR) was generated by subcloning the 168bp *NsiI* to *NsiI* (positions 324 to 492 in Figure 2.3) into Bluescript KS + at the *PstI* site.
Figure 2.1. Restriction maps of \( \lambda \text{FG2} \) and associated subclones

\[
\begin{array}{c}
\text{Short arm (9 Kb)} \\
\text{1 2 3 4 5 6 7 8 9} \\
\text{XbaI} \\
\text{XbaI} \\
\text{EcoRI} \\
\text{XbaI} \\
\text{XbaI} \\
\text{EcoRI} \\
\text{HindIII} \\
\text{HindIII} \\
\text{pFG2H1.1 (1.1 Kb)} \\
\text{39 40} \\
\end{array}
\]

\[
\begin{array}{c}
\text{FG2UTR (168 bp)} \\
\text{3' Untranslated region} \\
\text{pFG2E4.3 (4.3 Kb)} \\
\text{pFG2EX40 (200 bp)} \\
\text{pFG2Exb1 (1.0 Kb)} \\
\end{array}
\]

KEY
- Exons which have been sequenced and located to date
- 3' untranslated region

\[
\begin{array}{c}
\lambda \text{FG2} \\
(16 \text{ Kb}) \\
\text{Long Arm (20 Kb)} \\
\end{array}
\]

\[
\begin{array}{c}
pFG2H1.1 \\
(1.1 \text{ K b}) \\
\end{array}
\]

\[
\begin{array}{c}
pFG2EX40 \\
(200 \text{ bp}) \\
\end{array}
\]

\[
\begin{array}{c}
pFG2E4.3 \\
(4.3 \text{ Kb}) \\
\end{array}
\]

\[
\begin{array}{c}
pFG2Exb1 \\
(1.0 \text{ Kb}) \\
\end{array}
\]

\[
\begin{array}{c}
\text{FG2UTR} \\
(168 \text{ bp}) \\
\end{array}
\]

\[
\begin{array}{c}
3' \text{ Untranslated region} \\
\text{NeII} \\
\text{NeII} \\
\end{array}
\]
Figure 2.2 Restriction digests of λFG2

Single and multiple restriction digests of the genomic clone λFG2 were separated by electrophoresis on a 0.6% agarose gel (Panel A) and blotted to nylon membrane. The insert of the plasmid pFG2H1.1 was labelled with $^{32}$P α dATP (Amersham) as described in 2.2.5 and hybridised with the membrane under high stringency conditions (2.2.6). The washed membrane was then exposed to X-ray film (Fuji RX) at -70°C for 18 hours (Panel B). Lanes are as follows: 1. Marker DNA ($\lambda$ DNA HindIII cut). 2. EcoRI. 3. EcoRI plus HindIII. 4. HindIII. 5. HindIII plus BamHI. 6. Marker DNA (1 Kb ladder, Gibco BRL). 7. BamHI. 8. BamHI plus EcoRI. 9. EcoRI partial digest. 10. HindIII plus BamHI plus EcoRI. Size markers (Kb) are indicated by the arrows.
Figure 2.3 Nucleotide sequence of the 3' end of \( \lambda \text{FG2} \)

The sequence is numbered from the \( XbaI \) site of FG2Exb1 to the second \( Rsal \) site (see Figure 1.1). Deduced protein sequence of the exons is given in the one letter I.U.P.A.C code and a putative polyadenylation signal (AATAAA) highlighted in bold underlined text.

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**STOP**
2.3.2 The use of λFG2 derived subclones to isolate 3' untranslated regions from other carp myosin heavy chain isoforms

Since comparison of amino acid sequence data from the carboxyl termini region of mammalian MyoHC isoforms showed a relatively high degree of sequence homology (Figure 2.8), it was hypothesised that DNA subclones derived from the equivalent region of the carp λFG2 MyoHC isoforms would cross hybridise with other carp MyoHC isoforms at low stringency. Therefore, in order to locate the 3' ends of other carp MyoHC gene isoforms, restriction endonuclease digests of the remaining twenty seven carp genomic λ clones were probed under low stringency conditions with the insert of the plasmid subclone pFG2H1.1 and with pFG2EX40, a probe containing only the exon 40 sequence of λFG2 (see Fig 2.1). To eliminate those genomic clones which may have contained the same MyoHC isoform as λFG2, fragments which hybridised to the λFG2 exon 40 containing subclones were rescreened under high stringency with the insert of FG2UTR which contains only the

<table>
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<th>Endonuclease</th>
<th>Size of fragment (Kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FG4</td>
<td>SacI</td>
<td>6.0</td>
</tr>
<tr>
<td>&quot;</td>
<td>HindII</td>
<td>2.2</td>
</tr>
<tr>
<td>FG17</td>
<td>SacI</td>
<td>2.0</td>
</tr>
<tr>
<td>&quot;</td>
<td>RsaI</td>
<td>0.34</td>
</tr>
<tr>
<td>FG11</td>
<td>SacI</td>
<td>6.1</td>
</tr>
<tr>
<td>FG16</td>
<td>SacI</td>
<td>1.5</td>
</tr>
<tr>
<td>FG19</td>
<td>SacI</td>
<td>8.0</td>
</tr>
<tr>
<td>&quot;</td>
<td>HindII</td>
<td>0.5</td>
</tr>
<tr>
<td>&quot;</td>
<td>PstI</td>
<td>1.6</td>
</tr>
<tr>
<td>FG23</td>
<td>SacI</td>
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</tr>
<tr>
<td>FG50</td>
<td>SacI</td>
<td>1.8</td>
</tr>
<tr>
<td>FG47</td>
<td>HindIII</td>
<td>1.6</td>
</tr>
<tr>
<td>FG24</td>
<td>HindIII</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Table 2.1 Restriction fragments of λ Genomic clones that hybridise to probes containing exon 40 from λFG2
3' untranslated region sequence of the MyoHC gene contained in the genomic clone λFG2.

Out of the twenty seven genomic clones screened, a total of nine were found to contain restriction endonuclease fragments which hybridised to both pFG2H1.1 and pFG2EX40 (Table 2.1). Only one fragment, a 1.8 Kb SacI fragment from the clone λFG50, hybridised to the 3' untranslated region probe (FG2UTR), suggesting that this clone contains the same MyoHC isoform as the clone λFG2. These 9 fragments were subcloned into Bluescript KS⁺ and restriction digests of these subclones were re-screened, again under low stringency conditions, restriction mapped and partially sequenced. A 25mer oligonucleotide primer, FG2EXN40 (5' AGGAAGGTCCAGCACGAACTGGAGG 3') which corresponds to sequence obtained from λFG2 (positions 108 to 132 in Figure 2.3) was used as a sequencing primer to facilitate sequencing into the 3' ends of the subclones.

By this method of hybridisation followed by subcloning and DNA sequencing, a further three distinct MyoHC gene sequences were isolated from three genomic clones (λFG19, λFG17 and λFG16). The sequence data derived from these three clones is presented in Figures 2.4, 2.5, and 2.6. By comparison with the deduced amino acid sequence of the equivalent region from mammalian MyoHC isoforms (Figure 2.8), it was possible to assign putative exon/intron boundaries within the genomic sequences. Subsequent analysis at the cDNA level (Chapter 3) confirmed the exon intron boundaries for the genomic clone λFG17.
Figure 2.4 Nucleotide sequence obtained from the 3' end of λFG19

The deduced protein sequence of the exons is given in the one letter I.U.P.A.C code and a putative polyadenylation signal (AATAAA) highlighted in bold underlined text.
Figure 2.5 Nucleotide sequence obtained from the 3' end of λFG17

The deduced protein sequence of the exons is given in the one letter I.U.P.A.C code and the polyadenylation signal (ATTAAA) highlighted in bold underlined text. This polyadenylation site has been confirmed at the cDNA level (see Chapter 3).

10 20 30 40 50
CTTGAGTGTGAATTTTTTGACATTTTCTATTCAATACATTCAGTGCCATGAG
60 70 80 90 100
TGCAATGCTAAAGGGGTTTCTATCTGCTGTATCACTTACAGGAGGAGCAG E E Q

110 120 130 140 150
ACCAACACTCACCCTGTCCAAAGTTTACAGGAAGGTGACGCACGAGCTGGAGGAG

160 170 180 190 200
GCTCAGGAGCGTGTAGTTGCTAGTCACTAGCTACACAAAAGCTTAAGCC

210 220 230 240 250
AAGAGCGCAGCTGGAAGGTATAGTAAAGCAAATTTGCAGGGGGGTGGG

260 270 280 290 300
GGTGTTGGGATAATATTTTTTCAATTTTTGTTTTTGGTAA

310 320 330 340 350
CTTAAGGACAGAAGATGAGATGAGACAGACCACACACATCTAC

360 370 380 390 400
GCAAAGCATATAATATGACTTTCTGTGCTGTCTATATATGCAATTAAATAT

410 420 430 440 450
ACAGTTTCTGAGGACCTCTCGTTTTATGCTATTTGAAACTGGTAGAT

460 470 480 490 500 510
CTTGGAAGTGAGGATGCAAGGATATAGGATTTGCTACTGCTACTGCTG

520 530 540 550
AGGAAATCATGATTCCATCATCAACTATAGCAATTCTAAAT

57
The deduced protein sequence of exon 40 is given in the one letter I.U.P.A.C code. The sequence for exon 41 and the 3' untranslated region could not be located from the sequencing performed on this clone and is presumed to be further 3' than the sequence presented here.
2.3.3 Sequence data analysis

Nucleotide sequence alignment (Figure 2.7) was performed in order to demonstrate that the genomic sequences obtained from the clones λFG2, λFG16 λFG17 and λFG19 correspond to distinct carp MyoHC genes. The nucleotide sequence from within the putative exons of the clones show a high degree of homology between each of the individual clones. However, none of the corresponding amino acid sequences for this region were identical to each other (Figure 2.8). The intron between the putative exons 40 and 41 varies in length between clones and shows considerable sequence divergence between clones. Analysis of the three putative 3'UTRs isolated from the genomic clones λFG17, λFG2 and λFG19 showed that areas of homology also existed within this region. The 3'UTRs of the clones λFG2 and λFG17 show extensive regions of homology.

Figure 2.7 Multiple alignment of carp MyoHC sequences

Sequence data was aligned using the computer program "CLUSTAL" (Higgins and Sharp., 1988 and 1990) with the parameters set to an open gap cost of 10 and a unit gap cost of 10. The computer program has inserted gaps within the sequences in order to present the best alignment. The putative exon sequences are shown as bold text. The homology (percentage identity) between pairs of complete sequences is as follows.

FG2 and FG17=75%  FG2 and FG19=71.5%
FG2 and FG16=64.8%  FG17 and FG19=67.0%
FG19 and FG16=60.1%  FG16 and FG17=59.8%

FG16  ACAGGAAAGGTGCGAC
FG17  GAGGACAGACCAACACTCACCCTGTTCCAGGATCGTACGAC
FG2   GAGGACAGCCACAACACTCACCCTGTTCCAGGATCGTACGAC
FG19  GAGGACAGCCACAACACTCACCCTGTTCCAGGATCGTACGAC

-----------------------------------Exon 40-----------------------------------
FG16  GAGCTGGAGGAGCTGACATTTAGTGAGTCTCCAGGT
FG17  GAGCTGGAGGAGGCTCAGGAGCGCGCTGACATTGCTGAGTCCCAGGTC
FG19  GAACTGGAGGAGCTGAGGAGCGCGCTGACATTGCTGAGTCCCAGGTC

[-------------------Exon 40-------------------]

FG16  AACAAGCTGAGGCAAAGGCGGTAAGCTGGGAAGGTAACAAC
FG17  AACAAGCTGAGGCAAAGGCGGCGACGCTGGGAAGGTA----------------
FG2   AACAAGCTGAGGCAAAGGCGGATGCTGGGAAGGTA-----------
FG19  AACAAGCTGAGGCAAAGGCGGACGCTGGGAAGGTAACAAAGGCA

[-------------------Exon 40-------------------]

FG16  TATAGAAGTTTGCTAAAAATGAGACATTTGTCATTTAAAGTATATTTCTGAG
FG17  GATGAGCCATCACACCAGCTACAAGCAAGCATATAATATGACTTAC
FG2   AATGATGCATCAGACCAGCTACAAGCAAGCATATAATATGACTTAC
FG19  AAGAAG----------------ATACCAACACTACAAGCAAGCATATAATAGACTTAC

FG16  AGTATAAGCCAAGATCACTTTAAACTGTCATTTAAAGTATATTTCTGAG
FG17  GATGAGCCATCACACCAGCTACAAGCAAGCATATAATATGACTTAC
FG2   AATGATGCATCAGACCAGCTACAAGCAAGCATATAATATGACTTAC
FG19  AAGAAG----------------ATACCAACACTACAAGCAAGCATATAATAGACTTAC

FG16  GAGTACAATAAATCCTATTTTTCTGAGAAGT-------------------ACAT
FG17  TTGTGGCTGTGTCTTTATATACTGCAATTTAAATACAGTTTCTCAAGGCACT
FG2   TTGTGGCTGTCTTTAAATGCTTTAAATACAGTTTCTCAAGGCACT
FG19  CTGGTGGCTGTCTTTAAATGCTTTAAATACAGTTTCTCAAGGCACT

FG16  TAAAGCTAATTTTCTGAGAAGT-------------------ACAT
FG17  TCGGTGGCTGTCTTTAAATGCTTTAAATACAGTTTCTCAAGGCACT
FG2   TCTGTTCTTCTCTTTTCTGAGAAGT-------------------ACAT
FG19  TCTCTCTTTCTGAGAAGT-------------------ACAT
FG16 ATACTAATAGCAGACTTGAATAAACTTATTTTTGTGTAAGGGAACAGAT
FG17 -----------------------------------TTGAACTGGTAGATCTTGGAAGTAGGGAT
FG2 GCCATGATCACAAGGTTCAATTACCAGGGAATGCATGAACTAATA
FG19

FG16 GAGAATTATGTAGAC-------------------------------------------------
FG17 GCAACGATTATAGATTTGTGGTACGATTATAGTCTGAGGAAATCATGA
FG2 AATGCCTTTTAATGAAAAATAGATCTAAGTTTGT
FG19

FG16 ----------------------------------------------------------
FG17 TTCACGTTCATCACAATATAGCATTCCATTAATT
FG2 ----------------------------------------------------------
FG19 ----------------------------------------------------------

Figure 2.8 Comparison of carp MyoHC deduced amino acid sequences with MyoHC isoforms in other species

FG2 EEPANTHLSRYRKVQHELEEAQERADIAESQVNKLRAKSRDAGK___SKDEE
FG19 EEPANTHLSRYRKVQHELEEAQERADIAESQVNKLRAKSRDAGK___SKDEE
FG17 EEPANTHLSRYRKVQHELEEAQERADIAESQVNKLRAKSRDAGK___SKDEE
FG16 LEESQERADIAESQVNKLRAKSRDAGK
1. HUMANEMB DEQANHLSRYRKVQHELEEAQERADIAESQVNKLRAKSRDFTSSRMWHESEE
2. RABBIT EEPQNVLSPFRKQHELEEAQERADIAESQVNKLRAKTRFDFTSSRMWHESEE
3. HUMAN  EEPANTHLSRYRKVQHELEEAQERADIAESQVNKLRAKSRDAGK___SKDEE
4. BABOON  EEPANTHLSRYRKVQHELEEAQERADIAESQVNKLRAKSRDAGK___SKDEE
5. RATb   EEPANTHLSRYRKVQHELEEAQERADIAESQVNKLRAKSRDAGK___SKDEE
6. RABBITb EEPANTHLSRYRKVQHELEEAQERADIAESQVNKLRAKSRDAGK___SKDEE
7. HUMANa  EEPANTHLSRYRKVQHELEEAQERADIAESQVNKLRAKSRDAGK___SKDEE
8. RATa   EEPANTHLSRYRKVQHELEEAQERADIAESQVNKLRAKSRDAGK___SKDEE
9. RABBITa EEPANTHLSRYRKVQHELEEAQERADIAESQVNKLRAKSRDAGK___SKDEE
10. HAMSTERa EEPANTHLSRYRKVQHELEEAQERADIAESQVNKLRAKSRDAGK___SKDEE
11. RATEOM  EEPANTHLSRYRKVQHELEEAQERADIAESQVNKLRAKSRDAGK___SKDEE

Key

(*) indicates amino acid residues conserved in all the sequences.
2.3.4 Generation of plasmid constructs

With the sequence data obtained from the genomic clones λFG19 and λFG17, it was possible to subclone restriction endonuclease fragments containing only 3' untranslated region sequence. These subclones were named FG19UTR and FG17UTR respectively. This was an important step as it would allow the preparation of isoform specific probes for future expression studies. FG19UTR contained the sequence from position 446 (a FokI restriction site) to position 555 (a HindII restriction site) in Figure 2.4. FG17UTR contained the sequence from position 335 to 484 (both FokI restriction sites). Further cloning details of these plasmid constructs are given in Appendix 1.

Since the last coding exon and stop codon of λFG16 could not be located in the sequence available for this clone, it was not possible to generate a 3' untranslated region plasmid for this particular MyoHC isoform.
2.4 Discussion

Analysis of the deduced amino acid sequence data (Figure 2.8) obtained from the 3’ ends of the genomic clones λFG2, λFG16, λFG17 and λFG19 confirmed that these clones contain MyoHC gene sequences. Furthermore, the fact that the intron between the putative exons 40 and 41 showed little homology and varied in length between clones suggests that these four genomic clones are derived from four separate MyoHC isogenes. This is also supported by the fact that none of the deduced amino acid sequences of the clones were identical to each other. Analysis of the 3’UTRs isolated from the genomic clones λFG17, λFG2 and λFG19 showed that areas of homology existed within this region. Whilst small regions of homology (10-20 base pairs) between the 3’UTRs of MyoHC isogenes from mammalian species have previously been reported (Saez and Leinwand, 1986), the homology present between the carp MyoHC 3’ UTRs is more extensive. The 3’UTRs of the clones λFG2 and λFG17 showed exceptionally high homology with each other (85% sequence identity) raising the question of whether these two clones correspond to alleles of the same MyoHC gene. However, since the probe FG2UTR failed to hybridise to λFG17 in high stringency Southern blots (see section 2.3.2), both the FG2UTR and FG17UTR probes can be used in future hybridisation studies with confidence that they will not cross hybridise, providing the stringency is high enough. The possibility of λFG2 and λFG17 being alleles of the same gene is discussed in further detail in Chapter 6 where it is related to additional information regarding expression patterns obtained with the 3’UTR probes of these two genes and can be compared to the 3’UTR sequences from other carp MyoHC genes isolated in Chapter 3.

An interesting finding from the restriction mapping and nucleotide sequencing performed on the genomic clone λFG2 was the apparent size of the gene. Despite the fact that the insert of the genomic clone λFG2 is only 16kb in length, it was shown to contain a full MyoHC gene from promoter region and translational start site through to the stop codon and 3’ untranslated region (see Figure 2.1). The 5’ end of the clone including exons 1-7 and the equivalent to the rat embryonic MyoHC exon 19 were sequenced by L. Gauvry and E. Hansen (Personal communication) during their efforts to characterise the promoter region of this gene. The equivalents to the rat embryonic exons 40 and 41 were sequenced
in this current study (Figure 2.3) and were shown to be located in the correct orientation to belong to the same gene as the sequence information obtained by Gauvrey and Hansen indicating that the full coding region of the gene is localised to 12 kb of the clone. This is approximately half the size of the complete mammalian and chicken MyoHC genomic sequences published to date, which range from 22.8 Kb to 24.6Kb (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 12 exons of the gene which have been sequenced to date are all of similar size to the equivalent exon in the rat embryonic MyoHC gene (Strehler et al., 1986). The introns however, are consistently shorter. Further evidence supporting this theory of shorter introns is presented in Chapter 5, where Northern blot analysis revealed that the corresponding mRNA transcript is approximately 6 Kb in length, the same size as the mammalian and chicken mRNAs (Strehler et al., 1986; Jaenicke et al., 1990; Molina et al., 1987; McNally et al., 1989; Kraft et al., 1989; Stedman et al., 1990; Matsuoka et al., 1991).

Of the four complete genomic MyoHC gene sequences published, 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 three carp genomic clones, where the equivalent of these five amino acids could be located, were all similar to the rat embryonic MyoHC in that these five amino acids were 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.

The methods of genomic clone analysis employed in this current study allowed the cloning of the 3'UTRs of three separate carp MyoHC genes which could be used to characterise their expression patterns. However, genomic clone analysis has several disadvantages. Firstly, it does not allow the exact ending of the 3'UTR to be determined. Secondly, it is very time consuming and thirdly, analysis at the genomic level does not give any indication of where or when the gene is expressed, or indeed if it expressed at all since
non expressed pseudo genes, like those present in the actin multigene family (Gomez-Pedrozo et al., 1987), are possible. Therefore, further analysis of MyoHC isoform diversity in the carp was conducted at the cDNA level as described in the following chapter.
Chapter 3
Isolation of carp myosin heavy chain 3'UTR's by RACE PCR.
3.1 Introduction

The polymerase chain reaction (PCR) (Mullis and Faloona, 1987) enables the amplification of specific DNA sequences by over a million fold, thus allowing analysis of gene sequences of extremely low abundance. Since its advent in 1987 many modifications of the basic PCR reaction have been developed for specific applications. One such modification is RACE (rapid amplification of cDNA ends) PCR (Frohman et al., 1988). This technique allows the amplification of nucleic acid sequences from mRNA template between a defined internal site and unknown sequence at either the 5' or 3' end of the mRNA transcript.

The standard PCR reaction requires two sequence specific primers which flank the region of sequence to be amplified. However, when the sequence of the region to be amplified is unknown, it is not always possible to design two sequence specific primers. RACE PCR offers a possible solution in many cases since it requires only one sequence specific primer. The second primer anneals to a homopolymeric tail at either the 3' or the 5' end of the cDNA. In the case of 5' RACE this homopolymeric tail is added to the cDNA with terminal deoxynucleotidyl transferase enzyme, whereas in 3' RACE the naturally occurring poly (A) tail is utilised.

Viewed in basic terms the polymerase chain reaction appears to be quite simple, merely involving the combination of a DNA (or cDNA) sample with oligonucleotide primers, deoxynucleotide triphosphates and Taq DNA polymerase followed by appropriate temperature cycles. However, in reality the PCR is a complicated and poorly understood chemical mixture where constantly changing kinetic interactions determine the quantity, quality and specificity of the product. Various parameters within the reaction mix drastically effect the yield and quality of the product, notably the magnesium ion concentration of the buffer used and the annealing temperature. Such parameters need to be optimised for each set of primers used. It is also worth noting that Taq DNA polymerase misincorporates nucleotides at a rate of approximately one in ten thousand (Eckert and Kunkel, 1991). Therefore, any sequence data derived from PCR products should be verified by multiple subclones from different reactions.

The aim of the work presented in this chapter was to utilise the RACE PCR method
to amplify 3' sequences of different isogenes of the myosin heavy chain from carp muscle. Comparisons of MyoHC C terminal amino acid sequences (Figure 2.8) show that the penultimate exon of the gene (the equivalent to exon 40 in the rat MyoHC gene (Strehler et al., 1986)) is well conserved between different isoforms both within the same species and between different species. Furthermore, the analysis performed in Chapter 2 demonstrated that the sequence derived from exon 40 of the genomic clone λFG2 cross hybridises with multiple isoforms of the carp MyoHC gene. Therefore, it was hypothesised that a primer designed from this sequence would facilitate the amplification of carp MyoHC isoforms by 3' RACE PCR. Any amplified MyoHC products could then be further characterised by DNA sequencing and their 3' untranslated regions subcloned to facilitate hybridisation studies investigating the expression patterns of individual carp MyoHC isoforms.

### 3.2 Methods

#### 3.2.1 Oligonucleotides

The oligonucleotide primers, RoRidT17 and Ro were synthesised based on the sequence designed by Harvey et al., (1991). The Ro primer corresponds to the first 25 nucleotides of the RoRidT17 primer which was used to prime the cDNA synthesis. The oligonucleotide primer FG2EXN40 was synthesised based on part of the exon 40 sequence of the genomic MyoHC clone λFG2 (from position 108 to 132 in Figure 2.3). The expected size of the amplified PCR product from the FG2 MyoHC gene is approximately 250bp long when the FG2EXN40 and Ro primers are used.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
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<tr>
<td>RoRidT17</td>
<td>5' ATCGATGGTCGACGATGCGATCCAAAGCTTGAAT</td>
</tr>
<tr>
<td></td>
<td>TCGAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT 3'</td>
</tr>
<tr>
<td>Ro</td>
<td>5' ATCGATGGTCGACGATGCGATCC 3'</td>
</tr>
<tr>
<td>FG2EXN40</td>
<td>5' AGGAAGGTCGAGCATGAACTGGAGG 3'</td>
</tr>
</tbody>
</table>
3.2.2 cDNA synthesis

Single stranded cDNA was synthesized from total RNA, prepared as described in 4.2.2. Muscle samples from carp at various stages of development and carp acclimated to different temperatures were used to prepare cDNA (Table 3.1). In the initial studies cDNA was also prepared from poly (A) RNA which had been isolated with oligo dT magnetic beads (DYNAL), however since no difference in PCR products was observed between cDNA from total RNA and cDNA from poly (A) RNA (Figure 3.3), total RNA was used for subsequent amplifications. Total RNA (20 μg) was resuspended in diethylpyrocarboante (DEPC) treated water, heated to 65°C for five minutes, then rapidly cooled on ice. To this was then added 40 units of RNase inhibitor (Promega), 5 μl of 5x reverse transcriptase buffer (500mM Tris-HCL, 600mM KCL, 100mM MgCl₂, pH 8.15 at 42°C), 5 μl of dNTP stock (5mM for each nucleotide), 500ng of the oligonucleotide primer RoRidT17, 0.5 μl 1M DTT, 0.5 μl (7.5 units) Rous associated virus reverse transcriptase (Amersham) and DEPC treated water to a final volume of 25 μl. Control reactions, identical to the above but with no reverse transcriptase enzyme added, were also set up to determine whether or not contaminations of genomic DNA, which may also be amplified by subsequent PCR reactions, were present. The reactions were then incubated at 42°C for 2 hours and then stored at -20°C until required.

3.2.3 PCR amplification

PCR amplifications of first strand cDNA were performed using a recombinant Taq DNA polymerase (Bioline). A typical PCR reaction contained 1 μl of unpurified first strand cDNA (synthesised as in 3.2.2), 5 μl 10X Taq polymerase buffer (Bioline), 1.0mM MgCl₂, 25 pmol Ro primer, 25 pmol FG2EXN40 primer, 2 μl dNTP stock (5mM of each nucleotide in stock), water to a final volume of 49.5 μl and 50 μl of mineral oil. For each set of PCR reactions a control tube, with no template added, was used to check for contaminations in the solutions used. Reactions were placed on a thermal cycler (PREM) and heated to 95°C for five minutes. Taq DNA polymerase (0.5 μl, 2.5 units) was added and 20 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes were performed. After the
amplification cycles, samples were incubated at 72°C for 5 minutes in order to extend
incomplete products. PCR products were then analysed by gel electrophoresis on 2%
agarose gels made in TAE buffer.

3.2.4 Cloning of PCR products

PCR products were isolated from agarose gels using a "Prepagene" kit (BIO-RAD)
and subcloned into Bluescript PBS+ phagemid (Stratagene) by the T-A cloning method
described by Marchuk et al., (1991). This method utilises the fact that Taq DNA polymerase
adds a single nontemplated adenosine nucleotide to the 3' end of the double-stranded
amplified product creating a single "A" overhang. Plasmid DNA was cut with the restriction
enzyme Smal and given a "T" overhang to facilitate ligation with the PCR product.
Ligations, transformations, plasmid DNA preparations and DNA sequencing were
performed as described in section 2.2.
Figure 3.1 Schematic representation of the RACE PCR method used

1. **Total RNA Extraction**
2. **RNA**:
   - 5' AAAAAAAAAAAA
   - 3' TTTTTTTTTTTTTTTTTT
   - 3' Primer 5' Annel RoRidT17 primer to mRNA
3. **cDNA synthesis**:
   - 5' AAAAAAAAAAAA
   - 3' TTTTTTTTTTTTTTTTTT
   - 3' cDNA is synthesised from all the mRNA transcripts present in the tissue
   - 5' FG2EXN40 primer
4. **PCR**:
   - 3' TTTTTTTTTTTTTTTTTT
   - 5' FG2EXN40 primer anneals specifically to MyoHC isoforms
   - 5' The 3' ends of MyoHC isoforms are specifically amplified by PCR
5. **Subcloning and sequencing**:
6. **Isolation of isoform specific probes**
3.3 Results

3.3.1 Optimisation of PCR conditions

The effect of varying the concentration of magnesium ions in the PCR reaction was investigated over a range of 0.5 mM MgCl₂ to 3.0 mM MgCl₂. PCR reactions were performed on cDNA prepared from adult carp white muscle total RNA using the primers FG2EX40 and Ro. The cycles employed were as follows: 94°C 1 minute, 55°C 1 minute, 72°C 2 minutes for 20 cycles, then 72°C for 5 minutes. (Figure 3.2).

![Figure 3.2 Optimisation of Mg²⁺ concentration for RACE PCR.](image)

Concentrations of magnesium are as follows. Lane (1). 0.5 mM. Lane (2). 1.0 mM. Lane (3). 1.5 mM. Lane (4). 2.0 mM. Lane (5). 2.5 mM. Lane (6). 3.0 mM.
The use of 0.5mM MgCl₂ increased the stringency of the reaction as only one band at a size of approximately 250 bp was observed. From 1.0mM to 3.0 mM MgCl₂ three bands were observed, one at 250bp one at 550bp and one at 800bp. The amount of the 250 bp band synthesised slightly decreased as the concentration of magnesium ions increased. On the basis of these results a standard concentration of 1.0mM MgCl₂ was used for subsequent experimental PCR reactions.

Varying the annealing temperature from 50°C to 65°C and varying the number of PCR cycles from 20 to 35 did not alter the pattern or intensity of bands amplified (data not shown). Therefore an annealing temperature of 55°C and a repetition of 20 cycles were used in subsequent experimental reactions.

### 3.3.2 Preliminary PCR amplifications

In order to establish the best starting material for cDNA synthesis, amplifications on cDNA prepared from both total RNA and poly (A) RNA of adult carp white muscle were performed. Control reactions with only one primer were also set up in order to investigate the possibility of spurious amplifications.

cDNA prepared from both total RNA and poly (A) RNA produced amplification of DNA products (Figure 3.3). These products originated from cDNA rather than contaminating genomic DNA, as no amplification was observed in the control reactions which had no reverse transcriptase. The band observed at approximately 800bp (Figure 3.3 lanes 2, 5 and 7) was ignored as an artifactual unspecific amplification since it was also present in control PCR reactions which contained only the Ro primer (Figure 3.3 lane 7). The 550 bp band observed in amplifications from adult white muscle (Figure 3.3 lanes 2 and 5) was subcloned and after sequencing shown to be from a Troponin T gene isoform (sequence data not shown).

The most prominent amplified band in each sample was between 230bp and 250bp, the expected size for the amplification of the FG2 MyoHC isoform. Rather than being a discrete sharp band indicative of a unique product the band appeared disperse suggesting that it was made up of slightly different sized products. The 70bp band observed in lanes 1, 3 and 4 (Figure 3.3) is due to primer dimerisation.
Figure 3.3 PCR reactions on total RNA and mRNA (polyA')

Lanes are as follows. (M). Marker lanes (1kb ladder Gibco BRL) (1). Total RNA no reverse transcriptase control. (2). Total RNA with reverse transcriptase. (3). No template control. (4). mRNA no reverse transcriptase control. (5). mRNA with reverse transcriptase. (6). FG2EXN40 primer alone (cDNA prepared from total RNA as template). (7). Ro primer alone (cDNA prepared from total RNA as template).

3.3.3 PCR amplifications of cDNA from various carp muscle samples

In order to obtain a wide range of different MyoHC isoform types, total RNA was extracted from red and white muscle types from carp at a range of ages and also from fish which had been acclimated to either warm or cold temperatures (described in Table 3.1). The cDNA prepared from these RNA samples was amplified by RACE PCR using the optimised conditions described in 3.3.1.

All tissue samples investigated yielded amplified DNA products (Figure 3.4), the most prominent of which was between 230 and 250bp. None of the control reactions with no reverse transcriptase gave amplified products, verifying that amplification had not occurred from contaminating genomic DNA.

In each sample the 230-250 bp band was subcloned as a whole and a number of
insert containing colonies taken for nucleotide sequencing. Sequence data from these colonies (Figure 3.6) showed that this band was composed of isoforms of the MyoHC gene and that variation in the length of the poly (A) tail was mainly responsible for the variation in length of the PCR products present in the diffuse band.

Figure 3.4 Electrophoresis of carp muscle amplification products.


For a more detailed description of the muscle tissues types used see Table 3.1.
3.3.4 Analysis of sequence data

Sequence data from the MyoHC containing clones generated by RACE PCR was aligned using the CLUSTAL DNA alignment program (Higgins and Sharp, 1988) with the parameters set at an open gap cost of 10 and a unit gap cost of 10 (Figures 3.5 and 3.6). The dendrogram produced from such alignments (Figure 3.5) illustrates that out of a total of 20 clones derived by PCR from various tissue samples, five distinct myosin heavy chain gene sequence types were isolated (arbitrarily termed types 1-5 in Table 3.1 and Figure 3.5). Sequence data from the equivalent coding and untranslated regions of the genomic clones λFG2, λFG17 and λFG19 was also included in the dendrogram alignment. λFG17 showed identical sequence homology to the PCR isolated clone 15JUV. Therefore in accordance with the nomenclature adopted for the PCR clones, this genomic clone was also classed as the type 2 MyoHC gene. None of the PCR isolated clones showed identical sequence homology to the genomic clones λFG2 and λFG17. These two clones were therefore classed as belonging to an additional two types of MyoHC gene (types 7 and 6 respectively).

It should be noted that the numerical nomenclature adopted for the separate carp MyoHC gene types is purely arbitrary and does not relate in any way to the nomenclature of MyoHC genes adopted for mammals or the nomenclature adopted for the gross classification of myosin protein families (Goodson and Spudich, 1993).
Table 3.1 Tissue types used for cDNA preparation

The resulting subclones from PCR on each cDNA preparation were named and classed as various MyoHC types by virtue of sequence homology with each other.

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Names of subclones sequenced</th>
<th>Type of MyoHC gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>White axial muscle from 3 year old carp, 23 cm in length. Acclimated to 28°C for 5 weeks.</td>
<td>WHITEH1, WHITEH2, WHITEH3</td>
<td>Type 1</td>
</tr>
<tr>
<td>Red muscle from 3 year old carp, 23 cm in length. Acclimated to 28°C for 5 weeks.</td>
<td>REDH1, REDH2</td>
<td>Type 1</td>
</tr>
<tr>
<td>White axial muscle from 3 year old carp, 24 cm in length. Acclimated to 10°C for 5 weeks.</td>
<td>WHITEC1, WHITEC2</td>
<td>Type 1</td>
</tr>
<tr>
<td>Red muscle from 3 year old carp, 24 cm in length. Acclimated to 10°C for 5 weeks.</td>
<td>REDC1, REDC2</td>
<td>Type 1</td>
</tr>
<tr>
<td>White axial muscle from 14 month old carp, 7 cm long acclimated to 20°C for 5 weeks.</td>
<td>15JUV</td>
<td>Type 2</td>
</tr>
<tr>
<td>Unhatched fertilised carp eggs 26 hours after spawning.</td>
<td>EGGS19, EGGS20, EGGS22, EGGS24</td>
<td>Type 3</td>
</tr>
<tr>
<td>60 day old carp fry, 1.2 cm long, acclimated to 15°C for 2 weeks. 3 whole fish were used for RNA extraction.</td>
<td>FRY152, FRY155, FRY153</td>
<td>Type 3</td>
</tr>
<tr>
<td>60 day old carp fry, 1.2 cm long, acclimated to 28°C for 2 weeks. 3 whole fish were used for RNA extraction.</td>
<td>FRY2810, FRY288, FRY2811</td>
<td>Type 5</td>
</tr>
</tbody>
</table>
Figure 3.5 Dendrogram of PCR product sequence alignments. Sequences were aligned using the CLUSTAL alignment program (Higgins and Sharp, 1988). The equivalent sequence data from the genomic MyoHC clones isolated in chapter 2 was also used in this alignment (FG2cDNA, FG19cDNA and FG17cDNA)
Figure 3.6 Nucleotide sequence alignment of myosin heavy chain PCR products

The PCR products sequenced are aligned according to the type of MyoHC they were classed as in the dendrogram alignment shown in Figure 3.5. (*) denotes positions where all the products within a type are identical and (.) indicates positions where differences in sequence were observed. (-) indicates positions where the sequence was shifted in order to maintain alignment. The first 25 nucleotides of each sequence correspond to the 5' primer, FG2EX40, used for the PCR. The stop codon for each sequence is indicated in bold text. Sequence to the left of the stop codon corresponds to coding regions equivalent to exon 41 and part of exon 40 of the rat embryonic MyoHC gene whereas sequence to the right of the stop codon corresponds to 3' untranslated region.

Sequence alignment of PCR products classed as type 1 myosin heavy chain.

WHITEH1  AGGAAGTTCCAGCACGAATGGAGGAGTCTCATGAGCGCGCTGACATCGC 50
REDH2    AGGAAGTTCCAGCACGAATGGAGGAGTCTCATGAGCGCGCTGACATCGC 50
REDC1    AGGAAGTTCCAGCACGAATGGAGGAGTCTCATGAGCGCGCTGACATCGC 50
WHITEC1  AGGAAGTTCCAGCACGAATGGAGGAGTCTCATGAGCGCGCTGACATCGC 50
WHITEC2  AGGAAGTTCCAGCACGAATGGAGGAGTCTCATGAGCGCGCTGACATCGC 50
REDC2    AGGAAGTTCCAGCACGAATGGAGGAGTCTCATGAGCGCGCTGACATCGC 50
WHITEH3  AGGAAGTTCCAGCACGAATGGAGGAGTCTCATGAGCGCGCTGACATCGC 50
WHITEH2  AGGAAGTTCCAGCACGAATGGAGGAGTCTCATGAGCGCGCTGACATCGC 50
REDH1    AGGAAGTTCCAGCACGAATGGAGGAGTCTCATGAGCGCGCTGACATCGC 50

* * * * * * * * * * * * *  * * * * * * * *  * * * * * * * * *  * * * * *  * * * * * * * *  * *

WHITEH1  CGAGTCCAGGTCAAACAGCTGAAGCCAGGACGCTGAAGCTGGAAGA 100
REDH2    CGAGTCCAGGTCAAACAGCTGAAGCCAGGACGCTGAAGCTGGAAGA 100
REDC1    CGAGTCCAGGTCAAACAGCTGAAGCCAGGACGCTGAAGCTGGAAGA 100
WHITEC1  CGAGTCCAGGTCAAACAGCTGAAGCCAGGACGCTGAAGCTGGAAGA 100
WHITEC2  CGAGTCCAGGTCAAACAGCTGAAGCCAGGACGCTGAAGCTGGAAGA 100
REDC2    CGAGTCCAGGTCAAACAGCTGAAGCCAGGACGCTGAAGCTGGAAGA 100
WHITEH3  CGAGTCCAGGTCAAACAGCTGAAGCCAGGACGCTGAAGCTGGAAGA 100
WHITEH2  CGAGTCCAGGTCAAACAGCTGAAGCCAGGACGCTGAAGCTGGAAGA 100
REDH1    CGAGTCCAGGTCAAACAGCTGAAGCCAGGACGCTGAAGCTGGAAGA 100

* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
Sequence alignment of the PCR product 15JUV (classed as type 2 myosin heavy chain) and the deduced cDNA sequence from the genomic clone FG17 isolated in chapter 2.

15JUV
AGGAAGGTCCAGCACGAACTGGAGGAGGCTCAGGAGCGTGCTGACGTTGC 50
FG17 cDNA
AGGAAGGTCCAGCACGAACTGGAGGAGGCTCAGGAGCGTGCTGACGTTGC 50

15JUV
TGAGTCACAGGTCAACAAGCTTAGAGCCAAGAGCCGCGACGCTGGGAAGA 100
FG17 cDNA
TGAGTCACAGGTCAACAAGCTTAGAGCCAAGAGCCGCGACGCTGGGAAGA 100

15JUV
GCAAGGATGAAAGATGAGCCATCACAACCACTACATAGCAAGACGC 150
FG17 cDNA
GCAAGGATGAAAGATGAGCCATCACAACCACTACATAGCAAGACGC 150

15JUV
ATATAATATGACTTACTTGTGCTGCTTATATATGCAATTAAATACAGT 200
FG17 cDNA
ATATAATATGACTTACTTGTGCTGCTTATATATGCAATTAAATACAGT 200

15JUV
TTCTCAAGGC(A)n 210
FG17 cDNA
TTCTCAAGGC(A)n 210

80
Sequence alignment of PCR products classed as type 3 myosin heavy chain

**Stop**

---

---

---
Sequence alignment of PCR products classed as type 4 myosin heavy chain

<table>
<thead>
<tr>
<th></th>
<th>Sequence</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGGS24</td>
<td>AGGAAGGTCCACCGAATCTGGAGGAGCGTGCAGACATTGC</td>
<td>50</td>
</tr>
<tr>
<td>FRY153</td>
<td>AGGAAGGTCCACCGAATCTGGAGGAGCGTGCAGACATTGC</td>
<td>50</td>
</tr>
<tr>
<td>FRY288</td>
<td>AGGAAGGTCCACCGAATCTGGAGGAGCGTGCAGACATTGC</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>TGAGTCTCAGCTGAAATCTCATGAGCATTGC</td>
<td>100</td>
</tr>
<tr>
<td>FRY153</td>
<td>TGAGTCTCAGCTGAAATCTCATGAGCATTGC</td>
<td>100</td>
</tr>
<tr>
<td>FRY288</td>
<td>TGAGTCTCAGCTGAAATCTCATGAGCATTGC</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>GCAAAGAGCATGAGCAAATCTCATGAGCATTGC</td>
<td>150</td>
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<tr>
<td>FRY153</td>
<td>GCAAAGAGCATGAGCAAATCTCATGAGCATTGC</td>
<td>150</td>
</tr>
<tr>
<td>FRY288</td>
<td>GCAAAGAGCATGAGCAAATCTCATGAGCATTGC</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>ATTTGTATCTGAAAATGTGAAATTGTTCAATAAAGCATTACATTCATCA- (A)n</td>
<td>199</td>
</tr>
<tr>
<td>FRY153</td>
<td>ATTTGTATCTGAAAATGTGAAATTGTTCAATAAAGCATTACATTCATCA- (A)n</td>
<td>200</td>
</tr>
<tr>
<td>FRY288</td>
<td>ATTTGTATCTGAAAATGTGAAATTGTTCAATAAAGCATTACATTCATCA- (A)n</td>
<td>200</td>
</tr>
</tbody>
</table>

stop

Sequence of FRY2811 classed as type 5 myosin heavy chain.

<table>
<thead>
<tr>
<th></th>
<th>Sequence</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRY2811</td>
<td>AGGAAGGTCCACCGAATCTGGAGGAGCGTGCAGACATTGC</td>
<td>50</td>
</tr>
<tr>
<td>FRY2811</td>
<td>TGAGTCTCAGCTGAAATCTCATGAGCATTGC</td>
<td>100</td>
</tr>
<tr>
<td>FRY2811</td>
<td>GCAAAGAGCATGAGCAAATCTCATGAGCATTGC</td>
<td>150</td>
</tr>
<tr>
<td>FRY2811</td>
<td>AGAGTAAACATGAAATAAATCTTCAATTTC(A)n</td>
<td>179</td>
</tr>
</tbody>
</table>

stop

3.3.4 Summary of MyoHC isoforms isolated

From the 20 clones derived from RACE PCR a total of five distinct MyoHC sequence types could be distinguished. All the clones derived from adult carp muscle (white and red muscle from warm and cold acclimated carp) contained the same MyoHC isoform (type 1). The single insert containing colony obtained from 14 month old carp (designated as type 2) contained a MyoHC sequence identical to the isoform contained in the genomic clone λFG17 described in Chapter 2. Unhatched fertilized carp eggs yielded two different
MyoHC sequences designated as types 3 and 4. These two sequence types were also isolated from 60 day old carp fry at both warm and cold temperatures. An additional MyoHC sequence type, designated as type 5, was also isolated from 60 day old carp fry acclimated to 28°C.

For the MyoHC sequence types classed as type 1, type 3 and type 4 where multiple clones with slight nucleotide differences were isolated, one clone was chosen as a representative sequence for each type (see Table 3.2) and was subsequently used for hybridisation studies.

Table 3.2 Sequence identity between the 3' ends of seven carp MyoHC isoforms

<table>
<thead>
<tr>
<th></th>
<th>WhiteC1 Type 1</th>
<th>15JUV Type 2</th>
<th>EGGS22 Type 3</th>
<th>EGGS24 Type 4</th>
<th>FRY2811 Type 5</th>
<th>λFG19 Type 6</th>
<th>λFG2 Type 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>74%</td>
<td>37%</td>
<td>39%</td>
<td>43%</td>
<td>58%</td>
<td>73%</td>
<td>Type 1</td>
<td></td>
</tr>
<tr>
<td>87%</td>
<td>43%</td>
<td>35%</td>
<td>40%</td>
<td>63%</td>
<td>85%</td>
<td>Type 2</td>
<td></td>
</tr>
<tr>
<td>86%</td>
<td>88%</td>
<td>42%</td>
<td>44%</td>
<td>40%</td>
<td>39%</td>
<td>Type 3</td>
<td></td>
</tr>
<tr>
<td>83%</td>
<td>89%</td>
<td>96%</td>
<td>65%</td>
<td>34%</td>
<td>41%</td>
<td>Type 4</td>
<td></td>
</tr>
<tr>
<td>83%</td>
<td>88%</td>
<td>95%</td>
<td>99%</td>
<td>39%</td>
<td>52%</td>
<td>Type 5</td>
<td></td>
</tr>
<tr>
<td>90%</td>
<td>93%</td>
<td>86%</td>
<td>86%</td>
<td>85%</td>
<td>60%</td>
<td>Type 6</td>
<td></td>
</tr>
<tr>
<td>92%</td>
<td>94%</td>
<td>89%</td>
<td>90%</td>
<td>89%</td>
<td>95%</td>
<td>Type 7</td>
<td></td>
</tr>
</tbody>
</table>

Legend

Nucleotide sequences were aligned using the CLUSTAL DNA alignment program (Higgins and Sharp, 1988) (parameters = open gap cost of 10, unit gap cost of 10) and the percentage identities between each pair of sequences shown in the table. The top right hand diagonal of the table presents the sequence identities between the 3' untranslated region sequences (from the stop codon to the first "A" of the poly (A) tail). For the genomic clones λF2 and λFG19 the site of the poly (A) tail was estimated by comparison with the other five types of isoform. The bottom left hand diagonal of the table presents sequence identities of the last two coding exons of the gene (exons 40 and 41). The intronic sequences of the genomic clones λFG2 and λFG19 were omitted for the purpose of this alignment.
Together with the three genomic clones for which 3' UTR sequences were obtained (λFG2, λFG17 and λFG19) a total of seven distinct MyoHC sequences from which isoform specific 3' UTR probes could be generated were isolated. A description of how 3'UTR containing probes were generated from these clones for subsequent hybridisation studies is given in Appendix 1.

Sequence identity values (Table 3.1) demonstrate the divergence of the 3'UTR sequence between different isoforms. The sequence identities for the 3'UTR sequence between two isoforms ranges from 34% between type 4 and type 6 to 85% between type 2 and type 7. The sequence identities of the coding regions presented ranges from 83% between type 1 and types 4 and 5 to 99% between type 4 and type 5.

Table 3.7 Deduced amino acid sequence alignment of PCR products

The coding regions for PCR products shown in Figure 3.6 were translated and the deduced amino acid sequences aligned. Deduced coding regions for the last two exons of the genomic clones FG2, FG17 and FG19 are also shown in the table. Conserved amino acid positions throughout the whole group are indicated with (*).
3.4 Discussion

In this current application of RACE PCR the aim was not to amplify exclusively a single specific product but to amplify closely related isoforms which may differ slightly in sequence at the position of the 5' oligonucleotide. Therefore the stringency of the reaction conditions needed to be low enough to allow amplification of closely related isoforms but at the same time high enough to prevent unspecific amplification of non myosin cDNA's. By the nature of 3' RACE PCR the 3' oligonucleotide must consist of a long tail of thymidine nucleotides, to facilitate annealing with the poly (A) tail of the RNA molecule during first strand cDNA synthesis. However, the use of a 3' oligonucleotide consisting of exclusively thymidine residues would involve using relatively low annealing temperatures in subsequent PCR reactions due to the lower melting temperature associated with A-T hydrogen bonds. The 3' oligonucleotide used in this application consisted of 17 thymidine nucleotides followed by an additional sequence. The addition of an extra sequence to the end of the 3' oligonucleotide used for cDNA synthesis was first described by Frohman, (1989) and has a two fold benefit. Firstly, it enables the use of the additional primer in subsequent PCR reactions, thereby allowing more stringent annealing temperatures, and secondly the oligonucleotide sequence can contain convenient restriction enzyme recognition sites which can be used to facilitate cloning.

Certain criteria were used for the choice of the 5' oligonucleotide used in this study. Firstly, the primer had to cover an area of the gene which is highly conserved between different isoforms of the MyoHC, thereby facilitating the amplification of multiple isoforms. Secondly, the primer had to be far enough 5' to give sufficient sequence data to verify that the amplification products were in fact myosin heavy chain genes and far enough 3' to allow resulting subclones to be fully sequenced using primers from either end of the plasmid polylinker. Thirdly, the oligo had to satisfy standard criteria for primer selection such as a G-C content of 40-60% and lack possible secondary structures. The oligo FG2EXN40 satisfies all the above criteria and covers an area of eight amino acids in exon 40 (R K V Q H E L E) which was identical in the carp MyoHC genes isolated from the genomic clones described in chapter 2 and highly conserved in MyoHC sequences from other species, (see Figure 2.6).
The number of cycles during the PCR amplification was limited to 20 in an attempt to minimise the chance of the Taq polymerase introducing misread nucleotides into the sequence. However, slight variations in sequence were observed between some of the subclones within each type (Figure 3.5). These variations in sequence were observed in both the coding and 3' untranslated regions, however almost all the base substitutions occurring in the coding region are silent substitutions, i.e. they do not change the translated amino acid. The only case observed where a substitution changes the translated amino acid is in the group of clones classed as type 3 myosin heavy chain. Here two members of the group have an asparagine residue instead of lysine sixteen amino acids from the stop codon (Table 3.7).

One of the clones classed as type 1 MyoHC (REDH1) had two variations within the first 25 nucleotides. Since this sequence corresponds to the primer FG2EXN40 used in the PCR reaction, these differences were unexpected. One possible explanation could be that the synthesis of the oligonucleotide FG2EXN40 was not 100% pure and contained trace amounts of molecules with erroneous bases.

Two of the sequence variations within the coding regions cause a frame shift. WHITEC1, a clone classed as type 1 MyoHC, has an additional two adenosine nucleotides nine nucleotides before the stop codon whilst FRY2810, a clone classed as type 3 MyoHC has an additional cytosine residue just before the stop codon. These additions were assumed to be artifacts due to misreading of the Taq DNA polymerase enzyme. However, since estimates of misread bases are in the order of one in every ten thousand bases amplified (Eckert and Kunkel, 1991), the total number of base differences observed in all the clones is too great to be accounted for by Taq DNA polymerase misreading alone. Also, the fact that the PCR produced clone 15JUV is from the same gene as the λ clone FG17 and not a single nucleotide difference was observed between these two clones suggests that under the conditions used for these amplifications misreading of bases is unlikely to be extensive.

Some of the base mismatches within a MyoHC type could be accounted for by allelic differences. The carp, unlike mammals, is tetraploid and so has four alleles of each gene. Therefore allelic differences within the same gene could account for a number of the mismatches observed between clones from the same MyoHC type.

The sequence comparisons presented in Table 3.2 demonstrate that probes containing only 3'UTR sequence can be used with some confidence that they will be isoform
specific, providing the stringency is high enough. Taken in context with the sequence identities between the 3'UTRs of other carp MyoHC isoforms, the high sequence identity between the 3' UTR's of types 2 and 7 MyoHC isoforms appears to be atypical. However, it does highlight a need to give special consideration of stringency conditions when 3' UTR containing probes for these two isoforms are used in expression studies.

The 5' oligonucleotide primer (FG2EXN40) used in the RACE PCR reactions is likely to be more homologous to some MyoHC isoforms than others. The degeneracy of the genetic code means that even isoforms with identical amino acid sequence over the region covered by FG2EXN40 may have considerable divergence at the nucleotide level. Hence it is likely that the RACE PCR method employed here was selective for certain isoforms. It is therefore unlikely that the seven MyoHC isoforms isolated in this current study account the full MyoHC isoform family present in carp. Based on their restriction map studies of carp genomic MyoHC clones, Gerlach et al., (1990) estimate the number of MyoHC isoforms to be as many as 28, however the possibility that some of these genomic clones encode different regions of the same MyoHC isoform cannot be ruled out. In order to amplify MyoHC isoforms where the sequence homology with FG2EXN40 is not sufficient to facilitate amplification, degenerate oligonucleotides based on 3' MyoHC sequences could be used in the future.

Nevertheless, RACE PCR with the FG2EXN40 primer proved to be successful in that 3' UTR containing probes for a further five carp MyoHC isoforms could now be generated, making a total of seven isoforms on which expression studies can be performed.
Chapter 4

Characterisation of the expression patterns of carp MyoHC genes during post hatching development.
4.1 Introduction

The locomotor musculature of fish is divided into segments called myotomes which are separated by sheets of connective tissue known as myosepts. Superficially the myosepts form the shape of a broad "W" set across the long axis of the body with the central point directed towards the head. The myotomes themselves extend deep into the body and slot tightly into one another. In post larval fish, two main fibre types can be distinguished on the basis of their colouration and contractile properties: the red (slow) and white (fast) fibres (Figure 4.1). Unlike mammalian muscle, where the different fibre types are typically arranged in a mosaic like fashion, the fibre types of fish muscle tend to be arranged in discrete anatomical regions with a well defined septum separating the two main types (Bone, 1966). The red fibres form a small discrete wedge along the lateral line of the fish, just below the skin, and run parallel along the body axis (Sänger et al., 1988). The white fibres are arranged in complex three dimensional helical patterns with the fibre orientation varying with both the depth and position along the body axis. This arrangement of fibres is thought to reflect the need for constant amounts of sarcomere shortening at different body flexures during swimming (Alexander, 1969). The early stages of muscle development in fish occurs by the apposition of new fibre layers along proliferative zones. As a result of this, fibres produced at different times during development remain concentrated together in distinct areas (Veggetti et al., 1990; Brooks and Johnston, 1993). This apposition of distinctive fibre layers is largely completed by the end of the larval growth stage. During postlarval hyperplastic growth new muscle fibres are formed around, and in close proximity to, the larger more mature fibres formed during larval growth. In this respect post larval hyperplasia in fish resembles myogenesis in mammals where the primary fibres form the framework around which the secondary fibres develop.

Only the red muscle fibres are utilized for slow cruise swimming (Rome et al., 1988). They have a high oxidative enzyme activity and are capable of continuous aerobic metabolism and sustain prolonged contractile activities. Red fibres, in contrast to white fibres, are rich in succinate dehydrogenase (Sänger, 1991) and have a high glycogen (Akster, 1983) and myoglobin (Matsuura and Hashimoto, 1954) content. The mitochondria in the red fibres are numerous and are dispersed throughout the fibre, they are larger than white
muscle mitochondria, have more developed cristae and can occupy as much as 7% of the fibre volume (compared with less than 0.5% in white muscle) (Akster, 1985). Steady state exercise is fuelled mainly by the oxidation of pyruvate and fatty acids in the red muscle mitochondria. It has been hypothesised that the red muscle in fish has a metabolic role similar to the liver in that a non circulatory transfer of lactate from white to red muscle and glucose from red to white muscle occurs (Wittenberger et al., 1975). The carp (*Cyprinus carpio*) has been shown to have two distinct types of red muscle fibre (red a and red b) with the red b fibres having the highest succinate dehydrogenase activity (Akster, 1985).

White muscle fibres form the bulk of the myotomal muscle and are larger in diameter than red fibres. This tissue is specialised for burst swimming such that metabolic economy has been sacrificed in favour of maximal power output. Therefore, these fibres have a reduced vascularization, low mitochondrial density and low extracellular volume which allow for a very high density of myofibrils per mm$^3$ of muscle tissue. These adaptations however incur the penalty of an inability to maintain sustained aerobic metabolism except at a low level of activity. Fish white muscle has the capacity for rapidly increasing its glycolytic flux (Driedzic and Hochachka, 1976) and burst type swimming activities are fuelled by anaerobic glycolysis (Moyes et al., 1989).

Three types of white muscle fibre have been described in the carp (Akster, 1983) these being the true white, small pink and large pink fibres. The so called pink muscle fibres form a transitional zone between the true white and red fibres. They lie on the white muscle side of the septum dividing the two main fibre types and have been described in a number of species (Sänger, 1991). In carp the distinction between small and large pink fibres is based upon an inverse relationship between the cross sectional area and the reactivity with a rabbit-raised antiserum
against chicken pectoralis myosin (Akster, 1985). The stability of the myosin ATPase activity at high pH also varies between these two pink fibre types (Akster, 1983).

Histochemical studies on the white myotomal muscle of a variety of fish species have shown that there are species differences in the pH lability of the ATPase activity of the white muscle myosins (Carpene et al., 1982; Sanger et al., 1989) and that whereas some species have histochemically uniform white muscle consisting of similar sized fibres, others have mosaic white muscle consisting of very different diameters which may or may not differ in their histochemical characteristics (Rowlerson et al., 1985).

Additional fibre sub-types have been described in a number of other cyprinid species including tonic, intermediate, and transitional zone fibres, (reviewed by Sanger 1991). However, it is thought that the carp is atypical of the cyprinids in that its fibre types are less heterogenous, at least when studied by succinate dehydrogenase staining (Sanger et al., 1988).

Whether distinct isoforms of the MyoHC are associated with each of the different stages of carp myotomal muscle development and also in the adult fibre types remains to be determined. The aim of the work presented in this chapter was to characterise the expression patterns of the seven MyoHC isoforms isolated in Chapters 2 and 3 during post hatching development and in the adult. The general expression patterns of the isoforms are investigated by Northern blot hybridisation. In situ hybridisation was also used in some cases to investigate expression at the individual fibre level.
4.2 Materials and methods

4.2.1. Experimental animals

Common carp (*Cyprinus carpio*), (50-100g in weight) were purchased from a commercial fish farm and reared in 25 gallon tanks of circulating aerated tap water under a 12hr light : 12hr dark photoperiod. Fish were fed twice daily to satiation on a commercially available pellet diet (B.P. Nutrition U.K. Ltd). Fertilised carp eggs were also purchased from the same commercial supplier. Carp fry from these eggs were fed brine shrimp (*Artemia Sp.*) for 2 weeks after hatching followed by a commercially available fish fry pellet (B.P Nutrition). During rearing there was no control on the water temperature and fish were exposed to temperatures ranging from 10°C in winter to 20°C in summer. Fish which had been acclimated to warm and cold temperatures were also used for RNA extraction, such fish were raised or lowered to the desired experimental temperature at a rate of 1-2°C per day so as to minimise stress caused by temperature variations.

Regarding the gross nomenclature of fish used in this study, the terms carp fry, juvenile carp and adult carp are used. Fish from the time of hatching until they reached 3cm in length were classed as fry. Carp between 3cm and 9cm were classed as juvenile and fish over 9cm in length were classed as adult.

4.2.2. Extraction of RNA

With all work involving RNA, standard procedures were observed to prevent contamination with RNAses. All glassware, spatulas and magnetic fleas were previously baked at 250°C for 6 hours. Eppendorf tubes and pipette tips were autoclaved and baked overnight at 80°C. Solutions were treated with diethylpyrocarbonate (DEPC), with the exception of Tris buffer and other compounds containing primary amines which were made with DEPC treated water. Disposable gloves were worn at all times.

Total cellular RNA was extracted using an adapted version of the method described by Chomczynski and Sacchi (1987). Fish were sacrificed by a blow to the back of the head followed by decapitation and pithing. In the case of juvenile and adult carp, 0.25-0.75g of
tissue was dissected from each of three individual fish from the same group and pooled together. For carp fry, five whole fish were taken from each group since it was impractical to dissect out different muscle types in such small fish. Tissue samples were placed in 5ml of ice cold denaturing solution (D.S) (4M guanidium thiocyanate, 25mM sodium citrate pH 7.0, 0.1M 2-mercaptoethanol) and homogenised on ice using an Ultra Turrax T25 homogeniser (Janke and Kunkel) at maximum speed. Sarkosyl (125ul of a 20% solution), sodium acetate (330ul of 3M solution pH 4.0), phenol (5ml saturated with citrate buffer pH 4.5) and 1ml chloroform/isoamylalcohol (24:1 v/v) were added to the sample sequentially with mixing by inversion between each addition. The samples were vortexed vigorously for two ten second periods taking care to keep the tubes off ice for the minimum time possible. Samples were left on ice for 15 minutes before centrifugation at 10,000g for 20 minutes at 4°C. The upper aqueous phase was transferred to 10ml of ice cold ethanol, mixed well and precipitated at -20°C overnight. The RNA was then pelleted by centrifugation (10,000g, 4°C) and the supernatant carefully removed. Samples were redissolved in 1ml of D.S and 2ml of ice cold ethanol added before storing at -70°C.

When samples were required for analysis an aliquot was taken and spun down in a microcentrifuge for 10 minutes at 4°C, the supernatant was removed and the pellet washed three times in 70% ethanol. The RNA pellet was then dried in a Speedvac for five minutes and redissolved in DEPC treated water.

4.2.3 Electrophoresis of RNA

Electrophoresis of RNA was performed in 1.5% (w/v) agarose gels made in 3-(N-morpholino) propanesulfonic acid (MOPS) buffer (0.02M MOPS, 5mM sodium acetate, 1mM EDTA) pH 7.0. Formaldehyde (0.66M final concentration) and 3µg ethidium bromide per 100ml were added just before pouring. MOPS buffer was also the running buffer.

The concentration of RNA was determined by its optical density and the required volume to give 30µg of total RNA was taken and made up to 5ul with DEPC treated water. RNA loading buffer (25ul) (Sambrook et al., 1989) was added and the sample heated to 95°C for 2 minutes just prior to loading. Gels were then run at 30 volts constant voltage for 18 hours at 4°C.
4.2.4 Northern blotting of RNA

RNA was transferred to "Zeta Probe" nylon membrane (Bio-Rad) in 10x SSC buffer as per the recommendations of the manufacturer. The RNA was then fixed to the nylon membrane by baking at 80°C for 2 hours.

4.2.5 Slot blotting of RNA

Total RNA (30μg) was dissolved in 0.5ml of ice cold 10mM NaOH, 1mM EDTA and applied to nylon membrane assembled in a Schleicher & Schull Minifold II slot blot apparatus according to the manufacturers instructions. When the wells were just dry, a further 0.5 ml of the above solution with no RNA was applied. The membrane was then rinsed briefly in 2X SSC/ 0.1% SDS and dried at 80°C for 2 hours.

4.2.6 Labelling of DNA probes for Northern hybridisations

DNA probes used for Northern hybridisations were labelled by the "specific priming" method described in 2.2.5. Since the 3' UTR probes used tended to be A-T rich, α-32P dATP (3000 Ci/mmol) (Amersham International) was used as the radionucleotide in order to obtain higher specific activities. Details of how each plasmid construct was linearised and primers used to give antisense labelled DNA are given in Appendix 1.
4.2.7 Hybridisation conditions for Northern

Northern hybridisations were carried out at 25°C below the calculated melting temperature of the probe duplex with a sodium ion concentration of 0.5M and no formamide. Melting temperatures were calculated according to the equations of Britten et al., (1974) and Beltz et al., (1983) as follows:

\[ T_{m(DNA/DNA)} = 81.5 + 16.6 \times \log [Na^+] + 0.41 \times (G+C) - (500/probe \ length \ (bp)) \]

\[ T_{m(RNA/RNA)} = 79.8 + 18.5 \times \log [Na^+] + 0.584 \times (G+C) + 0.118 \times (G+C)^2 - (500/probe \ length \ (bp)) \]

\[ T_{m(DNA/RNA)} = \text{mean of } T_{m(DNA/DNA)} \text{ and } T_{m(RNA/RNA)} \]

where:
- \( T_{m} \) is the melting temperature of the probe duplex
- \([Na^+]\) is the concentration of sodium ions in the hybridisation buffer.
- \((G+C)\) is the percentage of guanine and cytosine nucleotides in the probe

<table>
<thead>
<tr>
<th>MyoHC Probe</th>
<th>Probe length (bp)</th>
<th>G-C content (%)</th>
<th>Melting Temp (°C) (DNA/DNA)</th>
<th>Melting Temp (°C) (RNA/RNA)</th>
<th>Melting Temp (°C) (DNA/RNA)</th>
<th>Hybridisation Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHITECl</td>
<td>151</td>
<td>37.1</td>
<td>88.39</td>
<td>97.5</td>
<td>92.95</td>
<td>67.95</td>
</tr>
<tr>
<td>FG17UTR*</td>
<td>88</td>
<td>35.2</td>
<td>85.25</td>
<td>96.25</td>
<td>90.75</td>
<td>65.75</td>
</tr>
<tr>
<td>15JUV</td>
<td>164</td>
<td>37.8</td>
<td>88.95</td>
<td>97.99</td>
<td>93.47</td>
<td>68.47</td>
</tr>
<tr>
<td>EGGGS22</td>
<td>112</td>
<td>26.8</td>
<td>83.02</td>
<td>90.72</td>
<td>86.87</td>
<td>61.87</td>
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<tr>
<td>EGGGS24</td>
<td>102</td>
<td>30.3</td>
<td>84.02</td>
<td>93.01</td>
<td>88.52</td>
<td>63.52</td>
</tr>
<tr>
<td>FRY2811</td>
<td>84</td>
<td>25.9</td>
<td>81.17</td>
<td>90.15</td>
<td>85.66</td>
<td>60.66</td>
</tr>
<tr>
<td>FG19UTR*</td>
<td>90</td>
<td>36.6</td>
<td>85.95</td>
<td>97.1</td>
<td>91.53</td>
<td>66.53</td>
</tr>
<tr>
<td>FG2UTR*</td>
<td>84</td>
<td>32.1</td>
<td>83.71</td>
<td>94.19</td>
<td>88.95</td>
<td>63.95</td>
</tr>
</tbody>
</table>

*For the genomic subclones FG2UTR, FG19UTR and FG17UTR the probe length is given as the length of DNA expected to bind to the mRNA, i.e. the total length of the labelled DNA fragment minus the length of the primer, any polylinker sequence and any genomic DNA which is after the putative polyadenylation site.
4.2.8 *In situ* hybridisations

Fish were killed as described previously and muscle samples dissected transversely as quickly as possible with a minimum amount of mechanical stretching. Tissue blocks for cryosections were mounted on cork tiles with OCT compound (Miles Scientific) and rapidly frozen in isopentane cooled in liquid nitrogen. Cryosections were cut at 12 µm thickness, mounted on aminopropyltrimethoxy silane (APTES) treated slides and fixed in PFA fixative (4% (w/v) paraformaldehyde in PBS, pH 7.4) for 20 minutes at 4°C. After two 20 minute washes in PBS (4°C) sections were dehydrated through a series of graded alcohols (50%, 60%, 70%, 85%, 95%, and 2x 100%), air dried and stored at -70°C with desiccant until required.

Tissue blocks for wax embedding were prefixed in PFA fixative at 4°C for four hours. Samples were then partially dehydrated by three 20 minute incubations in 50% ethanol followed by three 20 minute incubations in 70% ethanol. The tissue blocks were then transferred to an automatic wax embedding machine (Tissue TEK III) set to incubate in 70% ethanol for one hour, 90% ethanol for 1 hour, absolute ethanol for 1 hour (3 times), xylene for 1 hour (4 times) and 60°C wax until the following morning (with 3 changes of wax). Sections of 5µm thickness were cut on a sledge microtome, mounted on APTES treated slides and dried overnight at 37°C before storage at room temperature.

Prior to hybridisation, paraffin embedded sections were incubated in a 65°C oven for 10 minutes followed by two 10 minute incubations in xylene at room temperature. Sections were then rehydrated through a series of graded alcohols followed by a 20 minute incubation in 0.2M hydrochloric acid. Following acid denaturation, samples were heat denatured in 2x SSC at 70°C for 15 minutes. The sections were rinsed in PBS for 2 minutes and digested in pronase solution (20µg/ml in 50mM Tris, 5mM EDTA) for 20 minutes. Sections were then incubated in glycine solution (2mg/ml in PBS) for 30 seconds in order to arrest the pronase digestion. The sections were briefly refixed in PFA fixative for five minutes at room temperature followed by a 5 minute incubation in 3 x PBS to block the fixation.

In order to remove any static electricity, the sections were rinsed twice in PBS followed by a 10 minute incubation in 0.1M triethanolamine (pH 8.0), 0.25% acetic anhydride with gentle stirring. Finally sections were rinsed in 2x SSC for 5 minutes, dehydrated through...
graded ethanols and air dried before hybridisation on the same day.

For cryosections the same prehybridisation procedure as above was followed except the initial dewaxing in xylene and the 70°C heat denaturation steps were omitted.

4.2.9 Synthesis of labelled cRNA.

The DNA sequences to be used as probes for in situ hybridisations were transcribed with the appropriate RNA polymerase so as to give either sense or antisense labelled cRNA. Details of plasmid constructs used to generate cRNA probes are given in Appendix 1.

For non-radioactive hybridisations the probes were labelled with digoxigenin-11-UTP according to the manufacturers instructions (Boehringer Mannheim). For 35S labelling of cRNA probes a "Stratagene" RNA transcription kit was used with 35S UTP (1500 Ci/mmol, Amersham) according to the manufacturers instructions.

4.2.10. Hybridisation and washing conditions.

Probes were heated to 85°C for 2 minutes, rapidly cooled on ice and then diluted 1:10 in hybridisation buffer (50% formamide, 0.3M NaCl, 20mM Tris-HCl (pH8.0), 10mM NaPO4 (pH8.0), 10% dextran sulphate, 1x Denhardt's, 0.5 mg/ml yeast tRNA) so as to give a final probe concentration of 0.3ng per µl, for 35S labelled probes, or 5ng/ul for digoxigenin labelled probes. The hybridisation mixture (30µl) was then added to each section and a siliconised coverslip applied. Sections were incubated overnight in a humid chamber at 42°C.

After hybridisation, slides were dipped into 4x SSC in order to remove coverslips and the majority of unbound probe. The slides were then washed twice in 2x SSC (15 minutes each time at room temperature) followed by RNase A treatment at a concentration of 100µg per ml in 2x SSC for 30 minutes at 37°C. Following RNase A treatment slides were washed three times in 0.1 x SSC (twice for 20 minutes at 42°C and once for 10 minutes at room temperature.
4.2.11. Detection of bound probe on *In situ* hybridisation sections.

Bound digoxigenin probe was detected with either anti-digoxigenin alkaline phosphatase conjugated antibody or anti-digoxigenin- fluorescein antibody, both supplied by Boehringer Mannheim.

Slides were washed in digoxigenin Buffer 1 (0.1M maleic acid, 0.15M NaCl (pH 7.5)) for five minutes at room temperature followed by a 30 minute incubation in digoxigenin Buffer 2 (Boehringer Mannheim blocking reagent diluted 1:10 in Buffer 1). After dipping for 5 seconds in Buffer 1, slides were incubated with anti digoxigenin antibody for 2 hours at 37°C in darkness. For the alkaline phosphatase conjugated antibody the incubation buffer consisted of a 1:500 dilution of antibody in Buffer 2. With the fluorescein antibody, the reconstituted lyophilised antibody was diluted 1:6 in 1xPBS, 0.5% Bovine serum albumin, 1% Boehringer Mannheim blocking reagent, and 2% foetal calf serum. Approximately 500μl of antibody incubation buffer was used per slide.

Unbound antibody was removed with three five minute washes in 100mM Tris HCl (pH 7.4), 150mM NaCl at room temperature. Slides incubated with fluorescein antibody were then mounted with Citiflor mountant and stored darkness at -20°C until required. For detection of the alkaline phosphatase conjugated antibody the Boehringer Mannheim nucleic acid detection kit was used according to the manufacturers instructions. The colour precipitation reaction was left for 16 hours and the sections were dehydrated through graded ethanol, cleared in xylene and mounted with DPX.

Tissue sections hybridised to 35S labelled cRNA probes were dehydrated through a series of graded ethanol (50%, 70%, 80% 95%, each containing 0.3M ammonium acetate then twice in 100% ethanol), air dried and then exposed to X-Ray film for 24 hours. Autoradiography was subsequently performed with Amersham LM-1 emulsion according to the manufacturers instructions.
4.3 Results.

4.3.1 Northern hybridisation analysis of the expression patterns of seven MyoHC genes during post hatching development.

All seven of the carp MyoHC isoform probes investigated hybridised to RNA transcripts of approximately 6000 nucleotides in length. The patterns of expression however were different between isoform types with regards to muscle type and developmental stage. None of the MyoHC isoform specific probes hybridised to RNA transcripts present in the control tissues, carp spleen and liver. A summary of the hybridisation patterns of the seven MyoHC isoform probes used is given in Table 4.2.

The probe WHITECl, used to investigate the expression of type 1 MyoHC isoform, hybridised with both adult and immature carp (Figure 4.2, panel A). However, in the case of carp fry, expression of this isoform was only detected in fish which had been acclimated to a warm temperature (Figure 4.2, panel A, lanes 7 and 10). In adult fish, type 1 MyoHC isoform was detected in the white muscle of both warm and cold acclimated fish with no expression detected in the red muscle. Juvenile carp also showed expression of the type 1 MyoHC isoform but again only in the white muscle.

Type 5 MyoHC, as determined by the hybridisation pattern of the probe FRY2811, was also detected in both young and adult carp (Figure 4.3). However, the expression of this isoform type appeared to be less specific in that it was detected in both the red and white muscle of adult fish and in all the samples from young carp.

Three isoform types (types 2, 3 and 4) were detected only in immature carp. Type 3 MyoHC, as determined by the hybridisation pattern of the probe EGGS22, was detected in the white muscle of juvenile carp (12-14 months of age) as well as in carp fry which had been acclimated to either a warm or cold temperature. Type 4 MyoHC isoform however, as determined by the hybridisation pattern of the probe EGGS24, was absent in the juvenile carp and was only detected in carp fry (the Northern hybridisation analysis for this isoform type is presented in Chapter 5 Figure 5.5 Panel B).

Expression of the type 2 MyoHC isoform was similar to the type 3 isoform in that this isoform was detected in the white muscle of juvenile carp and in carp fry but not in adult
muscle. However the level of expression in juvenile carp appeared to be higher than in the carp fry.

The two genomic probes, FG19UTR and FG2UTR used to investigate the expression of types 6 and 7 MyoHC isoforms respectively were both only detected in warm acclimated adult fish (Figures 4.5 and 4.6). Furthermore, hybridisation of these two isoform probes only occurred in white muscle with no expression detected in the red. Hybridisation of the type 7 MyoHC isoform probe (FG2UTR) also occurred with RNA extracted from the pink muscle of warm acclimated fish.

Table 4.2 Expression patterns of seven carp MyoHC isoforms

<table>
<thead>
<tr>
<th></th>
<th>Adult warm acclimated</th>
<th>Adult cold acclimated</th>
<th>Juvenile carp (12-14 months old)</th>
<th>Carp fry (Whole fish)</th>
</tr>
</thead>
<tbody>
<tr>
<td>White</td>
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<tr>
<td>Type 1</td>
<td>*</td>
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<td>-</td>
</tr>
<tr>
<td>Type 2</td>
<td>-</td>
<td>-</td>
<td>*</td>
<td>n</td>
</tr>
<tr>
<td>Type 3</td>
<td>-</td>
<td>-</td>
<td>*</td>
<td>-</td>
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<tr>
<td>Type 4</td>
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<td>n</td>
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<tr>
<td>Type 5</td>
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<td>*</td>
</tr>
<tr>
<td>Type 6</td>
<td>*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Type 7</td>
<td>*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Legend.
(∗) Indicates that expression of the isoform was detected by Northern hybridisation under high stringency conditions
(-) Indicates that expression of the isoform was not detected by Northern hybridisation under high stringency conditions.
(n) Indicates that Northern hybridisation of the isoform with this particular muscle type have not been carried out.
"White" and "Red" refers to the muscle types dissected for RNA extraction.
"Warm" and "Cold" refers to the temperature the fish were acclimated to.
Northern hybridisation of carp RNA to the probes, WHITEC1, FG17UTR and HIIATP.

Total RNA (30µg) extracted from various carp tissue samples was separated by electrophoresis and transferred to nylon membrane. The membrane was then hybridised, under high stringency conditions, with ^32P labelled WHITEC1 probe. Panel A shows the autoradiograph (48 hours exposure) after this hybridisation. The membrane was then stripped and subsequently rehybridised, under high stringency conditions, with ^32P labelled FG17UTR probe (Panel B, 72 hour exposure of autoradiograph). Again the membrane was stripped and rehybridised to a control MyoHC probe, HIIATP, which contains the ATP binding site of the human β cardiac MyoHC gene, this time under low stringency conditions (Panel C, 72 hours exposure). Panel D shows the original RNA gel stained with ethidium bromide.

The RNA samples loaded in each lane are as follows:- (1). White muscle from 3 juvenile carp (12 months of age, 5.5-6cm in length). (2). Red muscle from the same fish in lane 1. (3). White muscle from 3 adult carp (4 years of age 19.0-22.0 cm in length), acclimated to 28°C for 5 weeks. (4). White muscle from 3 adult carp (4 years of age 18.5-21.3cm in length), acclimated to 16°C for 5 weeks. (5). Adult carp spleen. (6). White muscle from 3 adult carp (4 years of age 16.2-19.1cm in length), acclimated to 28°C for 5 weeks. (7). Five whole carp fry (60 days old, 1.2-1.6 cm in length), acclimated to 28°C for 5 weeks. (8). Red muscle from 3 adult carp acclimated to 28°C for 5 weeks (same fish as in lane 3). (9). White muscle from 3 adult carp acclimated to 28°C for 5 weeks, acclimated to 20°C for 5 weeks. (10). Five whole carp fry (60 days old, 0.8-1.1cm in length), acclimated to 16°C for 5 weeks. (11). Red muscle from 3 adult carp acclimated to 28°C for 5 weeks (same fish as in lane 6). (12). Red muscle from 3 adult carp acclimated to 16°C for 5 weeks (same fish as in lane 4).
Figure 4.2

A
WHITEC1

B
FG17UTR

C
HIIATP

D

MyoHC 6kb

28 S

18S
Figure 4.3

Northern hybridisation of carp total RNA to the probe FRY2811.

Total RNA (30μg) extracted from carp of different ages and acclimation temperatures was separated by electrophoresis and transferred to nylon membrane. The RNA loaded in each lane was extracted from the pooled muscle samples of three individual fish except where indicated otherwise. The membrane was hybridised, under high stringency conditions, with 32P labelled FRY2811 probe. Panel A shows the autoradiograph (48 hours exposure) after hybridisation. Panel B shows the original RNA gel stained with ethidium bromide.

The RNA loaded in each lane was from the following tissues: - (1) Carp spleen (from one adult fish). (2) and (3) White muscle from adult carp (4 years of age, 18.4-21.2 cm in length) acclimated to 10°C for 5 weeks. (4) and (5) White muscle from adult carp (4 years of age, 19-22cm in length) acclimated to 28°C for five weeks. (6) White muscle from juvenile carp 12 months of age, 5-6cm in length. (7) Whole carp fry (five fish, 60 days old, 1.2-1.6 cm in length acclimated to 28°C for 5 weeks). (8) Whole carp fry (five fish, 60 days old, 0.8-1.1 cm in length), acclimated to 16°C for 5 weeks. (9) and (10) Red muscle from adult carp acclimated to 28°C (same fish as in lanes 4 and 5). (11) Red muscle from adult carp acclimated to 10°C (same fish as in lane 4).
Northern hybridisation of carp total RNA to the probe EGGS22 (type 3).

Total RNA (30μg) extracted from various carp tissue samples was separated by electrophoresis and transferred to nylon membrane. The RNA loaded in each lane was extracted from the pooled muscle samples of three individual carp except where indicated otherwise. The membrane was hybridised, under high stringency conditions with 32P labelled EGGS22 probe and exposed to X-Ray film for 48 hours (panel A). Panel B shows the original RNA gel stained with ethidium bromide. Lanes are as follows. (1). Red muscle from juvenile carp (14 months of age, 5.6-6.5cm in length) (2). Whole heart from a 14 month old carp, 5.4cm in length. (3). Swim bladder from a 14 month old carp, 5.4cm in length. (4). Liver from an adult carp. (5). Pink muscle from adult carp (4 years old, 17.5-19.6cm in length) acclimated to 10°C for 5 weeks. (6). Adult carp spleen. (7). Pink muscle from adult carp (4 years old, 19.2-21.1cm in length) acclimated to 28°C for 5 weeks. (8). Whole heart from adult carp. (9). White muscle from juvenile carp 14 months of age, 5.6-6.5cm in length. (10). Five whole carp fry (20 days old, 0.5 cm in length) acclimated to 28°C for 2 weeks. (11). Whole carp fry (20 days old, 0.5 cm in length) acclimated to 16°C for 2 weeks. (12). Blank lane no RNA. (13). Adult pig soleus muscle control. (14). Red muscle from adult carp (4 years old, 18.9-22.0cm in length) acclimated to 28°C for 2 weeks. (15). Red muscle from adult carp (4 years old, 17.1-21.5cm in length) acclimated to 28°C for 5 weeks. (16). White muscle from adult carp (4 years old, 18.2-19.9cm in length) acclimated to 16°C for 5 weeks. (17). White muscle from adult carp (4 years old 16.5-18.5cm in length) acclimated to 20°C for 5 weeks. (18). White muscle from adult carp (4 years old, 18.9-22cm in length) acclimated to 28°C for 2 weeks. (19). White muscle from adult carp (4 years old, 17.4-21.5 cm in length) acclimated to 28°C for 5 weeks. (20). Whole heart from an adult carp acclimated to 20°C for 5 weeks.
Figure 4.4

[Image of a gel electrophoresis with labeled bands for MyoHC, 6Kb, 28S, and 18S]
Northern hybridisation of carp total RNA to the probe FG19UTR (type 6)

Total RNA (30μg) from various carp tissue samples was separated by electrophoresis and transferred to nylon membrane. The RNA loaded in each lane was extracted from the pooled muscle samples of three individual fish except where indicated otherwise. The membrane was hybridised, under high stringency conditions with 32P labelled FG19UTR probe and exposed to X-Ray film for 48 hours. Panel A shows the autoradiograph and panel B the original RNA gel stained with ethidium bromide.

Lanes are as follows: (1). Unhatched carp eggs (24 hours after fertilisation). (2). Adult carp Spleen. (3). Pig soleus muscle control. (4). Red muscle from adult carp (4 years old, 18.9-22cm in length) acclimated to 28°C for 2 weeks. (5). 12 day old carp fry (5 whole fish 0.5 cm in length). (6). 60 day old carp fry (five whole fish, 1.1-1.4 cm in length) acclimated to 15°C for 2 weeks. (7). White muscle from 14 month old juvenile carp, (5.6-6.5 cm in length). (8). White muscle from adult carp (four years old, 15.9-19.2 cm in length) acclimated to 10°C for five weeks. (9). White muscle from adult carp (four years old, 18.5-22 cm in length) acclimated to 28°C for two weeks. (10). White muscle from adult carp (4 years old, 18.1-1-20.3cm in length) acclimated to 28°C for three weeks. (11). White muscle from adult carp (4 years old, 17.4-21.5cm in length) acclimated to 28°C for five weeks.
Figure 4.6

Slot blot hybridisation of carp total RNA to the probe FG2UTR (type 7).

Total carp RNA was denatured as described in 4.2.5 and transferred to nylon membrane via a slot blot apparatus. The RNA loaded on each slot was extracted from the pooled muscle samples of three fish. The membrane was hybridised under high stringency conditions with \(^{32}\)P labelled FG2UTR and exposed to film for 24 hours (panel A). Subsequently the membrane was stripped of bound probe and rehybridised with the carp actin probe FGA101 (panel B, exposure 24 hours). Lanes are as follows: (1). White muscle from adult carp (3 years of age, 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). Pink muscle from adult carp (same fish as in slot 1). (4). Adult carp spleen. (5). Whole adult carp heart. (6). 20 day old carp fry (5 whole fish). (7). 12 day old carp fry (5 whole fish). (8). 8 day old carp fry (5 whole fish). (9). 4 day old carp fry (5 whole fish). (10). 2 day old carp fry (5 whole fish). (11). Red muscle from adult carp (3 years old 16.0-18.0 cm in length) acclimated to 10°C for five weeks. (12). White muscle from adult carp (same fish as in slot 11).
4.3.2 *In situ* hybridisation

4.3.2.1 Expression pattern of type 2 MyoHC isoform

X-ray film autoradiographs of *in situ* hybridisation experiments using $^{35}$S labelled FG17UTR sense and antisense probes on juvenile carp cryosections (Figure 4.7) showed a strong localised expression of carp type 2 MyoHC isoform in the pink muscle fibre layer of juvenile carp. Hybridisation of the antisense probe within the bulk of the white myotomal muscle was weaker than the hybridisation observed in the pink muscle fibre layer but was stronger than the hybridisation observed with the sense probe. Within the white myotomal muscle hybridisation was stronger around the borders of the myosepta. No specific hybridisation of the antisense labelled probe was observed in the red muscle fibre layer.

*In situ* hybridisations using the FG17UTR probe were also performed on carp fry. The pattern of hybridisation in the carp fry was very similar to that observed in juvenile carp, i.e. hybridisation of the antisense probe was localised to the pink muscle fibre layer. A distinctive "V" shaped localisation of silver grains was observed (Fig 4.8 plate A) marking the hybridisation of the antisense FG17UTR probe to the pink muscle fibres bordering the red muscle fibre wedge. These *in situ* hybridisations were performed with $^{35}$S labelled cRNA probes and fibre morphology was poor (see Fig 4.8 plates B and D). Also, the background hybridisation of the $^{35}$S labelled probes was high, as demonstrated by the amount of probe which bound to the sheep liver used to support the tissue block during cryosectioning. However, even under these sub-optimal *in situ* hybridisation conditions the FG17UTR antisense probe could still be clearly localised to the pink muscle fibre layer, so much so that the silver grains could also be viewed under lightfield microscopy (Figure 4.8, plate B).

Autoradiography using photographic emulsion proved unsuccessful in terms of visualising the individual fibres expressing the type 2 MyoHC isoform. The autoradiography technique used destroyed the morphology of the fibres making the identification of individual muscle fibres impossible. *In situ* hybridisations with digoxigenin labelled cRNA probes however proved to be superior to $^{35}$S labelled probes with regards to preservation of fibre morphology. Using the same sense and antisense probes for the type 2 MyoHC
isoform (FG17UTR), but labelled with digoxigenin, the individual fibres expressing the type 2 MyoHC isoform were visualised (Figure 4.9). Confirming the results of the $^{35}$S labelled probes, the fibres staining positive with the digoxigenin labelled antisense FG17UTR probe are shown to be within the pink muscle fibre layer. Most of the pink muscle fibres were of small diameter (10-25 μm) and these fibres showed a high level of expression of the type 2 MyoHC isoform. Deeper into the pink muscle fibre layer, the fibre diameters become more varied with small and larger diameter (25-50 μm) fibres interspersed. However, within this area of mixed fibre diameters it is the smaller diameter fibres which express more of the type 2 MyoHC isoform. Higher magnification of the positively staining fibres (Figure 4.9 plate D) indicate localisation of the type 2 MyoHC RNA transcripts to the borders of the fibres and discrete areas within the muscle fibres themselves. These areas within the muscle fibres are assumed to be intermyofibrillar spaces. Deeper into the white myotomal musculature no expression of the type 2 MyoHC isoform could be detected, this is demonstrated in Figure 4.9, plate C where the left hand side of the photomicrograph shows the border between the pink and white muscle fibre layers. Localisation of hybridisation around the borders between the myosepta as observed with the $^{35}$S labelled probe (Figure 4.7) was not detected with digoxigenin labelled probe. No specific hybridisation was observed with the sense digoxigenin labelled FG17UTR probe (Figure 4.9 plate B).
In situ hybridisation of $^{35}$S labelled FG17UTR probe with juvenile carp.

In situ hybridisations with $^{35}$S labelled FG17UTR cRNA sense and antisense probes were performed on cryosections from 14 month old juvenile carp (as described in sections 4.2.10-14). Tissue blocks were taken from the region of the fish depicted in Figure 4.1. After post hybridisation washes the sections were dehydrated and exposed to X-Ray film for one week at 4°C.

A and B: Autoradiographs of cryosections from two individual fish showing the hybridisation pattern of the antisense cRNA probe.

C: Autoradiograph showing hybridisation of the control sense labelled cRNA.
Figure 4.7 In situ hybridisation on 14 month old carp using $^{35}$S labelled FG17UTR probe.
**Figure 4.8**

*In situ* hybridisation of $^{35}$S labelled FG17UTR probe with carp fry.

Full cross sections were taken from the ventral region of 16 day old carp fry and embedded in sheep liver to aid cryosectioning. *In situ* hybridisations using $^{35}$S labelled antisense and sense FG17UTR cRNA probes were performed as described in 4.2.10-4.2.14. After post hybridisation washing the sections were dehydrated and autoradiography performed using Amersham LM-1 photographic emulsion. Sections were exposed to photographic emulsion for 1 month at 4°C.

**Plates A and B:** photomicrographs showing hybridisation of the antisense labelled FG17UTR cRNA probe (A: darkfield, B: lightfield, of the same region).

**Plates C and D:** photomicrographs showing hybridisation of the control sense labelled FG17UTR cRNA probe (C: darkfield, D: light field of the same region).

The region of tissue shown in the photomicrographs is shown bellow. Tissue to the right of the skin in the photomicrographs is sheep liver.
Figure 4.8 plate A: Antisense darkfield.

Figure 4.8 plate C: Sense darkfield.
Figure 4.8 plate B: Antisense lightfield.

Figure 4.8 plate D: Sense lightfield.
Paraffin wax embedded sections were prepared from the ventral region of 14 month old juvenile carp and subjected to \textit{in situ} hybridisation with digoxygenin labelled FG17UTR sense and antisense cRNA probes (as described in 4.2.10-4.2.14). Hybridised probe was detected with anti-digoxigenin-fluorescein antibody (Boehringer Mannheim) and photomicrographs taken on a fluorescent microscope. Serial photomicrographs were subsequently joined together and rephotographed. (Plates A and B, showing antisense and sense labelled probes respectively). Plate C shows the border between the pink and white muscle fibres. Plate D shows a higher magnification of antisense hybridisation to the pink muscle fibres, allowing the localisation of the probe to be visualised within and around individual muscle fibres.
Figure 4.9 plate A
Figure 4.9 plate B
Figure 4.9 plate C

Figure 4.9 plate D
4.3.2.2 *In situ* hybridisation analysis with a human β cardiac MyoHC derived probe, HIIATP.

The subclone HIIATP (Appendix 1) was created to act as a control probe which would bind to all carp MyoHC isoforms (the ATP binding site region contained in this probe is highly conserved between all vertebrate myosins). However, *in situ* hybridisation experiments with this probe revealed that the probe hybridised specifically to the red muscle fibres with no detectable hybridisation with white fibres at the stringency used. Hybridisations with the $^{35}$S labelled sense and antisense HIIATP probes, demonstrate clearly on a gross anatomical level this specific hybridisation to the red fibres. Figure 4.10 shows X-ray film autoradiographs of such hybridisations. As was the case with the FG17UTR $^{35}$S labelled probe, the protocol used for detection of the $^{35}$S labelled probe destroyed the morphology of the fibres (data not shown) making visualisation of individual fibres impossible with this method. However, the morphology of the muscle fibres was much better preserved with the protocol used with digoxigenin labelled HIIATP probe (Figure 4.11). With the digoxigenin labelled probe it was observed that the red muscle fibres closer to the skin hybridised with the HIIATP probe more strongly than the red fibres lying closer to the pink muscle fibre layer and the fibres closer to the lateral line nerve (Figure 4.11 plate A). Some of the pink muscle fibres stained slightly stronger than others with HIIATP probe (Figure 4.11 plate C) however this staining was still significantly less than that observed in the red muscle fibres.
Figure 4.10.

*In situ* hybridisation of $^{35}$S labelled HIIATP probe with adult carp muscle.

Muscle tissue blocks were taken just below the dorsal fin, extending down the full flank, from four year old adult carp (18-20 cm long). *In situ* hybridisation was performed on cryosections with $^{35}$S labelled HIIATP sense and antisense cRNA probes as described in 4.2.10-4.2.14. Sections were then exposed to X-ray film at 4°C for 24 hours. Autoradiographs of tissue sections are as follows:

**Plates A and B:** Hybridisation of the antisense labelled HIIATP cRNA probe on two individual fish.

**Plate C:** Hybridisation of the control sense labelled HIIATP cRNA probe.

Hybridisation of the antisense probe to the red muscle is clearly visible in plates A and B. Whereas absence of specific hybridisation with the sense labelled cRNA (Plate C) confirms the specificity of the probe.
Figure 4.10 *In situ* hybridisation on adult carp using $^{35}$S labelled HIIATP probe.
In situ hybridisation of digoxigenin labelled HIIATP probe with adult carp muscle.

Muscle tissue blocks were taken just below the dorsal fin from four year old adult carp (19-20 cm long). In situ hybridisation was performed on paraffin wax embedded sections with digoxigenin labelled HIIATP sense and antisense cRNA probes as described in 4.2.10-4.2.14. Hybridisation of the labelled probe was detected with alkaline phosphatase conjugated anti-digoxigenin antibody (Boehringer Mannheim) with the colour development left for 12 hours. Photomicrographs of tissue sections are as follows:

Plates A and C: hybridisation of the antisense labelled HIIATP cRNA probe (plate A shows the bulk of the red muscle block with surrounding pink and white muscle fibres and plate C shows the tip of the red muscle block at the top of the photomicrograph with the pink and red fibre populations bellow.

Plates B and D: Serial sections of the view shown in plates A and C respectively, hybridised with the sense labelled HIIATP cRNA probe.
Figure 4.11 plate C

Figure 4.11 plate D
4.4 Discussion

Results from the Northern hybridisation analysis performed in this chapter demonstrate that the expression of carp MyoHC isogenes is both muscle fibre type and developmental stage specific. Their expression also seems to be influenced by environmental temperature, however in the case of the developmental specific isoforms it is difficult to determine whether changes in their expression patterns are due to temperature per se or due to changes in the developmental stage induced temperature.

In mammals there is known to be a complex pattern of MyoHC gene expression during muscle growth and development (see Chapter 1). The hybridisation data obtained in this chapter shows that the situation regarding developmental isoforms in carp is also complex. Three of the seven MyoHC isogenes isolated in this current study (types 2, 3, and 4) were shown to be expressed exclusively in immature carp. Two of these isoform types (types 3 and 4) were isolated by RACE PCR (Chapter 3) from cDNA prepared from unhatched carp eggs, so it could be expected that these two isoforms would be involved in development. The type 2 MyoHC isoform was isolated from the genomic clone λFG17 and also by RACE PCR on juvenile carp cDNA.

Both types 3 and 4 MyoHC isoforms are expressed in carp fry but only the type 3 isoform is expressed in older carp 12-14 months of age. Therefore at some stage in the first year of development the type 4 MyoHC isoGene ceases to be expressed. In this respect the type 4 isoform appears to be analogous to the mammalian neonatal isoform. However, such analogies may not be completely relevant in species as widely divergent as fish and mammals.

The type 2 MyoHC isoform was shown, by in situ hybridisation, to be expressed in the pink muscle fibre layer of developing carp. No expression of this isoform could be detected in adult carp. Therefore in addition to the "small" and "large" pink muscle fibre types described by Akster (1985), there would appear to be a further developmental classification of pink muscle fibre types in the carp.

The type 1 MyoHC isoform was shown to be expressed in both young and adult carp. In the adult and juvenile carp, expression was only present in the white muscle fibres with no detectable expression, by Northern hybridisation, in the red muscle fibre layer. With the carp fry it was not possible to dissect out the red and white muscle fibre layers in order to
detect whether the expression of the type 1 isoform is restricted to the white fibres during the early developmental stages. In order to determine the fibre type specificity of the type 1 MyoHC isoform in carp fry in situ hybridisation experiments using the WHITECl probe would be necessary.

Although the type 1 MyoHC isoform was not detected in the red muscle fibres of adult carp by Northern blot analysis, the RACE PCR method described in Chapter 3 did reveal the presence of type 1 MyoHC transcripts in adult red muscle (see Table 3.1). The presence of these type 1 MyoHC transcripts could be due to a very low "background" expression of this gene in red muscle fibres, which was not strong enough to be detected by Northern hybridisation or alternatively the isolation of type 1 MyoHC from adult carp red muscle could be attributed to contamination of the red muscle sample with white muscle RNA during dissection. Due to the high sensitivity of the PCR, a contamination of only a few molecules of white muscle RNA would theoretically be enough to produce an amplification product.

Northern blot analysis of the expression of the type 1 isoform in carp fry could only detect expression in carp fry which had been acclimated to a warm temperature. Although the warm and cold acclimated fry used were from the same spawning it is likely that the warm acclimated fish entered a more advanced state of development than the cold acclimated fry. Visual inspection and physical size of the carp fry after the temperature acclimation period supported this theory. Therefore rather than being an acclimatory response to temperature, it is likely that this difference in expression of the type 1 MyoHC between the warm and cold acclimated fry is due to differences in developmental stage.

The type 5 MyoHC isoform was detected in all muscle types investigated from both young and adult carp. In situ hybridisation experiments using the 3' untranslated region probe of this isoform would be required to further investigate the expression of this isoform.

In order to determine more accurately the time scale for the expression of the different MyoHC isoforms during development, more extensive sampling is required on a group of carp from the time of egg fertilisation to at least one year of age. From the results obtained in this current study it can be concluded that at least 5 different isoforms of the MyoHC are expressed in carp skeletal muscle during the first year of development. Possible reasons for such a wide range of MyoHC isoform expression during development are discussed in
Chapter 6.

None of the seven isoform types isolated in this current study were shown to be expressed exclusively in the red muscle fibres of carp. The existence of at least one red muscle specific MyoHC isoform in carp was demonstrated by the expression pattern of the probe HIIATP. This probe was generated from the human β cardiac MyoHC isoform (Appendix 1), which is considered to be slow. When hybridised at low stringency the HIIATP probe hybridises to both red and white MyoHC isoforms. However at high stringency, as demonstrated by the in situ hybridisation data (Figures 4.10 and 4.11), the HIIATP probe hybridises specifically to the red muscle fibres. Therefore the human β cardiac MyoHC isoform is more homologous to the MyoHC isoforms present in carp red muscle fibres than it is to the isoforms expressed in the white muscle fibres. Moreover, this apparently high conservation of DNA sequence coding for the ATP binding site of the slow MyoHC isoforms in species as divergent as carp and human suggests that this region is very important in determining the functional properties of slow MyoHC isoforms.

The failure of the RACE PCR technique employed in chapter 3 to yield a red muscle specific isoform is probably due to the choice of the 5' oligonucleotide used in the PCR. This oligonucleotide (FG2EXN40) was designed from the sequence obtained from the genomic clone λFG2 and since the isoform encoded in this particular clone proved to be a white muscle, specific MyoHC isoform it is possible that the sequence of FG2EXN 40 is not sufficiently homologous to the slow red MyoHC isoform to allow amplification. Future RACE PCR experiments which aim to isolate red muscle specific carp MyoHC isoforms would perhaps be more successful if the 5' oligonucleotide sequence was based on the sequence of a known slow isoform, e.g. the human β cardiac isoform.

Two MyoHC isoforms (Types 6 and 7) were shown to be expressed exclusively in the white muscle fibres of adult carp. However, both isoform types were only detected in the white muscle from carp which had been acclimatised to warm temperatures indicating that these two isoform types are involved in the temperature acclimation process in carp. The temperature dependent expression of these two isoform types is investigated in more detail in Chapter 5.
Chapter 5

An investigation of the temperature dependent expression of carp myosin heavy chain genes.
5.1. Introduction.

Both acute and chronic changes in temperature have a profound effect on virtually all biological processes, not least the molecular events occurring during muscle contraction (Rome and Bennett, 1989). In order to minimise the biological effects of temperature change, many animals have evolved various physiological or behavioral means to maintain a constant body temperature. The body temperature of most species of fish however, is strictly dependent on the environmental water temperature.

Despite this dependence on ambient temperature, different species of fish have evolved to exploit extremes in temperature ranging from subzero in the polar regions to as high as 40°C in geothermal springs. Biochemical adjustments have minimised the effect of temperature on the catalytic processes of fish living at these widely different temperatures, thus when ecologically similar Antarctic and temperate zone fish are compared, the polar fish have increased specific myofibrillar ATPase activity, (Johnston et al., 1973) and increased activities of enzymes from the central pathways of aerobic energy metabolism (Crockett and Sidell, 1990), when assayed at cold temperatures.

In addition to this evolutionary adaptation, some species of fish possess the ability to seasonally acclimate to temperature extremes. Carp living in shallow inland lakes will seasonally experience temperatures ranging from 1°C in winter to as high as 25-30°C in summer, yet are able to maintain a relatively constant locomotory ability. During acclimation to cold temperatures, carp not only have to overcome the constraint of temperature on their rate of metabolism but have also to contend with the increased drag associated with colder water (water at 10°C is 64.3% more viscous than water at 28°C, (Newman and Wu, 1975)). Therefore a fish swimming in water at 10°C would require more power output from its muscles in order to equal the maximal cruising speeds and burst potential of a fish swimming at 28°C.

Since the maintenance of locomotory performance is critical to survival, in terms of escaping predation and searching for food, it is perhaps not surprising that evolution has provided eurythermal species with the ability to reduce their metabolic dependence on temperature after a period of exposure. The mechanisms underlying temperature acclimation are complex and as yet incompletely understood. They are known to involve changes in the
nervous system, muscle and other tissues. Johnston et al., 1985, showed that acclimation temperature affects the contractile properties of both fast and slow fibres in carp: the maximum isometric force, maximum contraction velocities and maximum power output are higher in cold (7-8°C) than in warm (23-24°C) acclimated carp. It has also been demonstrated that cold acclimated carp are able to compensate for lower temperatures by compressing their recruitment order of fibres into a narrower range of locomotor speeds, i.e. they recruit more slow muscle fibres than fast fibres at a given speed when compared to warm acclimated carp (Rome, 1990). Also, acclimation to cold temperature induces changes in the relative proportions of red to white muscle fibres with the numbers of red and intermediate fibres increasing in response to cold acclimation (Johnston and Lucking, 1983; Heap et al., 1985), thus enabling cold acclimated fish to maintain cruise swimming ability by producing more power from red muscle fibres.

A major determinant of muscle function is the myofibrillar ATPase activity and much research over the years has focused on the effects of temperature acclimation on this enzyme activity in fish. The first evidence that the contractile properties of fish muscle could be modified by thermal acclimation came from Johnston et al., 1975 who acclimated goldfish (Carassius auratus L.) to either 1°C or 26°C and showed that the myofibrillar ATPase activity was 2.8 times greater in cold acclimated compared to warm acclimated fish. Similar changes in ATPase activity with thermal acclimation have also been reported for other members of the cyprinid family, including carp (Cyprinus carpio), roach (Rutilus rutilus) and tench (Tinca tinca) (Heap et al., 1985). The changes in myofibrillar ATPase activity associated with warm and cold temperature acclimation occur within four weeks and have been shown to be totally reversible (Heap, 1985), they are not however observed in starved fish were rates of protein synthesis are low (Heap et al., 1986). Johnston et al., (1975) also reported that the myofibrils from cold acclimated carp were more susceptible to heat denaturation than those from warm acclimated carp, further suggesting that the protein structure of the myofibrils changes with temperature acclimation.

Two protein types have been postulated to be responsible for temperature acclimation dependent changes in myofibrillar ATPase: i) myosin and ii) the Ca^{++} regulatory proteins, tropomyosin and troponin. Johnston and coworkers (1979) demonstrated that desensitised actomyosin, from which the Ca^{++} regulatory proteins have been removed by high salt
concentrations, produces an ATPase activity which is almost identical in warm and cold acclimated goldfish (Carassius auratus). Furthermore, they showed that cross-hybridisation of regulatory proteins from 2°C acclimated goldfish to desensitised actomyosin from 31°C acclimated fish altered the ATPase activity towards that of the 2°C acclimated intact actomyosin. The converse of this was also demonstrated. These results were later confirmed by Penney and Goldspink, (1981) who also proposed that the Ca++ regulatory proteins play a more involved role in muscle contraction than a simple on/off switch. However, it might be expected that removal of the regulatory proteins will disrupt the orderly structure of the contractile apparatus and so these results may not be representative of physiological conditions within the myofibril. Guo and Watabe, (1993) carried out a similar experiment in carp (Cyprinus carpio) but were unable to demonstrate any acclimation temperature dependent effect of regulatory proteins on the activity or thermostability of actomyosins from warm and cold acclimated fish.

Both the heavy and light chains of myosin have been investigated with regards to elucidating their role in the temperature acclimation process in fish. Crockford and Johnston, (1990) showed that myofibrils from 8°C acclimated carp contain an additional myosin light chain band on SDS polyacrylamide peptide gels which was not present in warm (20°C) acclimated carp. Additionally, the MLC3/MLC1 ratio was significantly lower in 8°C than in 20°C acclimated carp. However, this data is not in accord with the results obtained by Hwang et al., (1990) who could find no difference in light chain composition between warm and cold acclimated carp.

Reports in the literature regarding the role of the myosin heavy chain in temperature acclimation are also contradictory. Johnston et al., (1990) purified MyoHC protein from the fast and slow myotomal muscle of both 8 and 20°C acclimated carp and digested with the proteolytic enzymes V8 protease and chymotrypsin. Although different peptide maps were obtained for fast and slow muscle MyoHC, no differences were observed with thermal acclimation. However, the extensive protein studies by Watabe and coworkers did show changes in MyoHC proteins in response to temperature acclimation. By using chymotrypsin proteolytic digests of MyoHC protein, differences were clearly demonstrated between the peptide maps of MyoHC from warm (30°C) and cold (10°C) acclimated carp (Hwang et al., 1990). The difference in results obtained by Johnston et al., (1990) and Hwang et al.,
(1990) may possibly reflect differences in technique or to the acclimation temperatures used in each experiment. The 20°C used by Johnston et al., (1990) may not be high enough to elicit the same acclimation response observed by Hwang et al., (1990) at 30°C. Hwang et al., (1990) also demonstrated that the thermostability of myosin isolated from warm and cold acclimated carp differed such that the inactivation rate constant ($K_p$) of myosin from cold acclimated fish is approximately three times greater than that of myosin from warm acclimated fish. This is in accordance with the difference in myofibrillar stability between warm and cold acclimated carp and suggests that differences in myosin maybe, at least in part, responsible for this. More recently Watabe et al.,(1994) have peptide mapped S1 MyoHC subfragments of warm (30°C) and cold (10°C) acclimated carp and demonstrated that cold acclimated carp contain four different types of S1 fragment whilst warm acclimated carp contain two S1 types, which were distinct from the cold isoforms.

The above data suggest that the changes observed in myofibrillar ATPase with temperature acclimation are due to the production of different isoforms of one or more of the contractile proteins rather than conformational changes in existing proteins. Such a change in isoforms would involve either the expression of different genes coding for different isoforms or alternative splicing of exons within the same gene. In order to investigate this question further, studies at the level of gene expression need to be carried out. Gerlach et al.,(1990) investigated the role of the MyoHC in temperature acclimation by constructing a carp genomic library and isolating 28 different $\lambda$ clones which contained MyoHC sequences. This work demonstrated for the first time the existence of a multigene family for MyoHC in a species of fish. From one of the genomic clones, ($\lambda$FG17), Gerlach and coworkers isolated a 1.7 Kb SacI restriction fragment (FG1706) which they showed to hybridise strongly at high stringency conditions to RNA isolated from the white muscle of warm acclimated carp but not to RNA from carp acclimated to 10°C or RNA from red muscle. At low stringency conditions the same probe bound equally to RNA from both warm an cold acclimated fish. From these data Gerlach and coworkers hypothesised that the temperature acclimation process in carp involves the differential expression of separate MyoHC genes.

The aim of the work presented in this chapter was to further investigate, at the level of gene expression, the role of MyoHC isoforms in temperature acclimation. The subclone
FG1706 generated by Gerlach et al., (1990) had already been proposed as a temperature dependent isoform, however the region of the MyoHC gene covered by this clone was not known. Therefore the first step in this current investigation was to determine the region of the gene covered by this clone. Such a region which differed between warm expressed and cold expressed isoforms would presumably be of functional significance and so give an indication as to which areas of the myosin molecule are important with regards to the differing myofibrillar ATPase activities observed with temperature acclimation.

Since a MyoHC 3' untranslated region sequence was also isolated from the genomic clone A-FG17 in this current study (chapters 2 and 3) it was also intended to compare the hybridisation profiles of the FG1706 probe isolated by Gerlach et al., (1990) and the 3' UTR probe FG17UTR.

Preliminary results presented in Chapter 4 suggest that two MyoHC isoforms, types 6 and 7, may be involved in the temperature acclimation process. In order to further investigate any temperature dependent expression of these MyoHC isoform types, two temperature acclimation experiments were carried out. Temperature acclimation experiment 1 (5.2.1) aimed to study the day to day expression of the type 7 MyoHC isoform when the environmental temperature was either increasing or decreasing. Temperature acclimation experiment 2 (5.2.2) investigated the persistence of MyoHC expressed in response to warm temperature. The work done by Heap, (1985) demonstrated that changes in myofibrillar ATPase activity associated with temperature acclimation are most pronounced after two to three weeks at a given temperature and are complete and stable after four weeks. Therefore, any changes in gene transcription would presumably start to occur before the second week of acclimation and this was borne in mind in the design of the two temperature acclimation experiments.
5.2. Materials and Methods

5.2.1. Experimental animals

Common carp (*Cyprinus carpio*), were obtained from a commercial supplier and kept in 25 gallon tanks of circulating aerated tap water under a 12hr light:12hr dark photoperiod. In order to minimise size-dependent variations (Somero and Childress, 1980; Heap, 1985), carp of standard body length (18±2cm), and weight (120±15g), were used for temperature acclimation experiments. Fish prior to commencement of the experiment were kept at a water temperature of 18±2°C and fed to station.

5.2.2. Temperature acclimation experiment 1

Forty six carp were split into two groups of twenty three. One group of carp (Group A) were gradually acclimatised to 10°C whilst the other (Group B) were gradually acclimated to 28°C. These experimental acclimation temperatures are within the thermal limits of myofibrillar ATPase compensation (Penney and Goldspink, 1981). Temperature changes were executed at a rate of 1-2°C per day in order to minimise temperature induced stress.

Once the experimental temperature was reached, each group of fish were left to acclimatise for a period of five weeks. Three fish from each group were then sacrificed (designated as day 0 in Table 4.1). The water temperature of Group A was then raised to 28 °C at a rate of 1-2°C per day whilst the water temperature of Group B was lowered to 10 °C at a rate of 1-2 °C per day. During this period of changing temperature two fish from each group were sacrificed each day. Fish were fed twice daily on a commercially available pellet diet (B.P. Nutrition U.K. Ltd). In order to minimise any effects of differential energy intakes between the two groups, Group A was fed to station whilst group B was fed the same amount as group A.

Muscle samples were dissected from the red and white muscle of each sacrificed fish. White muscle samples were taken from the region directly below the end of the dorsal fin whilst the whole of the red muscle block from one side of the fish was used for red muscle
samples. Muscle samples from fish sacrificed on the same day were pooled and total RNA extracted.

Table 5.1
Water temperatures for Groups A and B in Temperature acclimation experiment 1

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5.2.3. Temperature acclimation experiment 2

A group of 25 carp were acclimatised to 10°C for a period of eight weeks. Five fish were killed at this stage and the water temperature was then raised to 28°C over a period of seven 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 four weeks. The fish at 10°C were fed to station and this rate of feed intake was kept 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 sacrificed fish. Muscle samples were taken from the same region of each fish, i.e. the region directly below the end of the dorsal fin on the flank of the fish.

Total RNA was subjected to Northern blot analysis with probes for Type 4, Type 6, and Type 7 MyoHC genes. In situ hybridisations were performed on muscle sections from warm and cold acclimated fish with cRNA probes for Type 6 and Type 7 MyoHC.

5.2.4. RNA extractions and Northern hybridisations

Total RNA was extracted and Northern hybridisations performed at high stringency as previously described in Chapter 4.

5.2.5. In situ hybridisations

Non radioactive in situ hybridisations were performed with digoxigenin labelled probes on muscle sections from warm and cold acclimated carp described in 5.2.3. The method of in situ hybridisation was as described in Chapter 4, using alkaline phosphatase conjugated digoxigenin antibody for probe detection.

5.2.6 Analysis of the clone FG1706

The carp white muscle specific MyoHC gene probe FG1706 was previously isolated and restriction mapped by Gerlach et al., (1990). The plasmid was digested with the restriction enzymes PvuII and HindIII and the three resulting fragments subcloned into pBS+ (Stratagene) plasmid according to the methods described in Chapter 1. These subclones were then partially sequenced in order to determine the region of the MyoHC gene covered by the FG1706 subclone. Northern hybridisation analysis of this subclone was then performed on RNA from the carp described in 5.2.2.
5.3. Results

5.3.1 Analysis of the clone FG1706

When hybridised to RNA from carp subjected to the temperature regime described in 5.2.2, the probe FG1706 showed a pattern of hybridisation similar to that described by Gerlach et al., (1990), i.e. the probe hybridised strongest to the MyoHC RNA from carp which were sacrificed at the higher temperatures (Figure 5.2). However, unlike the observations of Gerlach et al., (1990) this probe also hybridised to RNA extracted from white muscle of carp acclimatised to 10°C for five weeks (lane 1, Figure 5.2). No hybridisation of this probe was observed on blots containing red muscle RNA from the same experiment (Data not shown).

Sequence data generated from the subclone FG1706 (Figure 5.1) revealed that this subclone covers a region of a carp MyoHC isoform equivalent to exons 25 to 30 in mammalian MyoHC isoforms. In all other vertebrate MyoHC gene sequences, these exons cover approximately 1020 base pairs inferring that the clone FG1706 consists of about 1000 base pairs of exon sequence and 700 base pairs of intron sequence. Furthermore, the region of the gene covered by exons 27 and 28 is known to code for the S2 hinge region of the MyoHC molecule in other vertebrates species (Jaenicke et al.,1990; Molina et al.,1987; Strehler et al.,1986). Therefore the preferential hybridisation pattern of the FG1706 probe to RNA extracted from warm acclimated carp observed by Gerlach et al., (1990) and in this current study could be taken as indicative that the MyoHC isoforms expressed during warm temperature acclimation differ from the isoforms expressed at cold temperatures in the region of the S2 hinge.
Figure 5.1

Restriction map and partial sequence of the subclone FG1706

i) Restriction map of FG1706.

Exons are numbered according to the equivalent exon in the human β cardiac MyoHC gene. The thick black lines indicate those regions of the clone which have been sequenced.

ii) The nucleic acid sequence with the deduced amino acid translation in the one letter I.U.P.A.C code is as follows. (--------) indicates intron sequence whilst (==========) indicates unsequenced regions.

GACCTGCAGCCCAAGCTTGAGGGTGATCTGAAACTGGCCCAGGAGTCCAGAAGTGA
DLQPKLEGDLKLAQESRSD exon 25

CCTGGAGAAGAAAAAACAGCAATCAGATGAGAAGATCAAAAAGTAAGCAGACATTGA
LENKQQSDEKIKK [---------intron

ATGTATTGAAAGTTTACACAATCAACATAAGTATTTGGCACAATCACAAGAAAACAA
---------------------------------------------------------intron-----------------------------------

TACTTTCAACAGGAAGGACTTTTGAAGATAAGTCACCTCTCAGCAAGATGAGGATG
-------------]KDFEISQLSLSKIED exon 26

AACAGTCTTTTGGGACGACGCTTTTGAGATAGTCACCTTCTCAGCAAGATGAGGATG
-------------EQLSKENQDLQV

==================================================================

=====End of exon 26 and start of exon 27 not sequenced ==

==================================================================
CAGGTGGCTGAACTCGGAGAACAGATCGACAACCTCCAGCGGGTCAAGCAGAAGCTGQVABLEQIDNLQRVKQKL
exon27

GAGAAGGAAAAGGTCGAATCAAGATGAGATTGATGACTTGACAAGCAACATGGAGEKEREKYEIKMMEIDDLTSNME
exon 27

GCTGTGGCTAAAAGCAAGGTAACATCACCCTGAGGATACATAACTTTAAAGGCAACTT
A V A K A K [------------intron-------------------
exon 27

TCAAAACATGTCTATATAATCTTGGATTTCTTTAGGCTAATTAGAGAAGATGTGCC
-------------------intron----------------------

========exons 28, 29 and start of exon 30 not sequenced=====
=================================================================================

AAGGCCAACAGTGAGGTGGCTCAGTGGCGAACCAAATATGAGACTGATGCCATCCAA
KANSEVAQRWTKYETDAIQ
exon 30

CGCCTGGAGGAGCTTTGAGGAAGCCAAATATCAACAAACGCAAAGGAGTTTTCAGTA
RTEELEBAK[------------intron-------------------

CCCTACTTTTAAATATCACAATATTAGTAA
-----------------------------------
Figure 5.2

Northern hybridisation of carp total RNA from fish subjected to a daily increase in temperature with the probe FG1706

Total RNA (30μg) prepared from the white muscle of carp described as "Group A" in 5.2.2 was separated by electrophoresis and transferred to nylon membrane. RNA was extracted from the pooled muscle samples of three fish on day 0 and two fish on each subsequent day. The 1.7 Kb insert of the subclone FG1706 was labelled with $^{32}$P α-dCTP by random priming (Feinberg and Volgstein.,1983) and hybridised to the RNA on the membrane under high stringency conditions (65°C hybridisation temperature and washes as described in 2.2.6). The blot was then exposed to X-ray film (Fuji RX) at -70°C for 72 hours. Lanes contain RNA extracted on individual days as indicated on the figure. The water temperatures for each day of the experiment are given in Table 5.1. Prior to commencing the increase in temperature this group of fish had been maintained at 10°C for five weeks.
5.3.2. Temperature acclimation experiment 1: Changes in MyoHC gene expression during high to low and low to high temperature transitions

Northern blots of carp white and red muscle RNA from groups A and B of temperature acclimation experiment 1 (5.2.2) were hybridised with FG2UTR, the isoform specific probe for type 7 carp MyoHC (Figures 5.3 and 5.4).

In group A, where the water temperature was increased each day, expression of the type 7 MyoHC gene was only detected after day seven when the water temperature had reached 23°C (Figure 5.3 panel A lanes 9, 10, 12, 13 and 14). No hybridisation was observed between the FG2UTR probe and red muscle RNA from the same fish (Fig 5.3 panel A lane 11 and unshown data). Hybridisation of the control MyoHC gene probe, HIIATP, (panel B) and the ethidium bromide staining of the original RNA gel (panel C) confirmed the integrity of the RNA. From the intensity of the ribosomal bands in lane 8 (panel C) it appears that the RNA from this sample is degraded which would explain why the HIIATP probe did not bind to this sample. The general MyoHC gene probe HIIATP hybridised strongly to RNA from red muscle and less strongly to RNA from white muscle, however this general probe showed no preference to RNA from carp at the warmer temperatures.

In group B, the water temperature started at 28°C and was lowered each day of the experiment. Expression of the type 7 MyoHC isoform was observed in fish on each day of the experiment up to day 7. A very weak expression of the isoform is detectable on days 9 and 10 (Figure 5.4 panel A). Hybridisation of the general myosin probe, HIIATP (Figure 5.4 panel B) and the ethidium bromide staining of the RNA gel (Figure 5.4 panel C) verified the integrity of the RNA.

The strong hybridisation of the FG2UTR probe with white muscle RNA from warm acclimated carp confirmed the preliminary results obtained in chapter 4 (Figure 4.6) by showing that carp type 7 MyoHC is a white muscle isoform specific to warm acclimated fish.
Northern hybridisation analysis of total RNA from carp subjected to increasing in environmental temperature (Group A) with the probes FG2UTR (type 7) and HIIATP.

Total RNA (30μg) from the carp described as "Group A" in 5.2.2 was separated by electrophoresis, transferred to nylon membrane and hybridised sequentially with the probes FG2UTR and HIIATP. Each lane contained RNA extracted from the pooled muscle samples of the fish sacrificed on each day of the experiment (3 fish on day 0 and 2 fish on subsequent days).

Panel A: The MyoHC isoform probe FG2UTR was hybridised to the membrane under high stringency conditions and the blot exposed to X-ray film (Fuji RX) at -70°C for 1 week.

Panel B: The membrane was stripped and reprobed under low stringency conditions with the general myosin heavy chain gene probe HIIATP. Again the blot was exposed to X-ray film (Fuji RX) at -70°C for 1 week.

Panel C: Ethidium bromide staining of the original RNA agarose gel.

Lanes are as follows: (1). White muscle from carp acclimatised to 10°C for five weeks (day 0). (2). White muscle from carp on day 1. (3). White muscle from carp on day 3. (4). White muscle from carp on day 4. (5). RNA from carp spleen. (6). White muscle from carp on day 5. (7). White muscle from carp on day 5. (8). White muscle from carp on day 6. (9). White muscle from carp on day 7. (10). White muscle from carp on day 8. (11). Red muscle from carp on day 10. (12). White muscle from carp on day 9. (13). White muscle from carp on day 10. (14). White muscle from carp on day 10.
Figure 5.4

Northern hybridisation of total RNA from carp subjected to decreasing environmental temperature (Group B) with the probes FG2UTR (type 7) and HIIATP

Total RNA (30μg) from the carp described as "Group B" in 4.2.15 was separated by electrophoresis, transferred to nylon membrane and hybridised sequentially with the same probes at the same times and under the same conditions as the membrane described in Figure 5.3. Each lane contains RNA extracted from the pooled muscle samples of the fish sacrificed on each day of the experiment (3 fish on day 0 and 2 fish on subsequent days)

Panel A: Autoradiograph (1 week exposure) after hybridisation with the probe FG2UTR.

Panel B: Autoradiograph (1 week exposure) after hybridisation with the probe HIIATP.

Panel C: Ethidium bromide staining of the original RNA agarose gel.

Lanes are as follows:
(1). White muscle from carp acclimatised to 28°C for five weeks (day 0.) (2). White muscle from carp on day 1. (3). White muscle from carp on day 2. (4). White muscle from carp on day 2. (5). White muscle from carp on day 4. (6). White muscle from carp on day 4. (7). White muscle from carp on day 7. (8). RNA from carp spleen. (9). White muscle from carp on day 7. (10). White muscle from carp on day 9. (11). White muscle from carp on day 10. (12). RNA from carp spleen. (13). RNA from carp liver.
Decreasing temperature

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<th>Lane</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<th>7</th>
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A

- MyoHc (6Kb)

B

- MyoHc (6Kb)

C

- 28S
- 18S
5.3.3 Temperature acclimation experiment 2: Maintenance of carp at warm temperature

i) Northern blot analysis

The hybridisation patterns of the type 7 MyoHC probe, FG2UTR with total RNA from carp described in temperature acclimation experiment 2 is presented in Figure 5.5. Expression of the type 7 MyoHC isoform was observed in adult white muscle of carp that had been exposed to an environmental temperature of 28°C. No expression was observed in white muscle from carp at 10°C (Lanes 3 and 4), or after 1 week at 28°C (Lane 5). Strong expression of the type 7 MyoHC isoform was detected through weeks 2, 3, and 4 at 28°C and a slightly weaker expression by week 5. After stripping, the membrane was rehybridised with the carp actin probe FGA101 (Gerlach et al., 1990). The actin probe hybridised with two isoforms of actin (α and β). When compared with the level of actin expression in carp which had been at 28°C for more than one week, the level of actin mRNA expression in carp sampled at 10°C and in carp after one week at 28°C was less. However the level of actin expression did not correlate with the amount of type 7 MyoHC mRNA expression in carp which had been at 28°C for more than one week (Figure 5.5 panel A lanes 7-12). Expression of the type 7 MyoHC isoform was not detected in carp red muscle, one year old carp fry or unhatched carp eggs (Figure 5.5. lanes 1, 13, 14, 15 and 16).

Northern blots of RNA from temperature acclimation experiment 2 were also probed with the Type 6 MyoHC probe, FG19UTR, (Figure 5.6). Expression of the Type 6 MyoHC isoform was only detected in the white muscle of adult carp which had been at 28°C for more than two weeks. No expression of this isoform was detected in red muscle, white muscle of carp at the starting temperature of 10°C, or the white muscle of carp which had been at 28°C for a period of 1 week. Hybridisation of the carp actin probe FGA101 and the ethidium bromide staining of the RNA gel confirm the integrity of the RNA samples.
Figure 5.5

Hybridisation of the probes FG2UTR (type 7) and EGGS24 (type 4) with RNA from carp maintained at warm temperature (acclimation experiment 2) and carp at different stages of development

Total RNA (30μg) from carp was separated by electrophoresis, transferred to nylon membrane and hybridised sequentially with the probes FG2UTR and EGGS24. Lanes contain RNA from the pooled muscle samples of a number of fish as indicated below.

Panel A: The membrane was hybridised under high stringency conditions with the probe FG2UTR and the blot exposed to X-ray film (Fuji RX) 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 (Fuji RX) at -70°C for 72 hours and superimposed on the autoradiograph of the FG2UTR hybridisation.

Panel B: The membrane was stripped of labelled probe, rehybridised with the probe EGGS24 under high stringency conditions and exposed to X-ray film for 1 week.

Panel C: Ethidium bromide staining of the original RNA agarose gel.

Lanes are as follows: (1). Red muscle from four carp acclimatised to 28°C for five weeks. (2) Carp spleen. (3). White muscle from three carp acclimated to 10°C for five weeks. (4). White muscle from two carp acclimated to 10°C for five weeks. (5). White muscle from four carp acclimated to 28°C for one week. (6). Blank lane no RNA. (7). White muscle from four carp acclimated to 28°C for two weeks. (8). White muscle from two carp acclimated to 28°C for three weeks. (9). White muscle from two carp acclimated to 28°C for three weeks. (10). White muscle from four carp acclimated to 28°C for four weeks. (11). White muscle from two carp acclimated to 28°C for five weeks. (12). White muscle from two carp acclimated to 28°C for five weeks. (13). White muscle from three juvenile (one year old) carp. (14). Five whole 60 day old carp fry, 1.2cm long, acclimatised to 28°C for 2 weeks. (15). Five whole 60 day old carp fry, 1.2cm long, acclimatised to 15°C for 2 weeks. (16). Unhatched carp eggs 24 hours after fertilisation.
Figure 5.6

Hybridisation of the FG19UTR (type 6) probe with RNA from carp maintained at warm and cold temperatures (acclimation experiment 2)

Total RNA (30µg) from carp described in 5.2.3 was separated by electrophoresis, transferred to nylon membrane and hybridised sequentially with the probes FG19UTR and FGA101.

Panel A: The membrane was probed under high stringency conditions with the Type 6 MyoHC probe, FG19UTR. The blot was then exposed to X-ray film (Fuji RX) 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 (Fuji RX) at -70°C for 72 hours and superimposed on the autoradiograph of the FG19UTR hybridisation.

Panel B: Ethidium bromide staining of the original RNA agarose gel.

Lanes are as follows: (1). Red muscle from four carp acclimatised to 10°C for five weeks. (2). Carp spleen. (3). Red muscle from four carp acclimatised to 28°C for one week (4). Red muscle from four carp acclimatised to 10°C for five weeks (5), (6), (7) and (8). White muscle from individual carp acclimated to 10°C for five weeks. (9) and (10). White muscle from two carp acclimated to 28°C for one week. (11) and (12). White muscle from two carp acclimated to 28°C for two weeks. (13) and (14). White muscle from two carp acclimated to 28°C for three weeks (15) and (16). White muscle from two carp acclimated to 28°C for four weeks. (17) and (18). White muscle from two carp acclimated to 28°C for five weeks.
Figure 5.6

- Red muscle 10°C
- Spleen
- Red muscle 28°C 1 week
- Red muscle 10°C 5 weeks
- White 10°C
- White 10°C
- White 10°C
- White 28°C 1 week
- White 28°C 1 week
- White 28°C 2 weeks
- White 28°C 2 weeks
- White 28°C 3 weeks
- White 28°C 3 weeks
- White 28°C 4 weeks
- White 28°C 4 weeks
- White 28°C 5 weeks
- White 28°C 5 weeks

18S
28S

MgoRC (65kb)
ii) *In situ* hybridisation analysis

In order to investigate which muscle fibre types were expressing the type 6 and type 7 MyoHC isoforms, *in situ* hybridisation experiments using cRNA probes synthesised from the plasmids FG19UTR and FG2UTR were carried out on muscle tissue sections from carp the carp described in temperature acclimation experiment 2. Hybridisation of the antisense cRNA from the FG2UTR plasmid showed that the expression of the Type 7 MyoHC isoform was restricted to white muscle fibres with a cross sectional area smaller than approximately 25μm (Figure 5.7). No expression of the type 7 isoform was detected in tissue sections from carp acclimated to 10°C or in the red muscle fibres from fish at either temperature. A few small diameter muscle fibres within the pink muscle fibre layer of warm acclimated fish were also shown to express the type 7 MyoHC isoform. However, the highest concentration of fibres expressing this isoform were observed within the deeper white myotomal region. Small diameter fibres within the white and pink muscle layers were also observed in the cold acclimated fish. However, no expression of the type 7 MyoHC isoform could be detected in these fibres.

Expression of the type 6 MyoHC isoform was not detected by *in situ* hybridisation experiments carried out with sense and antisense probes from the plasmid FG19UTR.
Figure 5.7

In situ hybridisation of FG2UTR probe with myotomal muscle from warm (28°C) and cold (10°C) acclimated adult carp

Paraffin wax embedded sections were prepared from muscle tissue blocks taken from warm and cold acclimated adult carp described in 5.2.3. Muscle blocks were dissected from just below the dorsal fin, extending down the flank of the fish and into the red muscle tissue. In situ hybridisation with digoxygenin labelled FG2UTR sense and antisense cRNA probes was then performed on the sections as described in 4.2.10-4.2.14. Hybridised probe was detected with anti-digoxigenin-alkaline phosphatase conjugated antibody (Boehringer Mannheim) according to the recommendations of the manufacturer with the colour reaction left to precipitate for 12 hours.

Plates A, B and E: White myotomal muscle from adult carp acclimatised to 28°C for five weeks hybridised with the FG2UTR antisense probe.

Plate C: White myotomal muscle from adult carp acclimatised to 28°C for five weeks hybridised with the FG2UTR sense probe.

Plate D: White myotomal muscle from adult carp acclimatised to 10°C for five weeks hybridised with the FG2UTR antisense probe.
Figure 5.7 plate A

Figure 5.7 plate B
Figure 5.7 plate C

Figure 5.7 plate D
Figure 5.7 plate E
5.4 Discussion

The thermal acclimation process allows carp and some other cyprinid fish to compensate for changes in environmental temperature which would otherwise be detrimental to muscle function. This thermal acclimation process has been well documented (Johnston et al., 1985; Heap et al., 1987; Crockford and Johnston, 1990; Gerlach et al., 1990; Johnston et al., 1990; Watabe et al., 1993; Watabe et al., 1994) but the mechanisms by which it is brought about still remain unclear. Several studies have suggested that the changes in myofibrillar ATPase activity in carp myotomal muscle following temperature acclimation result from changes in the MyoHC component of the myofibrils (Hwang et al., 1990; Hwang et al., 1991; Watabe et al., 1992). These changes in the MyoHC are thought to be brought about by the production of different protein isoforms and evidence for this theory has recently been supplied by differences in proteolytic digestions and SDS-PAGE analysis between MyoHC proteins from cold and warm acclimated carp (Watabe et al., 1992; Watabe et al., 1994).

The question of whether these different MyoHC proteins are produced from separate genes was first addressed by Gerlach et al., 1990 who showed that a restriction fragment (FG1706) from the carp MyoHC containing genomic clone λFG17 hybridised preferentially to RNA extracted from the white muscle of warm acclimated carp. The FG1706 probe was used in a similar experiment in this current study and although strongest hybridisation did occur in the warm acclimated carp (Figure 5.2), some hybridisation of the probe also occurred with white muscle RNA from fish which had been maintained at 10°C for five weeks. Nevertheless, it is interesting that the sequence data obtained in this current study showed that the FG1706 probe contained the exons which code for the S2 hinge region of the molecule. This region of the MyoHC molecule facilitates the formation of the crossbridge between the myosin of the thick filament and the actin of the thin filament during muscle contraction. Therefore the preferential hybridisation of the FG1706 probe to RNA from warm acclimated fish could be taken as indicative that the S2 hinge region plays a role in the differences in the myofibrillar ATPase activities observed between myosin from warm and cold acclimated carp.

A 3' untranslated region of a MyoHC gene (classed as type 2 MyoHC in this current study) was also obtained from the genomic clone λFG17, the sequence of which was verified at the cDNA level with the clone 15JUV (Chapter 3). However, the hybridisation
pattern obtained with this 3'UTR probe was very different from that of the subclone FG1706. The FG17UTR probe hybridised specifically to the pink muscle fibres of immature carp (Chapter 4) and did not hybridise to white muscle RNA from adult carp acclimated to either warm or cold temperatures. This discrepancy between the hybridisation patterns of two subclones derived from the same genomic clone could be due to one of two reasons.

Firstly, it is possible that the genomic clone \( \lambda \text{FG17} \) contains sequence from more than one MyoHC gene. In human and mouse it is known that the \( \alpha \) and \( \beta \) cardiac MyoHC genes are located on the same chromosome separated by only 4-5 Kb (Saez et al., 1987; Weydert et al., 1985; Gulick et al., 1991) and that the rest of the skeletal isoforms are also closely linked in a cluster on a different chromosome (Yoon et al., 1992). Therefore it is also possible that such clustering of MyoHC isoforms is also present in carp and taken in light of the finding that the type 7 carp MyoHC isoform (FG2) is only 12 Kb in length, it is quite possible that a genomic clone the size of \( \lambda \text{FG17} \) (17-20Kb) could overlap two different MyoHC isoforms. Thus it is possible that the subclones FG1706 and FG17UTR derive from separate MyoHC genes. Further mapping and sequencing of the genomic clone \( \lambda \text{FG17} \) would be necessary to determine this.

Secondly, it is possible the subclones FG1706 and FG17UTR are from the same MyoHC gene but the FG1706 probe cross hybridises with other MyoHC isoforms. Nucleotide sequence data obtained from FG1706 (Figure 4.1) showed that this subclone contains a series of introns and exons from a relatively well conserved part of the MyoHC gene (from exon 25 to 30). Therefore the hybridisation pattern of fragments produced from such a large clone by the random priming labelling reaction used by Gerlach et al., (1990) are likely to cross hybridise with several myosin isoforms and therefore may not necessarily reflect the actual expression patterns of the MyoHC gene encoded in the genomic clone \( \lambda \text{FG17} \). Should this second case be true, it would serve to illustrate the need to use specific probes, for example 3' untranslated region probes, in order to define the differential expression of separate isoforms.

This study has also highlighted another aspect of temperature adaptation, that of knowing whether the new protein resulting from changed gene expression can be regarded as myofibrillar remodelling or enhanced growth at higher temperatures. Muscle gene expression in carp, in relation to temperature acclimation per se, is difficult to study since at the warmer temperatures an increase in growth rate and hence a general upgrade in the expression of many genes can also be expected in addition to any functional adaptive
responses to increased environmental temperature. Thus when expression of a gene is seen to be increased after carp have been subjected to an increase in temperature, the question of whether this increase is due to a growth or an adaptational response arises. In the temperature acclimation experiments conducted in this study the energy intake of carp at warm and cold temperatures was kept constant in an attempt to minimise differences in growth rates. However this is not a fail safe measure to prevent differences in growth rates since the fish kept at the warmer temperatures may be able to utilise their energy intake more efficiently than the fish at cold temperatures. It has been shown quite conclusively (Watabe et al., 1994) that the MyoHC protein isoforms present in warm acclimated carp are different to the MyoHC isoforms present in cold acclimated carp. Thus it is logical that the changed MyoHC isoform composition is responsible for the measured differences in myofibrillar ATPase, contractile properties and swimming performance observed between warm and cold acclimated carp. However, concurrent to any adaptational changes in MyoHC gene expression, other MyoHC isoforms which are associated with muscle growth may also be expressed. Therefore, interpretation of hybridisation data investigating temperature adaptational responses in gene expression must be made with caution.

Northern blot analysis with the FG2UTR probe showed that the gene coding for the type 7 MyoHC isoform was expressed only in the white muscle of adult carp which had been subjected to an increase in environmental temperature (Figure 5.3. panel A). Expression of the type 7 MyoHC was observed after day 7 of the experiment when the water temperature had reached 23°C. This expression pattern was not a case of the fish at the lower temperatures having a generally low level of MyoHC expression since the hybridisation pattern of the HDATP probe (Figure 5.3 panel B) demonstrated that cold acclimated carp were expressing MyoHC genes. Therefore it can be concluded that exposure to warm environmental temperature induces adult carp to express a MyoHC isoform which they were not previously expressing at the colder temperatures.

Northern blot analysis performed on RNA from temperature acclimation experiment 2 (Figure 5.5) further demonstrated the temperature dependent expression of the type 7 MyoHC isoform. During this experiment the expression of the type 7 MyoHC isoform was shown to persist for at least 5 weeks following the initial increase in temperature. Hybridisation of the same blot with the MyoHC probe EGGS24 (Figure 5.5 panel B) demonstrated the existence of a developmental isoform of MyoHC which is expressed in carp fry but not in carp above 1 year of age. Failure of the FG2UTR probe to bind to the
RNA from carp fry or carp one year of age indicates that the type 7 MyoHC isoform is expressed specifically in adult fish. Given these results of Northern blot analysis one might hypothesise that the carp type 7 MyoHC isoform is involved in the temperature acclimation process. However, in situ hybridisation experiments using sense and antisense labelled FG2UTR probe revealed that the type 7 MyoHC isoform is expressed in a specific population of white and pink muscle fibres in fish that had been acclimated to a warm temperature. No hybridisation of the FG2UTR probe could be detected in the white or pink muscle from fish acclimated to cold temperature or in the red muscle fibres from any fish. The fibres shown to be expressing the type 7 MyoHC isoform in the warm acclimated carp were less than 25µm in diameter. These small diameter fibres were also present in the cold acclimated fish but in the cold fish no expression of the type 7 MyoHC isoform could be detected by either in situ hybridisation or Northern blot analysis.

Such small diameter fibres in the white muscle of carp have previously been described in a number of studies, (Rowlerson et al., 1985; Akster, 1983). Rowlerson and coworkers showed that the carp small white muscle fibres differ from the large white fibres both histochemically and immunohistochemically. The small white fibres had a higher glycogen content and were shown to react differently than the large fibres with a panel of anti-sera which had been raised against fast and slow myosins of the mullet. The small and large white fibres could also be distinguished from each other by using a mATPase staining technique after acid preincubation.

Unlike mammals where muscle growth is restricted to fibre hypertrophy after embryonic development, fish muscle can grow by both fibre hyperplasia and hypertrophy (Weatherley and Gill, 1984; Greer-Walker, 1983; Stickland, 1983). The source of the new muscle fibres causing fibre hyperplasia during growth however is still a matter of contention. Most authors prefer to assume that the new fibres arise from satellite cells (Egginton and Johnston, 1982; Johnston, 1982, Stickland 1983). Alternatively it has been hypothesised that the new fibres arise from splitting of existing fibres (Willemse and Lieuwma-Noordanus, 1983; Scapolo et al., 1984). Considering these two theories, it has been postulated (Rowlerson et al 1985) that if the splitting theory is correct, the small new fibres should contain the same myofibrillar isoforms as the fibre they split from and that if the satellite cell theory is correct then the small fibres may be expected to contain embryonic isoforms since it is known that mammalian satellite cells pass through a stage in which they contain the embryonic and neonatal isoforms of the MyoHC (Sartore et al., 1982).
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 had different histo- and immunohistochemical properties to 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, if the small fibres present in adult carp arise from myosatellite cells, then they would not necessarily have to contain embryonic or neonatal equivalents of the MyoHC.

The theory that if small diameter fibres arise from fibre splitting they should contain the same MyoHC isoforms as fibre they split from is not necessarily correct either. Some muscle fibres are known to have a non uniform expression of MyoHC isoforms along their length (Staron and Pette, 1987; Taylor and Bandman, 1989). This distinct molecular phenotype along the length of a muscle fibre represents different nuclear domains (Pavlath *et al.*, 1989). Therefore it is conceivable that a muscle fibre could express a different MyoHC isoform down a segment of its length and that fibre splitting is a consequence of this.

The results of Rowlerson and coworkers indicate that the MyoHC content of the small and large white muscle fibres differ. They therefore postulated that the small white muscle fibres are involved in fibre hyperplasia in fish growth and that the MyoHC isoform present in the small white muscle fibres is equivalent to the mammalian embryonic or neonatal MyoHC isoform. The results obtained by in situ hybridisation with the FG2UTR probe in this current study confirm the results of Rowlerson *et al.*, 1985 by showing directly that the small white muscle fibres express a MyoHC gene (type 7 MyoHC) that is not expressed in the large white muscle fibres. However, the hypothesis that this unique isoform in the small fibres is an embryonic or neonatal equivalent is incorrect since the Northern blot analysis performed with the FG2UTR probe showed that young carp fry and unhatched carp eggs do not express the type 7 isoform.

Considering the combined results of the Northern and in situ analysis done with the FG2UTR probe the question of the role of the type 7 MyoHC isoform now arises. Is the type 7 MyoHC isoform involved in muscle growth or is the isoform involved in the adaptational response to temperature? After five weeks at 28°C the adaptational response in terms of myofibrillar ATPase activity is complete (Heap, 1985). Therefore any MyoHC isoforms responsible for this change could be expected to be present in the majority of the white muscle fibres. However, after five weeks acclimation to 28°C, the type 7 MyoHC
isoform was only observed within the small diameter fibres which were in a minority when compared to the larger white muscle fibres. Therefore, it seems more likely that the role of the carp type 7 MyoHC isoform is involved in temperature induced muscle growth rather than temperature acclimation per se. However it is still not clear from these results whether the small muscle fibres have arose from fibre splitting or from myosatellite cells. Visual inspection of the small fibres expressing the type 7 MyoHC isoform (Figure 5.7 plate E) gives the impression of fibre splitting since in some cases a clear boundary between the small "splitting" fibre and the larger "parent" fibre can be seen whilst in others the area expressing the type 7 MyoHC isoform looks like it is attached to a larger fibre. *In situ* hybridisations using the FG2UTR probe on longitudinally cut sections would possibly give a better indication as to whether the small fibres expressing the type 7 MyoHC isoform are splitting from larger fibres.

A second MyoHC isoform type isolated in this current study (type 6 carp MyoHC) also showed a hybridisation pattern in which expression was only observed in the white muscle of adult carp which had been acclimated to a warm temperature (Figure 5.6). *In situ* hybridisation experiments using the 3' untranslated region probe (FG19UTR) for this isoform however showed a negative result for both the sense and antisense labelled probes (data not shown). This lack of hybridisation was attributed to sub-optimal conditions for *in situ* hybridisation of this probe rather than absence of gene expression since the sections for *in situ* hybridisation experiments were taken from the same fish which had shown strong expression of the type 6 MyoHC isoform by high stringency Northern hybridisation.

In summary, the use of isoform specific 3' UTR probes in this study has demonstrated the dynamic nature of MyoHC isoform expression in carp subjected to changes in environmental temperature. Expression of both type 6 and type 7 carp MyoHC isoforms was clearly shown to be upregulated in response to an increase in environmental temperature. *In situ* hybridisation with the type 7 MyoHC isoform probe shows expression is limited to small diameter fibres suggesting that the role of this MyoHC isoform is in temperature induced muscle growth. The fibre types expressing the type 6 MyoHC isoform remain to be determined by *in situ* hybridisation. Knowledge of which fibre population express the type 6 MyoHC isoform during acclimation to warm environmental temperature is necessary to establish whether this isoform is involved in the temperature acclimation process or growth process.
Chapter 6

General Discussion
6.1 Summary of results

The objective of the work described in this thesis was to characterise the family of MyoHC isoforms of carp by investigating the expression patterns of their corresponding genes. In order to achieve this goal, DNA probes specific for individual MyoHC isoforms had to first be isolated. Using genomic clone analysis and 3'RACE PCR, seven distinct carp MyoHC 3' untranslated regions were isolated (Chapters 2 and 3) and subsequently used in hybridisation studies to map the expression patterns of the individual isoforms (Chapters 4 and 5). Whilst it is unlikely that the seven MyoHC sequences isolated in this current study account for the full family of MyoHC isoforms in the carp, the molecular analysis performed in this study demonstrated interesting aspects of MyoHC polymorphism in a species of fish.

One interesting finding from the genomic clone analysis described in Chapter 2 was the size of the MyoHC gene contained in the genomic clone λFG2. Restriction mapping and DNA sequencing demonstrated that this gene is approximately half the size (12Kb) of mammalian and chicken MyoHC isoforms sequenced to date. Since the size of the mRNA transcript for this gene was shown to be 6Kb, the size of mammalian and chicken MyoHC mRNA transcripts, the difference in size at the genomic level can be attributed to shorter intron sequences.

The hybridisation analysis performed in this study showed that, like mammalian species, the expression of MyoHC isoforms in carp is developmentally regulated. Of the seven 3'UTR probes investigated in this study, five (types 1 to 5) hybridised to total RNA extracted from the skeletal muscle of carp under 1 year of age. One of these probes, type 4, only hybridised to RNA from carp fry and two, types 1 and 5, hybridised to skeletal muscle RNA from both adult and immature carp. The type 1 probe hybridised exclusively to RNA extracted from white muscle fibres whereas the type 5 probe hybridised to both red and white muscle RNA.

*In situ* hybridisation studies with the type 2 carp MyoHC gene probe demonstrated the fibre type specific expression of this isoform. Expression was only observed in the pink muscle fibre layer of juvenile carp and carp fry. Whilst the existence of distinct isoforms of myosin in the pink muscle fibre layer of carp has been
demonstrated indirectly in adult fish by histochemical and immunohistochemical techniques (Akster, 1983) the results presented in this study provide the first direct evidence that developmentally regulated fibre type specific MyoHC gene expression also occurs in the pink muscle fibre layer.

Two of the 3'UTR sequences isolated (types 6 and 7) hybridised exclusively to white muscle RNA from adult fish. Furthermore, the expression of both these isoform types was only observed in fish which had been acclimated to a warm environmental temperature. The possible role of these two MyoHC isoforms in the temperature acclimation process was investigated in Chapter 5. Whilst both types 6 and 7 carp MyoHC were clearly shown to have a temperature dependent expression pattern, their role in temperature acclimation per se still remains to be determined unequivocally. The expression of the type 7 MyoHC isoform was shown by in situ hybridisation to be exclusively in the small diameter (25μm) muscle fibres suggesting that this isoform may be involved in muscle growth by hyperplasia rather than temperature acclimation. If different MyoHC isoforms are preferentially expressed at warm and cold temperatures then one would expect to see such isoforms expressed in the majority of the muscle fibres after five weeks since at this time the changes observed in the myofibrillar ATPase activity are complete (Heap, 1985). Determination of the fibre population which expresses the type 6 carp MyoHC isoform is required before its role in temperature acclimation can be further elucidated.

6.2 Do the seven 3' MyoHC sequences isolated correspond to seven separate MyoHC genes?

The question of whether the seven MyoHC 3'UTR sequences isolated in this current study correspond to seven distinct MyoHC genes, rather than some types being alleles of the same gene, can be addressed by assessing both sequence comparison data (Chapters 2 and 3) and the expression patterns obtained by hybridisation of 3'UTR containing probes (Chapters 4 and 5).

Comparison of the deduced amino acid sequences corresponding to the last two exons of the MyoHC gene (Table 3.7), showed a high degree of homology between all
seven of the MyoHC types isolated. Indeed, two of the carp MyoHC types isolated in this study (types 4 and 5) have identical deduced amino acid sequence in the carboxyl terminal region. Such high sequence homology over this region of the gene is not uncommon between different MyoHC isoforms within the same species. A similarly high sequence homology is also present in the rat MyoHC isoforms where the amino acid sequence of the fast IIa and IIb isoforms are identical to one another over the known carboxyl terminal region and the IIX isoform has only one amino acid difference (DeNardi et al., 1993). Thus, for five of the seven MyoHC types deduced amino acid sequence differences can be taken as indicative that they correspond to separate genes, especially in view of the fact that no vertebrate MyoHC gene to date has been found to undergo alternative splicing of exons.

The 3'UTR nucleotide sequences of the seven carp MyoHC types show, in most cases, sufficient sequence divergence from each other (Table 3.2) to strongly suggest that they correspond to separate genes. The two carp MyoHC types which showed the highest sequence homology in their 3'UTR sequences (85% identity) were type 7 (\(\lambda\)FG2) and type 2 (\(\lambda\)FG17 and 15JUV). The homology between the 3'UTR sequences of these two MyoHC types is sufficiently high to raise suspicion that they correspond to alleles of the same MyoHC gene. However, two lines of evidence suggest that these two MyoHC types do in fact correspond to distinct genes. Firstly, analysis of the corresponding genomic clones revealed that the intron between the penultimate and final exon is different in both sequence and length (Figure 2.7). Secondly, these two carp MyoHC types show a completely different pattern of expression when the 3'UTR is used as probe (Chapters 4 and 5). The type 2 MyoHC 3'UTR probe hybridised exclusively to the pink muscle fibres in immature carp whereas the type 7 MyoHC 3'UTR probe hybridised only to the small diameter muscle fibres in adult carp acclimated to a warm temperature.

Therefore, considering the results of sequence comparison analysis and expression studies, it is reasonable to assume that all seven carp MyoHC types isolated in this study correspond to distinct MyoHC genes rather than some being alleles of the same gene. Thus, the research described in this thesis provides the first direct evidence that carp possess and express multiple MyoHC isoform genes.
6.3 Possible mechanisms involved in creating a MyoHC multigene family

In order to determine the evolutionary relationships between MyoHC isoforms of different species, sequence comparisons at both the amino acid and nucleotide levels have been performed by a number of workers (Moore et al., 1993; Eller et al., 1989; Stedman et al., 1990; Moore et al., 1992; Matsuoka et al., 1991). Moore and coworkers (1993), compared the LMM nucleotide and derived amino acid sequences of five chicken fast MyoHCs, eight mammalian fast MyoHCs and six mammalian slow MyoHCs. The data resulting from these comparisons confirmed the results of previous investigations (Stedman et al., 1990; Stewart et al., 1991) by demonstrating that the mammalian MyoHC isoform types, for example the rat IIb gene and the mouse IIb gene, are more similar to each other than they are to other MyoHC isoforms within the same species. The chicken MyoHC isoforms however were shown to be more similar to each other than they are to MyoHC isoforms from other species leading Moore and coworkers (1992; 1993) to hypothesise that the chicken MyoHC multigene family has arose from an evolutionary recent expansion by gene conversion. Alternatively, or in conjunction with gene conversion events, molecular mechanisms associated with concerted evolution may have played a role in slowing the divergence of the chicken MyoHC isoforms.

The term "gene conversion" has to be used loosely when referring to MyoHC gene families of higher eukaryotic organisms since in such species it has not been possible to determine whether "classical" gene conversion as defined in yeast and fungi has occurred (Moore et al., 1993). Thus regarding MyoHC multigene families the term gene conversion has been used to describe short nonreciprocal exchanges between closely related genes. In the highly conserved chicken MyoHC gene family and the mammalian cardiac MyoHC genes, both the size and distribution of the sequences which show 100% homology are consistent with gene conversion like events (Moore et al., 1993).

The high sequence homology between the 3'UTR sequences of the type 7 and type 2 MyoHC isoforms could also be taken to be indicative that evolutionary recent gene conversion events have also occurred in the carp. Furthermore, given the fact that these two isoform types have very different expression patterns, one might hypothesise
that such a gene conversion event led to the splicing of the 3' end of a common ancestral gene to different 5' ends which have different regulatory (promoter) regions, hence the different expression patterns of the resulting duplicated genes. If such gene conversion events were responsible for the high sequence homology observed between the 3'UTR sequences of the carp type 2 and type 7 MyoHC genes then one would also expect to see a similarly high degree of homology in the intron sequence in the 3' ends of these two genes. Whilst only limited sequence data for these two MyoHC genes is available at this time, the intron between putative exons 40 and 41 has been sequenced for both these genes (Figure 2.7) and this shows only limited sequence homology at the 3' splice site.

For many years the 3'UTR sequences of genes have been "structures in search of a function" however, a number of recent experiments have shown that they may play a role in controlling cell growth and differentiation (Rastinejad and Blau 1993) and may also modulate the efficiency of translation by influencing rates of mRNA degradation (for review see Jackson and Standart 1990). Therefore, one possible explanation of why the 3'UTR sequences of the carp types 6 and 7 MyoHC isoforms should show more homology than the preceding introns is that the 3'UTR plays some role in the regulation of gene expression or RNA processing and this role has led to selective pressure for the 3'UTR sequence to be more conserved than the intron sequence. More extensive sequence data from the genomic clones λFG2 and λFG17 would be required to further investigate the evolutionary relationships between these two isoforms. The promoter sequence of the λFG2 clone (type 7) has already been isolated, sequenced and partially characterised (L. Gauvrey personal communication) and comparison of this sequence with the promoter region of λFG17 may yield some interesting data.

6.4 Sequence comparisons between the 3'UTR sequences of MyoHC isoforms from a variety of species

The 3'RACE PCR method utilised in this current study to isolate the 3' ends carp MyoHC genes (Chapter 3) has been recently been employed by the author and colleagues to isolate MyoHC sequences from a range of mammalian species. The new MyoHC gene sequences isolated include the human IIa gene (Ennion et al., 1994), the IIa and IIX
MyoHC genes from the pig (S. Ennion and S. Yang unpublished data) and the IIX, IIa, IIb MyoHC genes from the rabbit (G. McCoy, unpublished data). Comparisons of the 3'UTR sequences of these and previously published MyoHC genes from a variety of species (Figure 6.1) demonstrate sequence homology relationships between the 3' UTR sequences of MyoHC isoforms across different species.

The mammalian 3'UTR sequences show higher homology between equivalent isoforms across different species than between other isoforms from the same species. In contrast, the 3'UTR sequences from carp, Xenopus and chicken show more homology intraspecies than interspecies. These relationships are demonstrated in the dendrogram produced by the CLUSTAL alignment program (Higgins and Sharp, 1988) (Figure 6.1). Interestingly, two of the carp 3'UTR sequences show more homology to the Xenopus MyoHC 3'UTRs than the other carp isoforms. However, this homology is low at 40% sequence identity. The type 3 carp MyoHC is positioned alone in the dendrogram indicating that it does not have any homology with the other sequences. The expression patterns of the individual carp MyoHC isoforms does not bear any relationship to the grouping of the isoforms in the dendrogram alignment.

The homologies between the 3'UTR sequence are likely to reflect evolutionary relationships between species and it will be interesting in the future to compare the carp chicken and Xenopus MyoHC sequences with isoforms from other species of fish, birds and amphibians to see if a relationship similar to that present in the mammalian isoforms is also present in these species.

The 3'UTR sequences of the rat fast MyoHC isoforms (IIa, IIb and IIX) have been shown to contain small homologous stretches of sequence (DeNardi et al, 1993) and these have also been highlighted between the human and rat fast MyoHC (Saez and Leinwand 1986). The MyoHC sequences aligned to produce the dendrogram in Figure 6.1 also showed some regions of homology between MyoHC isoforms. Whilst no sequence was found to be present in all the 3' UTR sequences aligned, a ten nucleotide sequence "AAAATGTGAA" was found to be present in twelve of the isoforms aligned (Table 6.1). Moreover, this sequence was present in MyoHC 3'UTRs from a wide range of species where it appeared in approximately the same position (38-45 nucleotides after the stop codon) in the 3'UTR. It is unlikely that this motif is present purely by chance.
since the surrounding sequence in divergent species showed no homology. Furthermore, the fact that it occurs at approximately the same position after the stop codon in each gene suggests that it has a functional role which has caused its conservation through evolution. Areas of sequence identity were also present in the carp MyoHC 3'UTR sequences, the most noticeable of which was a 15 nucleotide motif which was present in four of the seven carp isoforms (Table 6.1).

The significance of these conserved sequence motifs in the 3'UTR is unclear at this time and requires further investigation. Band shift assays with cytoplasmic extracts from muscle cells would need to be performed in order to establish whether such motifs are binding recognition sequences for proteins which may be involved in RNA processing.
Dendrogram alignment of MyoHC 3'UTR sequences from a variety of species. Sequence data from the first nucleotide of the stop codon to the first A of the Poly (A) tail were aligned using the CLUSTAL alignment program of Higgins and Sharp, (1988) with an "open gap" cost of 10 and a "unit gap" cost of 10. The EMBL accession numbers of the source sequences are as follows.

Table 6.1 Conserved sequence motif in MyoHC 3'UTRs

A)

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carp type 4</td>
<td>45 to 54</td>
<td>ttgtatatcg AAAATGTGAA attgattcaat</td>
</tr>
<tr>
<td>Chicken fast</td>
<td>40 to 49</td>
<td>aggcagctcg AAAATGTGAA cctctgcttt</td>
</tr>
<tr>
<td>Chicken embryonic 1</td>
<td>40 to 49</td>
<td>agaattgcac AAAATGTGAA attctatcacc</td>
</tr>
<tr>
<td>Chicken embryonic 2</td>
<td>40 to 49</td>
<td>agaattgcac AAAATGTGAA attctatcacc</td>
</tr>
<tr>
<td>Human IIIX</td>
<td>41 to 50</td>
<td>agaattgcac AAAATGTGAA aacttattgc</td>
</tr>
<tr>
<td>Pig IIa</td>
<td>42 to 51</td>
<td>agagagccgc AAAATGTGAA gtctttgctt</td>
</tr>
<tr>
<td>Rabbit IIb</td>
<td>40 to 49</td>
<td>agaattgcac AAAATGTGAA gttcaaaagt</td>
</tr>
<tr>
<td>Rabbit IIIX</td>
<td>40 to 49</td>
<td>agaattgcac AAAATGTGAA actctttgc</td>
</tr>
<tr>
<td>Rat IIa</td>
<td>42 to 51</td>
<td>agagaagccgc AAAATGTGAA gcttattgc</td>
</tr>
<tr>
<td>Rat IIIX</td>
<td>40 to 49</td>
<td>agagagacctc AAAATGTGAA gatcattgc</td>
</tr>
<tr>
<td>Xenopus E19</td>
<td>38 to 47</td>
<td>tgaaatttgc AAAATGTGAA ttcttttctt</td>
</tr>
<tr>
<td>Xenopus E15</td>
<td>38 to 47</td>
<td>tgaaatttgc AAAATGTGAA ttcttttctt</td>
</tr>
</tbody>
</table>

B)

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carp type 1</td>
<td>21 to 35</td>
<td>aaactagaca TACAAGCAAGCATAT gactgacttgt</td>
</tr>
<tr>
<td>Carp type 6</td>
<td>21 to 35</td>
<td>ataccagcgc TACAAGCAAGCATAT aataagacttg</td>
</tr>
<tr>
<td>Carp type 2</td>
<td>26 to 40</td>
<td>acaccagcgc TACAAGCAAGCATAT aatatgacctt</td>
</tr>
<tr>
<td>Carp type 7</td>
<td>26 to 40</td>
<td>agaccagcgc TACAAGCAAGCATAT aatatgacctt</td>
</tr>
</tbody>
</table>

Legend.

A) Sequence motif found in the 3'UTR across range of isoforms in a range of species.
B) Sequence motif found in four of the seven carp MyoHC 3'UTRs.
The sequence motif is given in bold uppercase text and the flanking sequences in lowercase. "Position" corresponds to the number of nucleotides after the stop codon that the motif appears. The source of the sequence data is given in Figure 6.1.
6.5 The biological relevance of multiple MyoHC isoforms

Since all vertebrate species studied to date have been shown to possess multiple isoforms of the MyoHC which are encoded by a multigene family it is difficult to avoid the question of why so many isoforms should exist. A number of theories have been proposed for the reasons why animals have so many different MyoHC isoforms and the evidence available to date is not sufficient to completely rule any of them out.

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 is a consequence of various functional demands on the molecule dictating evolution by selective pressure. Such functional demands may possibly reside in the S1 region of the molecule where differences in primary structure could affect the actin binding properties and ATPase activity of the molecule. The tail beat frequency of fish changes dramatically with development and is far higher in fry when compared to adult fish. Therefore, developmental changes of MyoHC gene expression observed in this study may confer the necessary ATPase activities or actin binding properties necessary for these differing modes of swimming. Alternatively the functional demands which dictate evolutionary pressure for multiple MyoHC isoforms may reside in the 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). This theory is supported by the fact that during situations of muscle regeneration in adult mammals, the embryonic and neonatal isoforms are expressed (see Chapter 1). Also, if the expression of the carp type 7 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 which facilitated the correct formation of thick filaments could be important since it is difficult to envisage changes in ATPase 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 changes in protein function. Thus the MyoHC isoforms themselves would be very similar in their functional properties but differ significantly in the regulatory regions of their genes.
It has also been proposed that isoforms are a consequence of evolutionary dictated specialisation based on chromosomal geography (Gros and Buckingham, 1987). Hence two identical genes which were originally generated by a gene duplication event may have diverged independently depending on their chromosomal location. However, due to the location of the MyoHC genes in the mammalian genome such an argument could only be used to explain the divergence between the cardiac and skeletal isoforms.

6.6 Future studies

The isolation of isoform specific carp MyoHC gene probes in this study has provided the molecular tools for many possible future lines of investigation. One interesting finding from this work which warrants further investigation is the hybridisation pattern of the type 7 MyoHC probe. The MyoHC gene corresponding to this probe was shown, by in situ hybridisation, to be expressed exclusively in the small diameter white muscle fibres of adult carp which had been acclimated to a warm environmental temperature. As discussed previously (Chapter 5) such small diameter muscle fibres have been implicated to be involved in the fibre hyperplasia process. A fundamental question regarding fibre hyperplasia in fish however, remains unanswered and this is the source of the new fibres i.e. do they arise from fibre splitting or from myosatellite cells. One approach to this question is to culture carp white muscle satellite cells (Koumans et al., 1990) and using the probes isolated in this current study, investigate which MyoHC isoforms are expressed when the cells start to differentiate. If expression of the type 7 MyoHC isoform is observed in such differentiating satellite cells then this can be taken to suggest that the small diameter fibres have arose from satellite cells rather than fibre splitting. Such studies are currently being performed.

Whilst the work described in Chapter 4 of this thesis showed that the post hatching development of carp fry involves the expression of at least five MyoHC isoforms, further investigations which more accurately map the sequential expression of MyoHC isoforms during fry and postlarval development are required. Such a study could easily be performed by Northern blot and in situ hybridisation analysis with the isoform specific probes isolated in this study and will be undertaken on fry from the next

With regards to the role of MyoHC isoforms in the temperature acclimation process in carp, the results presented in this study demonstrate that expression certain MyoHC isoforms can be upregulated in response to an increase in temperature. Whilst it seems likely that the type 7 MyoHC gene is involved in hyperplasic growth rather than an acclimatory response, the type 6 MyoHC still remains a candidate for a MyoHC gene involved in acclimation to warm temperature and the expression of this isoform requires further investigation. None of the seven MyoHC isoforms isolated in this study were shown to be expressed specifically in response to cold temperature acclimation. Isolation of such a MyoHC gene in which expression is upregulated in response to cold temperatures would provide more conclusive evidence that MyoHC isoforms facilitate temperature acclimation since the expression cannot be confused with a growth response. The use of degenerate oligonucleotides in RACE PCR on cDNA prepared from cold acclimated carp may be more suited to isolation of such an isoform than the FG2EXN40 oligonucleotide employed in this study.
Appendix 1

Plasmid constructs used for generation of DNA and cRNA probes
1. MyoHC Type 1

The clone WHITECl was used to generate the isoform specific probe used to investigate the expression pattern of MyoHC Type 1 isoform. This clone was generated by RACE PCR on cDNA prepared from the white axial muscle of a 3 year old carp, 24cm in length, which had been acclimated to 10°C for five weeks (as described in Chapter 3).

Cloning details

The PCR product was cut with the restriction enzyme *EcoRI*, which cuts within the 3' PCR primer RoridT17, and then ligated into PBS° phagemid (Stratagene) at the *HindIII* and *EcoRI* sites. The *HindIII* site in the plasmid polylinker was lost after ligation.

Probe Preparation for Northern hybridisations

The clone was cut with *HindIII* to produce linearised DNA. Oligo dT with a cytosine nucleotide at the 5' end (17 mer in total) was used to prime specifically from the poly (A) tail producing a 154bp labelled antisense probe containing the 3'UTR.
2. MyoHC Type 2

The subclones FG17UTR and 15JUV were used to generate the isoform specific probes for investigation of the expression pattern of MyoHC Type 2 isoform. The subclone FG17UTR was derived from the genomic clone λFG17. The clone 15JUV was generated by RACE PCR on cDNA prepared from the white axial muscle of a 14 month old carp, 7cm in length, which had been acclimated to 20°C for five weeks (as described in Chapter 3).

A. FG17UTR

Cloning details

The 2.0 Kb SacI restriction fragment of the genomic clone λFG17 was subcloned into Bluescript II SK+ phagemid (Stratagene) at the SacI site and named FG17S2.0. This subclone was then cut with FokI restriction enzyme and a 149bp fragment isolated. The 149 bp restriction fragment was treated with mung bean exonuclease to create blunt ends and then ligated into alkaline phosphatase treated, Smal cut pBS phagemid (Stratagene).

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 Probe Preparation for Northern and In Situ hybridisations

For Northern hybridisations the subclone FG17UTR was cut with EcoRI to produce linearised DNA. The T3 oligonucleotide was then used to specifically prime antisense labelled DNA containing 149 bp of insert DNA with 60bp (including the T3 oligo) of plasmid polylinker at the 3' end. For In Situ hybridisations the subclone FG17UTR was cut with EcoRI and the bacteriophage T3 RNA polymerase enzyme used to transcribe antisense labelled cRNA. For sense cRNA the plasmid was cut with HindIII and RNA transcribed with the bacteriophage T7 RNA polymerase.
B. 15JUV

Cloning details

The PCR product 15JUV was cloned directly into the pCR™ II TA cloning vector (Invitrogen) according to the manufacturers instructions. Subsequently the insert of this clone was transferred to pBS(+) (Stratagene) phagemid by cutting the pCR™ clone with SacI and XbaI enzymes and ligating to SacI/XbaI cut pBS(+) phagemid.

Probe preparation for In Situ hybridisations

For in situ hybridisations the subclone 15JUV in pBS(+) was cut with HindII and the bacteriophage T7 RNA polymerase enzyme used to transcribe antisense labelled cRNA. For sense cRNA the FG17UTR plasmid was used (cut with HindIII and RNA transcribed with the bacteriophage T7 RNA polymerase).
3. MyoHC Type 3

The clone EGGS22 was used to generate the isoform specific probe used to investigate the expression pattern of MyoHC Type 3 isoform. This clone was generated by RACE PCR on cDNA prepared from unhatched fertilised carp eggs 26 hours after spawning (as described in Chapter 3)

Cloning details

The PCR product EGGS22 was cloned directly into the pCR™ II TA cloning vector (Invitrogen) according to the manufacturers instructions.

Probe Preparation for Northern hybridisations

The clone was cut with MboII which produced multiple DNA fragments of vector and insert. The 1262 bp MboII fragment was isolated and oligo dT with a cytosine nucleotide at the 5' end (17 mer in total) was used to prime specifically from the poly (A) tail of the insert producing a 112 bp labelled antisense probe.
4. MyoHC Type 4

The clone EGGS24 was used to generate the isoform specific probe used to investigate the expression pattern of MyoHC Type 4 isoform. This clone was generated by PCR on cDNA prepared from unhatched fertilised carp eggs 26 hours after spawning (as described in Chapter 3)

Cloning details

The PCR product EGGS24 was cloned directly into the pCR™ II TA cloning vector (Invitrogen) according to the manufacturer's instructions.

Probe Preparation for Northern hybridisations

The clone was cut with PvuII which produced multiple DNA fragments of vector and insert. The 426 bp PvuII fragment was isolated and oligo dT with a guanine nucleotide at the 5' end (17 mer in total) was used to prime specifically from the poly (A) tail of the insert producing a 97 bp labelled antisense probe.
5. MyoHC Type 5

The clone FRY2811 was used to generate the isoform specific probe used to investigate the expression pattern of MyoHC Type 5 isoform. This clone was generated by PCR on cDNA prepared from 60 day old carp fry, 1.2cm in length, which had been acclimated to 15°C for two weeks (as described in Chapter 3).

Cloning details

The PCR product FRY2811 was cloned directly into the pCR™ II TA cloning vector (Invitrogen) according to the manufacturers instructions.

Probe Preparation for Northern hybridisations

The clone was cut with PvuII which produced multiple DNA fragments of vector and insert. The 407 bp PvuII fragment was isolated and oligo dT with a guanine nucleotide at the 5' end (17 mer in total) was used to prime specifically from the poly (A) tail of the insert producing a 84 bp labelled antisense probe.
6. MyoHC Type 6

The subclone FG19UTR was used to generate the isoform specific probes for investigation of the expression pattern of MyoHC Type 6 isoform. The subclone FG19UTR was derived from the genomic clone λFG19.

Cloning details

The 1.6 Kb PstI restriction fragment of the genomic clone λFG19 was subcloned into Bluescript II SK+ phagemid (Stratagene) at the PstI site and named FG19P1.6. This subclone was then cut with HindIII restriction enzyme and a 320bp fragment isolated. This 320 bp restriction fragment was subcloned into the HindIII site of Bluescript II SK+ phagemid and named FG19h320. The insert DNA of FG19h320 was then isolated and digested with FokI enzyme. After treatment with mung bean exonuclease to produce blunt ends the 114bp fragment from this digestion was subcloned into pBS phagemid at the Smal site to produce the subclone FG19UTR.
Probe Preparation for Northern and *In Situ* hybridisations.

For Northern hybridisations the subclone FG17UTR was cut with *EcoRI* to produce linearised DNA. The T3 oligonucleotide was then used to specifically prime antisense labelled DNA containing 114 bp of insert DNA with 60bp (including the T3 oligo) of plasmid polylinker at the 3' end.

For *In Situ* hybridisations the subclone FG19UTR was cut with *EcoRI* and the bacteriophage T3 RNA polymerase enzyme used to transcribe antisense labelled cRNA. For sense cRNA the plasmid was cut with *BamHI* and RNA transcribed with the bacteriophage T7 RNA polymerase.

7. MyoHC Type 7

The subclone FG2UTR was used to generate the isoform specific probes for investigation of the expression pattern of MyoHC Type 7 isoform. The subclone F2UTR was derived from the genomic clone λFG2 as previously described in Chapter 2.

![Diagram](image)

**FG2UTR**

Probe Preparation for Northern and *In Situ* hybridisations.

For Northern hybridisations the subclone FG2UTR was cut with *EcoRI* to produce linearised DNA. The T7 oligonucleotide was then used to specifically prime antisense labelled DNA containing 168 bp of insert DNA with 61bp (including the T7 oligo) of plasmid polylinker at the 3' end. For *in situ* hybridisations the subclone FG2UTR was cut with *EcoRI* and the bacteriophage T7 RNA polymerase enzyme used to transcribe antisense labelled cRNA. For sense cRNA the plasmid was cut with *XbaI* and RNA transcribed with the bacteriophage T3 RNA polymerase.
8. General MyoHC probe

The subclone HIIATP was used as a general MyoHC probe. This subclone contains part of the human β MyoHC gene which codes for the ATP binding site. Since this region is highly conserved between isoforms, it was postulated that this probe would bind to all MyoHC isoforms present in the carp thereby acting as a control.

Cloning details

A plasmid containing the 1581bp NcoI-NcoI fragment of the human β MyoHC gene (EMBL Ac M30605) was a kind gift from Dr Thomas Jaenicke. From this plasmid the 183 bp HindII-HindII restriction fragment which contains part of exons 5 and 6 was subcloned into pBS(+) phagemid at the Smal site

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Probe Preparation for Northern and In Situ hybridisations.

For Northern hybridisations the subclone HIIATP was cut with BamHI to produce linearised DNA. The T7 oligonucleotide was then used to specifically prime antisense labelled DNA.

For in situ hybridisations the subclone HIIATP was cut with BamHI and the bacteriophage T7 RNA polymerase enzyme used to transcribe antisense labelled cRNA. For sense cRNA the plasmid was cut with EcoRI and RNA transcribed with the bacteriophage T3 RNA polymerase.
Appendix 2

Publications


REFERENCES

Aigner S. and Pette D. (1990) In situ hybridisation of slow myosin heavy chain mRNA in normal and transforming rabbit muscles with the use of a nonradioactively labeled cRNA. Histochemistry 95, 11-18.


190


202


