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**INVESTIGATION INTO THE  
TRANSCRIPTIONAL REGULATION OF PORCINE  
MUSCLE FIBRE TYPE**

*by*

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A thesis completed in partial fulfilment of the degree of Doctor of Philosophy,  
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*For Erica*

## SUMMARY

Skeletal muscle is a highly organised yet adaptive tissue, capable of changing aspects of fibre phenotype in response to demand. While the external signals mediating these changes, such as patterns of motor neuron activity or hormone signalling, are well studied the manner by which these external stimuli are converted into specific patterns of gene expression is less understood. GATA-2 and NFAT-2 are two transcription factors implicated in this process. In this study, conserved regions of the GATA and NFAT transcription factor families were used to design primers for a homology-based PCR-cloning approach to isolate the porcine orthologues of GATA-2 and NFAT-2. The pig was chosen as the target species for this investigation, given the importance of porcine muscle both as a large animal model for muscle development and as a commercially important meat animal.

The long-term goal of the work was to determine what effects these transcription factors might have in the regulation of stage- and isoform-specific gene expression. A porcine orthologue of GATA-2 was cloned, and the sequence submitted to GenBank (Accession N<sup>o</sup> AY251012). Though only a truncated cDNA clone was obtained from efforts to clone porcine NFAT-2, sufficient sequence data was recovered for the design of isoform-specific primers for quantitative real-time RT-PCR. GATA-2 and NFAT-2 were found to have a wide tissue distribution. A novel finding was that GATA-2 was found to be highly expressed in uterine smooth muscle. This suggests that the segregation of GATA-family members between haemopoietic functions and mesodermal tissues may not be as distinct as was previously thought.

In addition to studying *trans*-acting factors, this study also looked at potential targets for factors modifying fibre phenotype, in particular the isoforms of myosin heavy chain (MyHC). The different MyHC isoforms expressed in a muscle fibre greatly influence aspects of phenotype such as shortening velocity and ATPase activity, and are indicative of fibre type. One such isoform, the embryonic MyHC (emMyHC), was one of the last remaining uncharacterised isoforms in the pig. The first intron and immediate upstream region of the porcine embryonic MyHC were isolated and sequenced, and it was found that the translation start site of the porcine isoform was

located on exon 2, something which had not been previously reported in any other isoforms or orthologues of the gene. A GATA-2 binding site was found, 4 kb upstream of the transcriptional start site. Reporter constructs were generated for use in conjunction with the GATA-2 clone.

Through *in vitro* transcription-translation, the GATA-2 clone was found to encode a functional reading frame for a protein of the expected size and was deemed suitable for functional studies. Technical difficulties frustrated the efforts to quantify changes in MyHC expression in porcine primary myoblasts through quantitative real-time RT-PCR. However, a preliminary *in vitro* study in C2C12 murine myoblasts suggested that emMyHC reporter activity was reduced by GATA-2 overexpression. Together with developmental expression patterns showing an upregulation in GATA-2 coinciding with the developmental downregulation of emMyHC, and the presence of a putative GATA-2 binding site in the 5' UTR, this was taken as evidence of a possible role for GATA-2 in negatively regulating emMyHC. Some possibilities for studies to further explore these findings are discussed.

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## DECLARATION

The studies described in this thesis were carried out in the Molecular Medicine Laboratory, in the Department of Veterinary Pathology at the University of Glasgow Veterinary School between September 1999 and February 2004. The author was directly responsible for all the work described herein, with the exception of the following: Sequencing using the ABI PRISM dGTP BigDyc Sequencing kit described in Chapter Three was carried out by staff at the MBSU department at Glasgow University. The characterisation of the porcine emMyHC isoform 5' regulatory region, described in Chapter Four was done as part of a collaboration between the Molecular Medicine Laboratory and Dr Yuh-Man Sun of the Roslin Institute, Edinburgh. The three GFP-promoter constructs resulting from this work were kindly donated by Dr Yuh-Man Sun. Western blotting and *In Vitro* Transcription Translation described in Chapter Five were carried out by Dr Hadi Alzuherri, also in the Molecular Medicine Laboratory at the University of Glasgow Veterinary School. Pooled cDNA used in the investigation of GATA and NFAT expression in development and in different dietary conditions was graciously supplied by Mr Nuno da Costa, Molecular Medicine Laboratory. The desmin immunostaining described in Chapter Five was carried out by Mr Collin Nixon. The humane slaughter of the animals used in the study was carried out by Veterinary Faculty pathologists.

## ABBREVIATIONS

°C	degrees Celsius
μl	microlitre(s)
μM	micromolar
A <sub>260/280/600</sub>	absorbance at 260/280/600 nm
Akt	<i>see</i> PKB
APS	ammonium persulphate
bHLH	basic helix-loop-helix
BMP-4	bone morphogenetic protein-4
bp	base pair
CaM	calmodulin
cDNA	complementary deoxyribonucleic acid
Cain	calcineurin inhibitor
CaMK	calcium/calmodulin-dependent protein kinase
Ci	curie
Cn	calcineurin
CsA	cyclosporin A
C <sub>T</sub>	threshold cycle
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra acetic acid
EGFP	enhanced green fluorescent protein
emMyHC	embryonic myosin heavy chain
ERK	extracellular signal regulated kinase
EtBr	ethidium bromide
FG	fast-glycolytic
FOG	friend of GATA or fast oxidative-glycolytic
FCS	foetal calf serum

GATA	<i>family of transcription factors recognising the consensus binding site 'WGATAR'</i>
GFP	green fluorescent protein
Grb2	growth factor receptor bound protein 2
GSK3	glycogen synthase kinase 3
GSP	gene specific primer
IIATs	histone acetyltransferases
HDACs	histone deacetylases
HRP	horseradish peroxidase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IGF	insulin-like growth factor
IGF-1R	insulin-like growth factor-1 receptor
IRS-1	insulin receptor substrate-1
kDa	kilodalton
L	litre(s)
LD	<i>longissimus dorsi</i>
LiCl	lithium chloride
LiDS	lithium dodecyl-sulphate
LB	Luria-Bertani medium
MAPK	mitogen-activated protein kinase
MADS domain	MCM1, Agamous, Deficiens and SRF domain
M	molar
MEF-2	myocyte enhancer factor 2
MEK	mitogen-activated protein kinase/ERK kinase
MESA	MOPS-EDTA-sodium acetate
ml	millilitre(s)
mg	milligram(s)
MOPS	3-morpholinopropanesulphonic acid
MPC	magnetic particle collector
Myf	myogenic regulatory factor
mTOR	mammalian target of Rapamycin
MyHC	myosin heavy chain
mRNA	messenger RNA
NCAM	neural cell adhesion molecule

NFAT	nuclear factor of activated T-lymphocytes
NFκ-B	nuclear factor kappa beta
nm	nanometre
oligo(DT)	oligodeoxythymidylic acid
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDK1	phosphoinositide-dependent protein kinase 1
PEG	polyethylene glycol
periMyHC	perinatal myosin heavy chain
pfu	plaque forming units
PGC-1α	peroxisome proliferator-activated receptor γ coactivator 1α
PI3-K	phosphatidylinositol 3-kinase
PIP <sub>2</sub>	phosphatidylinositol (4,5)-bisphosphate
PIP <sub>3</sub>	phosphatidylinositol (3,4,5)-trisphosphate
PKB	protein kinase B
PMSF	phenylmethylsulfonyl fluoride
poly(A) <sup>+</sup>	polyadenylated (mRNA)
PPARγ	peroxisome proliferator-activated receptor γ
PP2A	protein phosphatase 2A
PVDF	polyvinylidene fluoride
PTEN	phosphatase and tensin homologue on chromosome 10
RACE	rapid amplification of cDNA ends
RT-PCR	reverse-transcriptase polymerase chain reaction
SDS	sodium dodecyl-sulphate
Shh	sonic hedgehog
SMART	switching mechanism at 5' end of RNA transcript
SoS	son of sevenless
SO	slow-oxidative
SRF	serum response factor
SURE	slow upstream regulatory element
TCA	trichloroacetic acid

TEMED	tetramethyl-1-,2-diaminomethane
Tris	tris (hydroxymethyl) aminomethane
Tris HCl	tris hydrochloride
TBS	tris buffered saline
TSR	template suppression reagent
UTR	untranslated region
Wnt	wingless/int

## **CHAPTER ONE - GENERAL INTRODUCTION**

## **1.1 INTRODUCTION**

Skeletal muscle-fibres may be divided into a number of subtypes that exhibit markedly different physiological properties, such as contraction velocity or resistance to fatigue. These properties are conferred by fibre-specific patterns of gene expression that control both the enzymes involved in energy metabolism and the characteristic protein isoforms of the contractile apparatus. The population of fibre types within a muscle is not fixed, however, and can be altered by extrinsic factors such as motor nerve activity, mechanical load or hormonal levels, allowing the muscle to adapt to changes in physiological demand.

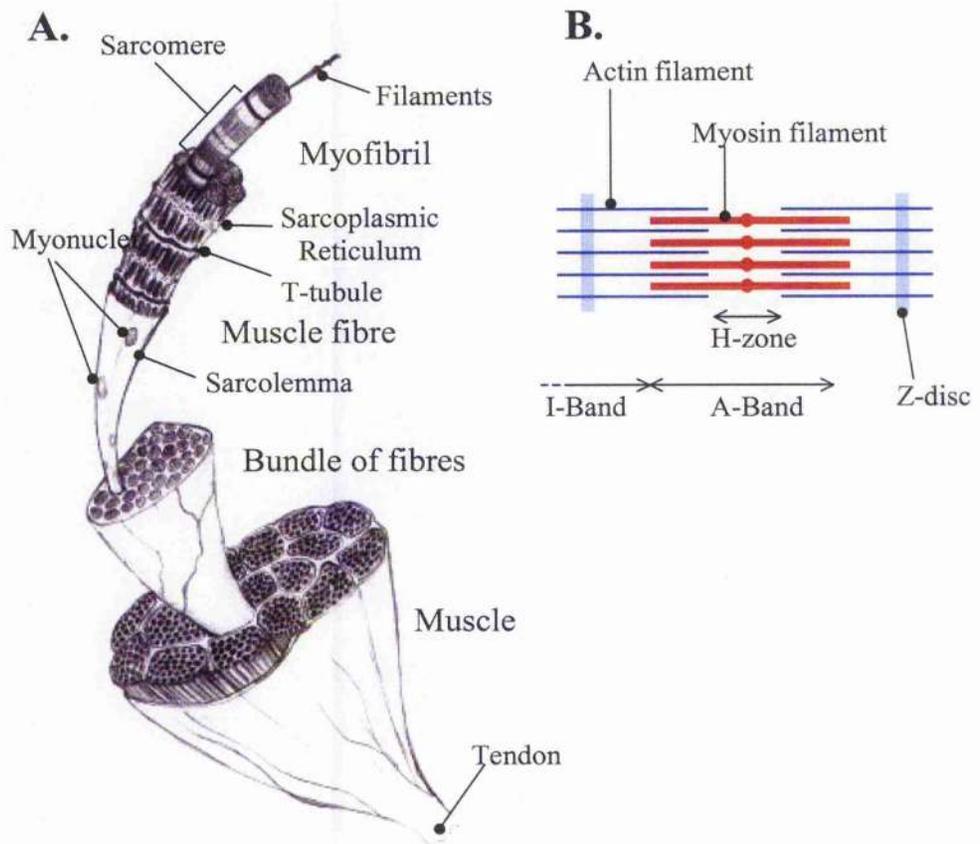
Currently, the mechanisms of transcriptional control by which these extrinsic factors are transduced into changes in fibre-specific patterns of gene expression remain incompletely understood. One pathway recently implicated in the acquisition and maintenance of fibre-phenotype is the  $\text{Ca}^{2+}$ /calcineurin/NFAT pathway. While this pathway is well known and studied in a range of tissue types, its role in muscle has been the subject of considerable, recent debate. It is the investigation into the role of the transcription factors involved in this pathway that forms the basis of the study described herein.

It is the aim of this chapter, then, to provide an overview of the following: Firstly, the different fibre-types and the underlying muscle-biology that gives rise to them. Secondly, the manner in which various transcriptional pathways, particularly calcineurin/NFAT, are thought to regulate fibre-specific patterns of gene expression. Finally, to relate this background information to the study undertaken and the immediate strategies employed.

## **1.2 AN OVERVIEW OF MUSCLE FIBRES**

### **1.2.1 MUSCLE FIBRES AND FIBRE TYPES**

Skeletal muscle is a hierarchical structure made up of bundles of long, terminally-differentiated cell-syncytia, called muscle fibres (Figure 1.1). These fibres contain the



**Figure 1.1.** Levels of organisation in skeletal muscle. **A.** Cross-section of an anatomical muscle, showing the composition of individual muscle fibres and their relationship to whole muscle. **B.** Detail of an individual sarcomere, showing the arrangement of the contractile actin (thin) and myosin (thick) filaments.

protein filaments of the contractile apparatus, arranged in a series of functional units, or sarcomeres, of overlapping thick (myosin) and thin (actin) filaments. During contraction, cross-bridges are formed between these myosin and actin filaments in a cyclical, ATP-dependent manner. The thin filaments are drawn in over the thick filaments, resulting in a shortening of the sarcomere and the generation of force (Huxley, 1969).

Muscle fibres are not identical, however, but may be classified into different types according to physical or metabolic properties (Pette and Staron, 1990), such as power output or resistance to fatigue. Traditionally, fibre types are classified into three main groups, these being slow-oxidative (SO), fast oxidative-glycolytic (FOG) and fast-glycolytic (FG), based on histochemical stainings (Brooke and Kaiser, 1970; Handel and Stickland, 1987). These are also sometimes referred to as type I, type IIa and type IIb fibres. The remainder of this section will consider the different fibre properties that are a consequence of factors intrinsic to the different fibre type. That is to say, independent of the properties conferred by extrinsic factors, such as neuronal or hormonal control, which are covered in Section 1.2.2.

#### **1.2.1.1 Fibre Properties**

Power output, measured as work done per unit time, is largely dependent on the velocity of sarcomere contraction ( $V_{MAX}$ ), which is in turn related to the specific activity of the myosin ATPase (Barany, 1967). A greater rate of ATP hydrolysis results in a greater rate of actin-myosin cross-bridge formation and a higher  $V_{MAX}$ . Fast fibres possess myosin that hydrolyses ATP quickly, and thus have a faster cycle time and greater  $V_{MAX}$  and power output. The downside of this is an increased demand for ATP. ATP may be supplied at high rate by glycolysis, and fast glycolytic fibres typically possess large stores of glycogen. This process results in the build-up of lactic acid, however, a by-product that inhibits muscle function (Brooks and Mercier, 1994). Slow fibres, on the other hand, have a lower velocity of contraction and hence a lower power output, but as a result are more economical in ATP usage and capable of sustaining isometric force for longer periods of time. Low intensity force generation may be supplied by oxidative metabolism of carbohydrate or lipid stores, which is a more efficient process than glycolysis (Hargreaves, 2000). Oxidative fibres are characterised by their larger lipid stores, and higher mitochondrial and oxidative enzyme content

(Goldspink, 1985). Oxidative metabolism is limited, however, by the requirement for adequate perfusion of oxygen and nutrient substrate from the blood, which limits the size of predominantly oxidative fibres. This places another limit on the amount of force that slow oxidative fibres can generate, since larger fibres have more contractile proteins in parallel (see also Section 1.2.2.5 on muscle fibre hypertrophy). Oxidative metabolism is less useful during high intensity exercise, when the levels of oxygen and substrate in the blood become limiting. Oxidative metabolism, and the fibres best adapted for it, therefore tends to be used for low-intensity activity. As the force requirement increases, fast fibres and glycolytic metabolism are recruited for the generation of the necessary speed and power (Brooks and Mercier, 1994; Goldspink, 1996). A summary of the properties of different fibre types is presented in Table 1.1.

Fibre Type	SO	FOG	FG
V <sub>MAX</sub>	Slow	Intermediate	Fast
Size	++	(+)	+++
Glycogen	(+)	(+)	+++
Lipid	+++	(++)	(+)
Fatigue	Resistant	Intermediate	Sensitive

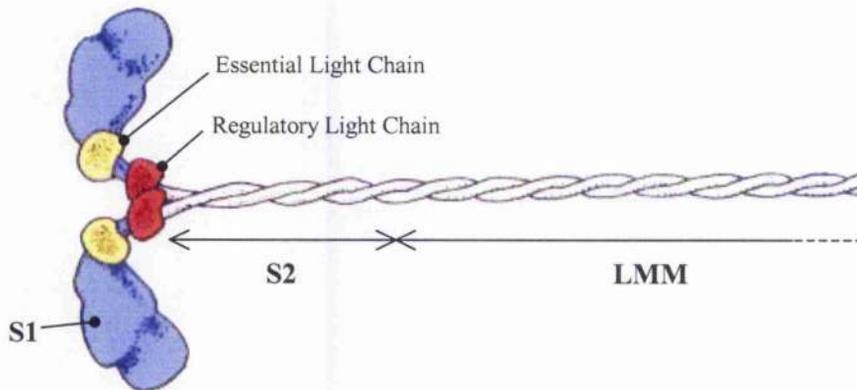
**Table 1.1.** The properties of different fibre types. ( ) indicates variable levels.

### 1.2.1.2 Fibre Distribution

Each anatomical muscle is composed of a mosaic of these different fibre types, but the predominant type depends on the muscle's physiological role. Postural muscles such as the *soleus* are activated continuously during standing or walking, and are characterised by efficient, slow oxidative fibres better suited for producing slow, repetitive movements (Hnik *et al.*, 1985). Conversely, the muscles used in locomotion are composed of FG or FOG fibres, with their greater capacity for force generation, but lower fatigue resistance. Given the mosaic nature of muscle tissue, accurate determination of the fibre content within muscle has proved difficult with more traditional methods. Fibre type may be more accurately identified by reverse transcriptase-PCR (RT-PCR), characterising fibre-specific expression of different isoforms of metabolic and contractile proteins (Ennion *et al.*, 1995). Of these, perhaps the most useful and most commonly used markers for fibre type are the isoforms of myosin heavy chain (MyHC) (reviewed in Schiaffino and Reggiani, 1996).

### 1.2.1.3 Myosin Heavy Chain

The sarcomeric MyHC proteins are a component of the thick filament of the contractile apparatus (reviewed in Weiss and Leinwand, 1996). The MyHCs expressed in muscle are a subset of the class II, or conventional two headed myosins, which are composed of two non-identical pairs of myosin light chains (MyLC) and two, paired MyHC subunits. It is the MyHC component that is of most interest when considering muscle fibre type, since it is these proteins that mediate both the myosin motor function and filament formation. Each MyHC is made up of two functional domains, as illustrated in Figure 1.2; a globular head domain (the S1 proteolytic fraction), and a long, helical rod domain (S2 and LMM proteolytic fractions). The head contains the actin binding domain and ATPase, while the rod domain is required for filament formation.



**Figure 1.2.** Myosin heavy chain dimer with bound myosin light chains (from Weiss and Leinwand, 1996). The blue-shaded S1 proteolytic fragment/myosin head domain contains the actin binding and ATPase sites. S2 and LMM proteolytic fragments both correspond to the alpha helical coiled coil rod domain.

There are eight MyHC genes, expressed in skeletal muscle in a temporal-spatial specific manner. These are the  $1/\text{slow}/\beta$ , 2a, 2x/d, 2b, embryonic, perinatal,  $\alpha$  and extra-ocular MyHCs. The properties of the actin binding domain and ATPase, and therefore the speed of the filament cross-linking cycle, are unique to each MyHC isoform. The MyHC isoform present in a muscle fibre therefore plays a large part in conferring its speed of contraction and power output. Of the eight skeletal muscle MyHCs, the  $1/\beta$ , 2a and 2x/d isoforms (listed in ascending order of  $V_{\text{MAX}}$ ) are of the greatest importance in post-natal skeletal muscle, the others being developmental or

specialist forms. A fourth, faster adult isoform, the 2b, is expressed in the fast fibres of rodent and porcine muscle (Pette and Staron, 2000), but is absent from many other mammalian species such as the cow or horse (Chikuni *et al.*, 2004a, 2004b). The expression of 2b MyHC in humans is limited to the extraocular and masseter muscles, with only mRNA detectable in the latter (Horton *et al.*, 2001). The presence of 2b MyHC in porcine muscle makes the pig, at the time of writing, somewhat unique among large animals. It has been suggested that the high expression of the 2b isoform in porcine muscle is related to intensive breeding for leaner meat (Chang *et al.*, 2003b), a subject that shall be covered in more detail in Section 1.2.4.2.

It should be noted that the expression of MyHC isoforms do not always correlate with the traditional, histochemical-based fibre-type nomenclature of the fibres in which they are expressed. In addition to fibres expressing a single MyHC isoform there exists a continuous spectrum of intermediate forms, from combinations of type 1/ $\beta$  with 2a, 2a with 2x/d, and 2x/d with 2b (Schiaffino and Reggiani, 1994). The properties of the fibre types in which these four isoforms are predominantly expressed are summarised in Table 1.2.

MyHC Isoform	Fibre Type
Slow/1/ $\beta$	SO
2a	FOG
2x	FOG
2b	FG

**Table 1.2.** Fibre-type specific expression of MyHC isoforms in porcine, adult muscle.

It is worthwhile mentioning the four remaining MyHC family members. Two of these isoforms (embryonic and perinatal MyHC) are expressed during development (Lu *et al.*, 1999; see also Section 1.2.2.3). They are normally downregulated after birth, but are activated in muscle satellite cells during the regeneration of damaged muscle (Webster *et al.*, 1988; Karsch-Mizrachi *et al.*, 1989) and are therefore also expressed in differentiating myoblasts in primary cell culture (Torgan and Daniels, 2001). The last two isoforms are found in the specialised fibres of the extra-ocular and mandibular muscles (coMyHC and MyHC- $\alpha$ , respectively). As a final note, the  $\alpha$  and 1/ $\beta$  isoforms are also the major MyHCs in cardiac muscle. Cardiomyocytes and slow skeletal muscle

fibres share more than just the  $1/\beta$  MyHC, however, and in fact a number of other muscle-gene isoforms found specifically in slow muscle are also expressed in the heart.

## **1.2.2 MUSCLE GROWTH AND DEVELOPMENT**

The complex, hierarchical structure of skeletal muscle is derived from its undifferentiated, mesodermal precursors by a tightly regulated cascade of events, triggered in response to a variety of signalling molecules secreted by neighbouring tissues. This chain of events begins with the commitment of progenitor cells to the myogenic lineage, followed by withdrawal from the cell cycle and terminal differentiation. Differentiation events include the fusion of myocytes into myotubes and their subsequent maturation into muscle fibres, with the development of the contractile apparatus and the complex structures already discussed. The same process of embryonic myogenesis is also, to some extent, reiterated in adult muscle stem cells during the adaptive responses to muscle training or regeneration following injury (reviewed in Parker *et al.*, 2003).

Since the myogenic process plays a role in post-natal muscle adaptation, it is worthwhile considering the events of embryonic myogenesis. The purpose of this section, then, is to introduce key transcription factors central to the myogenic process, namely the members of the myogenic regulatory factor (MRF) family, and to summarise the developmental networks that give rise to committed muscle progenitors.

### **1.2.2.1 MRF Proteins**

Perhaps the best studied of all muscle transcription factors (reviewed in Pownall *et al.*, 2002), the MRF family are central to the development of muscle, both during embryogenesis and in post-natal regeneration and repair. The MRFs are basic helix-loop-helix (bHLH) transcription factors consisting of four known proteins, MyoD, myf-5, myogenin and MRF-4, all noted for their ability to initiate the expression of myoblast markers in virtually any cell-type (Davis *et al.*, 1987; Braun *et al.*, 1989; Rhodes and Koniczny, 1989; Wright *et al.*, 1989; Braun *et al.*, 1990; Miner and Wold, 1990). Experiments with loss-of-function knockout mice have demonstrated a division of the four MRF proteins into two groups, each operating at different stages of myogenesis summarised in Table 1.3 below. MyoD and myf-5 are important in the initial myogenic

specification and for this reason are known as muscle determination factors (Choi *et al.*, 1990; Weintraub, 1993). Myogenin and MRF-4 act downstream of the other two factors, and are responsible for mediating terminal differentiation and the expression of structural and functional proteins.

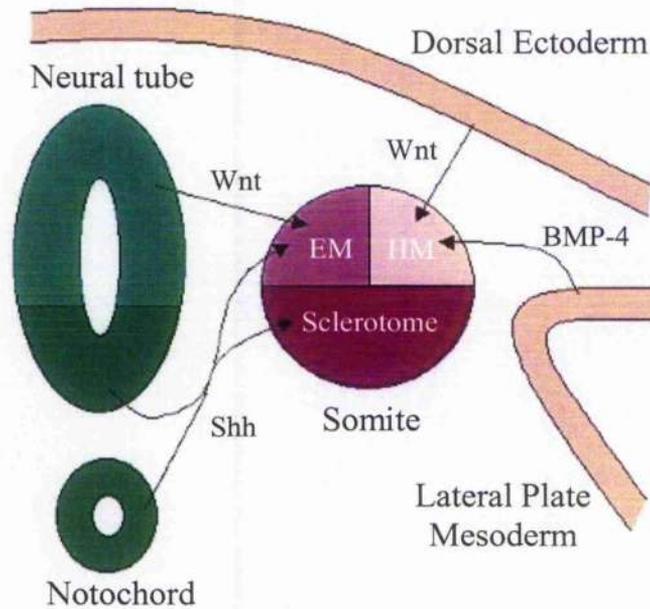
Gene Knockout	Phenotype	References
MyoD	No skeletal muscle defects	(Rudnicki <i>et al.</i> , 1992)
Myf-5	No skeletal muscle defects, but abnormalities in rib formation.	(Braun <i>et al.</i> , 1992)
MyoD & Myf-5	Embryos devoid of myoblasts and differentiated muscle	(Rudnicki <i>et al.</i> , 1993)
Myogenin	Defects in skeletal muscle differentiation	(Hasty <i>et al.</i> , 1993; Nabeshima <i>et al.</i> , 1993)
MRF4	No skeletal muscle defects, but mild abnormalities in rib formation.	(Zhang <i>et al.</i> , 1995)

**Table 1.3.** Summary of the investigation into the roles of MRF proteins in myogenesis discovered through the generation of homozygous knockout mice.

MRF proteins are controlled through the formation of heterodimers with various Helix-loop-helix enhancer or repressor molecules (reviewed in Molkenin and Olson, 1996). For example, MyoD is transcriptionally inactive until it can form a heterodimer with various E-proteins, splice products of the E2A gene (Lassar *et al.*, 1991). The active complex binds to the E-box enhancer (CANNTG), a common motif in the control regions of muscle-genes (Blackwell and Weintraub, 1990). An indirect inhibitor of MyoD is the Inhibitor of Differentiation (Id) protein, which competes with MyoD for binding with the E-proteins (Benezra *et al.*, 1990). MRF proteins display a degree of auto regulation, in that once activated they are capable of promoting their own expression. This positive feedback is reinforced by co-factors such as myocyte enhancer factor-2 (MEF-2) family of transcription factors (Section 1.3.4). In this way, the cell maintains its developmental choice even after the signal that triggered it is no longer present.

#### 1.2.2.2 Embryonic Myogenesis

The majority of skeletal muscles, with a few exceptions such as those of the head, originate from transient, mesodermal structures called somites. These are paired, spherical blocks of epithelial cells formed early in embryogenesis, adjacent to the neural tube from the paraxial mesoderm (reviewed in Pourquie, 2001). As shown in



**Figure 1.3.** Compartmentalisation in the somite (from Molkenin and Olson, 1996). **EM**, epaxial dermomyotome. **HM**, hypaxial dermomyotome. Factors controlling cell determination include: **Shh**, sonic hedgehog, **Wnt**, wingless/int family members, **BMP-4**, bone morphogenetic protein-4.

Figure 1.3, the somites are rapidly compartmentalised into groups of cells with very different developmental fates. The lower, or ventral half, becomes the sclerotome and will form the axial skeleton. The dorsal half gives rise to the dermomyotome from which skeletal muscle and dermis are formed. The dermomyotome is further compartmentalised into different muscle lineages: the epaxial dermomyotome that gives rise to the muscles of the deep back, the hypaxial dermomyotome that forms the muscles of the body wall, and a migratory sub-population of hypaxial dermomyotome that forms the limb muscles.

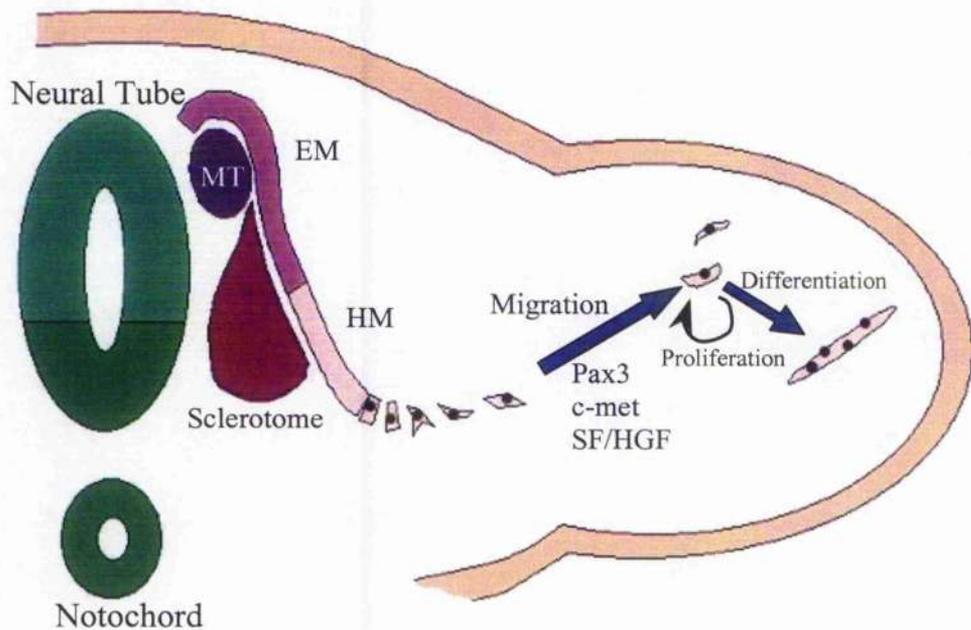
At this stage of muscle development the muscle precursors are highly proliferative, with epaxial and hypaxial cells extending under the dorsal and ventral lip of the epithelial dermomyotome layer respectively (Denetclaw *et al.*, 1997). Here they form the first pre-muscle mass, the myotome, which eventually forms the trunk musculature. As the myotome condenses, myoblasts exit the cell cycle, fuse and differentiate. Other pre-muscle masses require the chemotactic migration of precursor myoblasts from the

somite to their eventual site of differentiation (reviewed in Buscher and Izpisua Belmonte, 1999).

Experiments in explanting somitic tissue and altering somite orientation in chick embryos (reviewed in Brand-Saberi and Christ, 1999) have shown that the cells in the early somite are not yet committed to specific cell fates. The specification of somitic cells is mediated by a network of different signalling factors secreted from neighbouring tissues (reviewed in Pownall *et al.*, 2002). The ventral half of the somite, the sclerotome, forms the axial skeleton and cartilage, and is determined by signals from the floor plate of the neural tube (Pourquie *et al.*, 1995), most notably sonic hedgehog (Shh). The dermomyotome, from which skeletal muscle is derived, is formed largely in response to Wnt family signals from the neural tube and dorsal ectoderm. Myogenic precursors in the dermomyotome express the homeobox transcription factors Pax-3 and Pax-7, and low levels of the MRF member, Myf5. In differentiating cells of the developing myotome, this is quickly followed by MyoD expression, which regulates the onset of the myogenic cascade. In myoblasts of the hypaxial lineage, however, MyoD expression is down-regulated by bone morphogenetic protein-4 (BMP-4) signalling from the lateral plate mesoderm (Pourquie *et al.*, 1996). The opposing signals of Wnt and BMP-4 are a part of the balance required during embryonic muscle growth, between proliferation and withdrawal from the cell cycle and differentiation (Amthor *et al.*, 1999), which is more apparent still during the migration of limb-muscle myoblasts.

### **1.2.2.3 Limb-muscle formation**

The migration of limb-muscle precursors from the somitic dermomyotome to the pre-muscle mass in the limb bud is shown in Figure 1.4. Myoblasts delaminate from the epithelium of the hypaxial dermomyotome opposite the limb buds, following a path delineated by non-somitic mesodermal cells (reviewed in Buckingham *et al.*, 2003). This is thought to be an evolutionary holdover from fish, whose paired fins are controlled by long myotome processes extended from the trunk (Grim, 1973). The migration of limb-muscle precursors is a modification of this simpler scheme, to generate the increased complexity of higher vertebrate limbs.



**Figure 1.4.** Delamination of limb-muscle precursors and migration. Cells migrate in response to SF/HGF secreted by mesodermal cells within the limb field, sensed through the receptor c-met. Expression of c-met is, in turn, dependent on the myogenic marker Pax-3. SF/HGF, scatter factor/hepatocyte growth factor. MT, myotome. EM, epaxial dermomyotome. HM, hypaxial dermomyotome.

Pax-3 plays an important role in the migration, as demonstrated in mice homozygous for the *Splotch* mutation that lack a functional Pax-3 gene and which fail to develop limb musculature (Bober *et al.*, 1994; reviewed in Birchmeier and Brohmann, 2000). Pax-3 is essential for the expression of c-met, a tyrosine kinase receptor, the ligand for which being hepatocyte growth factor (HGF), otherwise known as scatter factor (SF). It is SF/HGF secretion by mesodermal cells that defines the migratory pathway through the limb field (Dietrich *et al.*, 1999). The importance of both c-met and HGF is illustrated by null-mutants for both genes, either of which result in an absence of limb muscles (Bladt *et al.*, 1995; Schmidt *et al.*, 1995). The phosphorylation of tyrosine residues by tyrosine kinases such as c-met is important in a variety of protein-protein interactions with factors such as phosphatidylinositol 3-kinase (PI3K) and Grb2. PI3K is involved in many cellular responses, including muscle fibre hypertrophy as will be discussed later (Section 1.3.3.2), and cytoskeletal organisation and cell motility. Grb2 is part of the Ras-signalling pathway involved in mitogen activated protein (MAP)

kinases, and ultimately in cell growth and proliferation (Rodrigues *et al.*, 1997). C-met mediated signalling is important for maintaining myoblast populations as well as moving the cell along the correct path.

Other factors involved in limb-muscle formation include Lbx1, another homeobox transcription factor, though its role is less completely understood. In Lbx1 mutant embryos the progenitors of dorsal limb muscles delaminate from the somite, but exhibit abnormal migration into the limb bud (Schafer and Braun, 1999; Brohmann *et al.*, 2000). Specifically, muscles in the distal, dorsal portion of the limb failed to form, while ventral muscles were formed but in an abnormal manner. It may be that Lbx1 has a role in recognising the cues that guide chemotaxis along the correct migratory pathway (Brohmann *et al.*, 2000). A third homeobox factor, Mox2, is also required for the correct formation of limb muscle groups (Mankoo *et al.*, 1999). While mutations in Mox2 do not appear to cause problems with the delamination of cells from the somite epithelium or cell migration, the levels of both Pax-3 and Myf5 are lower. This may affect the proliferation and survival of certain populations of migrating myoblasts.

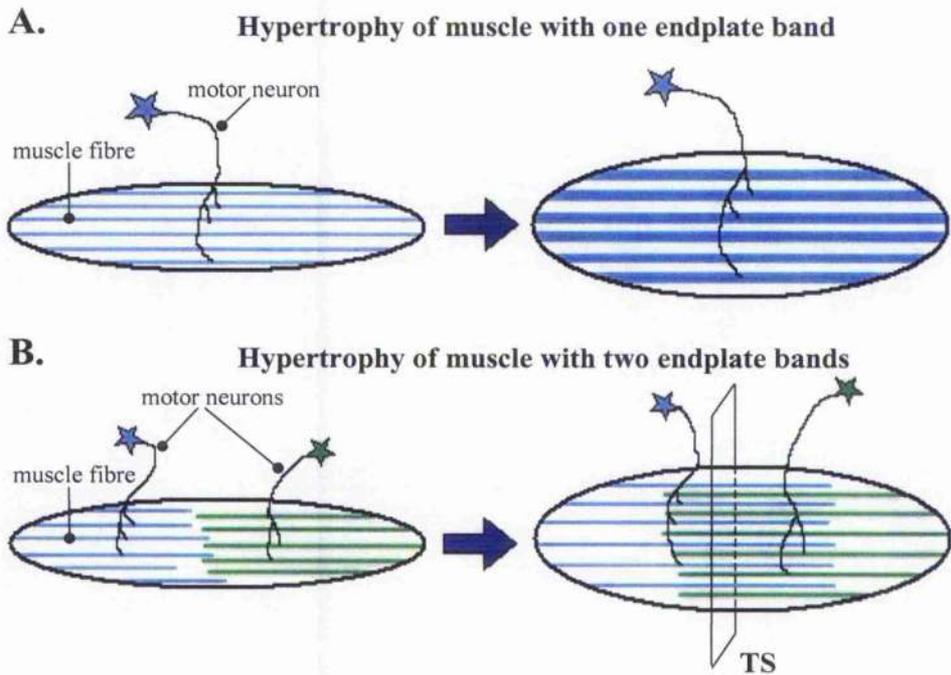
#### **1.2.2.4 Fibre Formation**

The waves of migrating cells aggregate to form the pre-muscle masses, exit the cell cycle and fuse to form multinucleated myotubes. Down-regulation of Pax-3 would seem to be essential in the differentiation process. During cell migration, myogenic markers such as *Myf-5* and *MyoD* are repressed (Tajbakhsh and Buckingham, 1994), but are both upregulated if Pax-3 is artificially repressed (Pourquie *et al.*, 1995). The formation of myotubes, and ultimately muscle fibres, occurs in two waves to form the primary (embryonic) and secondary (foetal) muscle fibres. The secondary fibres form in parallel to the primary fibres and are the predominant fibres in both later stages of development and in postnatal muscle (Ashmore *et al.*, 1972; Swatland and Cassens, 1973; reviewed by Stockdale, 1992). The two waves of fibre formation is a means of generating the highly ordered structure of fibre bundles, with the primary fibres forming a scaffold for the orderly assembly of subsequent waves of myoblasts (Wigmore *et al.*, 1996). Myoblasts have been shown to line up with substrate grooves *in vitro* (Evans *et al.*, 2003), which would suggest that topographic cues are used for the correct alignment of myoblasts.

During embryonic development, the predominant MyHC isoforms are the embryonic and perinatal forms (Weydert *et al.*, 1987). Primary myotubes express emMyHC first, then co-express perinatal and  $\beta$ /slow MyHCs (Harris *et al.*, 1989; Condon *et al.*, 1990). Secondary myotubes similarly express emMyHC first, and then subsequently co-express the perinatal isoform (Harris *et al.*, 1989). Not all secondary fibres co-express the  $\beta$ /slow isoform, and this may possibly reflect a developmental commitment to a post-natal slow fibre type (Gunning and Hardeman, 1991). More recently, the expression of the adult 2a, 2x and 2b MyHCs was also characterised in developing murine and porcine muscle, though at much lower levels than the developmental forms (Lu *et al.*, 1999; Da Costa *et al.*, 2003). Later in gestation, the perinatal isoform predominates over the embryonic, but is in turn downregulated at birth and replaced by adult isoforms, 2a, 2x, 2b and, in slow fibres, the slow/ $\beta$  isoform. In skeletal muscle culture, in the absence of innervation, the embryonic, perinatal and 2b isoforms accumulate (Weydert *et al.*, 1987).

#### **1.2.2.5 Muscle Fibre Hypertrophy**

Hypertrophy is the primary mechanism of post-natal growth in muscle and an adaptive response to increased muscle activity or the removal of atrophic stimuli. Hypertrophy may be induced as an adaptive response to resistance training, such as weight training, consisting of high-intensity, low-repetitive exercise. The hypertrophic response results in an increase in muscle volume and contractile protein, and greater muscle strength (MacDougall *et al.*, 1979). While all fibre types tend to increase in size during hypertrophy, the greatest increases in muscle mass are associated with type 2 fibres (Hortobagyi *et al.*, 2000). A similar situation is observed in cardiomyocytes, in which hypertrophy may be observed under a number of pathological conditions that impose stress upon the heart (reviewed in Frey and Olson, 2003). While initially a compensatory response, this hypertrophy can rapidly lead to a loss of cardiac function if the conditions underlying the response are sustained. In cardiomyocytes, hypertrophy is defined as an increase in cytoplasmic volume without increase in the number of nuclei. In multinucleated skeletal muscle fibres, however, the situation is a little more complex (Paul and Rosenthal, 2002). In muscle fibres, hypertrophy involves both an increase in size and an accompanying increase in the number of myonuclei.



**Figure 1.5.** Different modes of skeletal muscle hypertrophy (from Paul and Rosenthal, 2002). **A.** Skeletal muscle with a single endplate zone, showing hypertrophy through increase in fibre diameter. **B.** Muscle with multiple endplate bands and interfascicularly terminating fibres, showing hypertrophy through fibre elongation, causing an increase in the number of fibres in transverse section (TS).

This may occur by one of two distinct mechanisms, depending on the number of motor-neuron endplate bands present in the muscle (Figure 1.5).

Muscles may be innervated either singly or by multiple endplate bands (Paul, 2001). In singly innervated skeletal muscles, the fibres extend throughout the length of the muscle from one tendon to the other and hypertrophy occurs through an increase in fibre diameter (Gollnick *et al.*, 1981; Paul and Rosenthal, 2002). Singly innervated muscles are the norm for rodents and similar small mammals, but in larger mammals the increased length of the muscle presents a problem with regard to the slow conduction of action potentials along the fibre. A large number of studies on various large mammals (summarised by Paul, 2001) have shown that the longer muscles are multiply innervated, with fibres that do not extend the entire length between the two

tendons. In such muscles, hypertrophy occurs through the elongation of the fibres, resulting in an increase in the number of fibres in transverse section in a form of indirect hyperplasia. No studies have been done in the pig, but it would seem likely that porcine muscle shares the same architecture with other animals of similar size. Humans and primates have muscles with singly innervated, extremely long fibres, though this seems to be a unique feature, possibly conferring finer motor control (Paul, 2001). Both mechanisms of hypertrophy result in an increase in the number of myofibrils in parallel, and an increase in muscle volume and mass.

The increase in the fibre size associated with hypertrophy requires an increase in the number of myonuclei, since a constant ratio of nuclei to cytoplasmic volume is maintained throughout the hypertrophic response (McCall *et al.*, 1998; Allen *et al.*, 1999). Since muscle fibres are terminally differentiated, this nuclear increase must come from the recruitment of newly proliferated myoblasts (Rosenblatt *et al.*, 1994). These are generated from the proliferation of non-differentiated muscle cells known as satellite cells.

#### **1.2.2.6 Skeletal Muscle Satellite Cells**

Satellite cells are found in the spaces and grooves between the basal lamina and sarcolemma of muscle fibres (Mauro, 1961). Under normal conditions, satellite cells remain in a quiescent, non-proliferative state, but muscle trauma or hypertrophic stimuli induce extensive proliferation and the expression of muscle markers. Following multiple rounds of cell division the progeny myoblasts undergo differentiation and fusion to form mature muscle fibres, either with each other or through incorporation into existing fibres as post-mitotic nuclei. In addition to contributing to hypertrophy and repair, satellite cells provide the primary source for post-natal muscle growth (Schultz, 1989, 1996), and comprise 32% of muscle nuclei at birth, in the mouse (Bischoff, 1994). This drops to a reserve of 5% of muscle myonuclei in the adult mouse.

The pathway of commitment, proliferation and differentiation of satellite cells largely parallels that of embryonic muscle precursors in the dermomyotome. As with other myogenic precursors, satellite cells are thought to originate in the somite, though there is some evidence skeletal myogenic cells originating from vascular tissue, in particular

the dorsal aorta (De Angelis *et al.*, 1999). The homeobox factor Pax-7 is essential for the development of the satellite cell lineage, as *Pax-7* deficient animals show a complete lack of satellite cells (Scale *et al.*, 2000). As with embryonic myogenesis, the c-met/HGF pathway is also important in the activation of satellite cells, and mediates the chemotaxis to sources of injury or hypertrophy (Allen *et al.*, 1995; Tatsumi *et al.*, 1998).

MRF expression is largely absent in quiescent satellite cells, with the exception of low levels of *myf-5* (Beauchamp *et al.*, 2000). The role of Myf-5 would appear to be maintaining the cells in a proliferative state (Parker *et al.*, 2003). MyoD is required for the activation of the differentiation pathway, and differentiated satellite cells are characterised by an accumulation of MyoD protein. In mutants deficient in MyoD the differentiation of satellite cells is severely impaired, as evidenced by an increased number of satellite cells produced in response to injury without subsequent regeneration (Megency *et al.*, 1996). Differentiating satellite cells initially express the embryonic and perinatal forms of MyHC (Miller, 1990; LaFramboise *et al.*, 2003), as do muscle myoblast cell lines, such as mouse C2C12 myoblasts, that form the basis of many *in vitro* studies. Such *in vitro* studies suggest that satellite cells do not inherit the fibre-type specific phenotype from their host muscle, predominantly expressing MyHC 2a or slow. This suggests that further specialisation in fibre-type specific isoforms is doubtless dependent on external factors such as nerve stimulation or muscle activity (LaFramboise *et al.*, 2003).

Satellite cell populations are self-renewing (reviewed in Parker *et al.*, 2003), as demonstrated by the maintenance of the satellite cell pool in old muscle after repeated cycles of degeneration and regeneration. Proliferating satellite cells are characterised by an asymmetric distribution of the Numb protein (Conboy and Rando, 2002), a regulator of asymmetric division in the central nervous system (Shen *et al.*, 2002). Numb is an antagonist of the Notch-1 receptor, the *Notch* genes being a family of surface receptors important in determining cell fate in a number of systems (reviewed in Kimble and Simpson, 1997; Lai, 2004). Activated Notch receptors are cleaved, such that the Notch internal component (NIC) is released into the cytoplasm where they form complexes with transcription factor targets. These complexes are then nuclear translocated, driving patterns of gene expression associated with proliferation and the down-regulation of

differentiation. Conboy & Rando (2002) found that Notch-1 was activated in satellite cells in response to injury, while attenuation of Notch-1 by Numb accumulation led to the expression of differentiation and myoblast commitment markers, such as Pax-7 and MRFs. The presence of the Notch-1 system in satellite cells implies that these precursors are able to generate distinct daughter cells for differentiation or maintenance of the satellite cell pool.

While the regenerative capacity of satellite cells is well studied, there remain some questions regarding the presence of populations of truly multipotent muscle stem-cells. Both Ferrari *et al.* (1998) and Gussoni *et al.* (1999) demonstrated the ability of cells derived from bone marrow to take part in the regenerative program. Ferrari *et al.* (1998) further demonstrated the presence of low, but detectable, levels of blood-borne myogenic cells. These cells have been shown to differentiate into satellite cells (La Barge and Blau, 2002). The converse is true in that cells derived from muscle have been shown to differentiate along a number of non-muscle pathways, such as adipogenesis (Csete *et al.*, 2001), haematopoiesis (Jackson *et al.*, 1999) and osteogenesis (Lee *et al.*, 2000). It has been proposed that a distinct population of stem cells, resident either in muscle or in non-muscle tissue, can participate in muscle regeneration and replenish the existing satellite cell population (Parker *et al.*, 2003). However, the possibility that the satellite cells themselves may be multipotent has also been raised (Seale and Rudnicki, 2000). The study by Csete *et al.* (2001) observed adipogenesis in satellite cells specifically, and it may be that the distinction between satellite cell and stem is something of an artificial one.

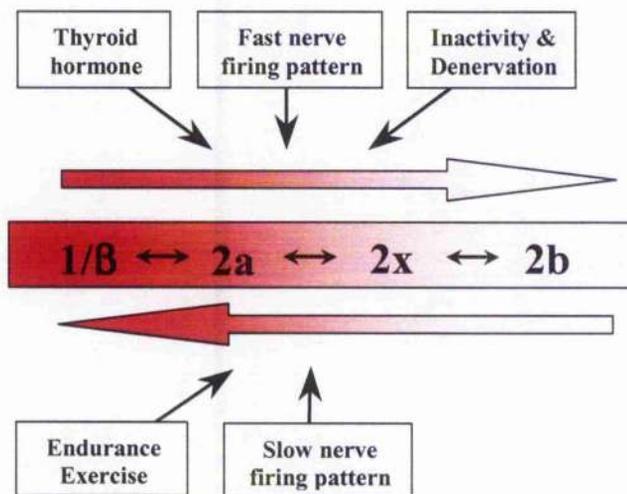
### **1.2.3 SKELETAL MUSCLE ADAPTATION**

Skeletal muscle is a highly adaptive tissue, capable of radical changes in fibre composition, with consequent alteration of physical and metabolic properties, as a response to changes in functional demand (reviewed in Fluck and Hoppeler, 2003). This allows muscle tissue to adopt the most energy-efficient configuration for the demands placed on it at any given time. For example, in humans the most extensively studied fibre transformation is that resulting from changes in exercise regime (reviewed in Pette and Staron, 1990). As might be expected, endurance training regimes increase the proportion of slow fibres (Gollnick *et al.*, 1972; Jansson and Kaijser, 1977), while

sprint training results in a slow to fast fibre transformation (Jansson *et al.*, 1990; Esbjornsson *et al.*, 1993). Changing requirements brought about by factors such as exercise regime, nutrient intake and even disease conditions are converted into changes in muscle physiology through the response to extrinsic signalling factors. The best studied of these extrinsic factors are innervation (Buller *et al.*, 1960), patterns of nerve stimulation (Pette, 1998) and hormone signalling (Florini, 1987; Florini *et al.*, 1991a). A summary of these factors and their effect on fibre expression of MyHC isoform is shown in Figure 1.6.

### 1.2.3.1 Innervation

Perhaps the most important influence on fibre-type specific expression is innervation by motor neurons, first demonstrated by Buller *et al.* (1960) through cross-innervation experiments. Grafting the motor neurons associated with fast-twitch muscles to postural slow muscles, and vice versa, resulted in a switch to the phenotype associated with the transplanted motor neuron. This crucial role of nerve activity in the acquisition and maintenance of fibre type, along with the long term survival of muscle fibres, was confirmed by further studies (Lomo, 2002; Schiaffino and Serrano, 2002). These included the observation that muscle inactivity following spinal cord injury results both in muscle atrophy and in a slow to fast switch in fibre phenotype.



**Figure 1.6.** Summary of the extrinsic factors influencing MyHC expression and fibre type (from Fluck and Hoppeler, 2003).

Electrical activity is the main mechanism by which the nervous system exerts control over fibre phenotype, though there are also the local effects of neurochemicals, such as neural agrin and neuregulin, at the neuromuscular junction. Direct electrical stimulation of denervated muscle, using implanted electrodes, has been found to mimic the effects of cross-innervation in mediating a fast-slow or slow fast fibre transition (Eken and Gundersen, 1988). Low-frequency (10-20 Hz), continuous (tonic) stimulation, which mimics the firing pattern observed in slow motor neurons, results in slow-fibre specific gene expression. Conversely, short bursts (phasic) of high-frequency (100-150 Hz) stimulation pattern, characteristic of fast motor neurons, results in a slow-to-fast fibre transition.

A likely pathway for transforming such patterns of electrical stimulation into control of gene expression is the detection of fluctuating, intracellular calcium ( $\text{Ca}^{2+}$ ) levels (reviewed in Talmadge, 2000). Calcium influx is an essential part of muscle function, required for the activation of muscle contraction through the troponin-tropomyosin, and for secondary roles such as the regulation of ATP provision (Tate *et al.*, 1991). However, different patterns of nerve stimulation result in different concentrations of  $\text{Ca}^{2+}$  in the cytosol. Slow-type signalling patterns result in a sustained intracellular  $\text{Ca}^{2+}$  concentration of 100 to 300 nM (Chin and Allen, 1996), while fast patterns result in short-lived  $\text{Ca}^{2+}$  elevations of up to 1  $\mu\text{M}$ . The role of  $\text{Ca}^{2+}$  concentration has been further demonstrated by Kubis *et al.* (1997), in experiments using the calcium ionophore A23187. This group found that the expression of slow myosin was activated in primary muscle cells cultured in the presence of the ionophore, and hence subjected to sustained elevation of  $\text{Ca}^{2+}$ .

Changes in  $\text{Ca}^{2+}$  concentration are sensed by calcium binding proteins such as calmodulin, a primary component of a number of calcium-response pathways. A major target for calmodulin is the serine-threonine phosphatase calcineurin, and the role of this signalling intermediary and its associated signal transduction pathway is a matter of some considerable debate, discussed in detail in Section 1.4.

### 1.2.3.2 Hormones

Hormonal signalling exerts a major influence on skeletal muscle in development, but also plays a significant role in post-natal adaptation (reviewed in Florini, 1987; Florini *et al.*, 1991a). Hormonal signals are particularly important in regulating changes in protein turnover, and consequent changes in muscle mass. Anabolic hormones, including growth hormone, insulin-like growth factors (IGFs) and testosterone, contribute greatly to increases in muscle mass by increasing protein synthesis or preventing protein breakdown (Rooyackers and Nair, 1997). Conversely, catabolism of protein and loss of muscle mass are mediated by such factors as glucagon, glucocorticoids and catecholamines. In the control of fibre-type, two hormones in particular stand out for their effect on the isoform-specific expression of different MyHCs. These are the IGFs, IGF-1 and IGF-2, and the thyroid hormone, triiodothyronine (T3).

#### 1.2.3.2.1 IGFs

IGF-1 and IGF-2 are involved both in muscle development (Florini *et al.*, 1991a) and in post-natal hypertrophy and regeneration (Barton-Davis *et al.*, 1999). Both are primarily synthesised in liver, in response to growth hormone, but are also synthesised locally in muscle tissues, acting in an autocrine/paracrine manner in response to damage, mechanical loading or stretch (Adams, 2002; Fiorotto *et al.*, 2003). The IGFs are stabilised in serum through binding to six known IGF binding proteins (IGFBPs), which also serve to regulate their access to target tissues (reviewed in Jones and Clemmons, 1995; Rosenfeld *et al.*, 1999)

IGF-1 is particularly well-studied in muscle growth and adaptation (reviewed in Florini *et al.*, 1996; Singleton and Feldman, 2001). There are two IGF-1 isoforms known to be of major importance in muscle. In addition to the systemic IGF-1Ea form synthesised both in the liver and locally, a splice variant termed mechano growth factor (MGF) is also induced in muscle following tissue damage (Yang and Goldspink, 2002; Hill and Goldspink, 2003). IGF-1 function, together with that of its binding proteins and receptors, has been uncovered through studies using knockout mice (reviewed in Kitamura *et al.*, 2003). Mice deficient in IGF-1 fail to develop muscle and die at birth, in a similar manner to mice deficient in *myf-5* (Liu *et al.*, 1993). *In vitro* experiments (Florini *et al.*, 1977; Ewton and Florini, 1981) have shown that the IGF-1 is important

both for the survival and proliferation of myoblasts, but also, unlike other mitogenic factors, for their subsequent differentiation into non-proliferating fibres (Quinn *et al.*, 1994). MGF, in contrast, is activated early in the response to muscle damage, and is likely to be more of a contributing factor in satellite cell proliferation (Hill and Goldspink, 2003).

This dual ability of IGF-1 to promote both proliferation and differentiation in myoblasts underlies its role as a potent enhancer of hypertrophy. This role has been demonstrated *in vivo*, where direct infusion or overexpression of IGF-1 in muscle results in hypertrophy (Adams and McCue, 1998). IGF-1 mediated hypertrophy has been associated with a slow to fast-glycolytic shift in fibre type both *in vivo* (Semsarian *et al.*, 1999a; Semsarian *et al.*, 1999b) and *in vitro*, in C2C12 myoblast culture (Semsarian *et al.*, 1999a). The basis for this reasoning is the observation that the viral transfection of IGF-1 prevents the age-related atrophy of type 2x fibres (Barton-Davis *et al.*, 1998), and Semsarian *et al.* (1999a) did indeed note an increase in glycolytic enzymes such as lactate dehydrogenase (LDH) following treatment with IGF-1. However, a direct, causative role for IGF-1 in the promotion of the fast fibre phenotype, as opposed to general hypertrophy, is still far from clear.

IGF-2 plays a similar role to IGF-1 in the differentiation of myoblasts (Florini *et al.*, 1991b; Prelle *et al.*, 2000; Wilson *et al.*, 2003) and the regeneration of damaged muscle (Kirk *et al.*, 2003). In myoblast cell culture, IGF-2 is secreted in an autocrine/paracrine manner following inducement of the differentiation pathway through transfer to low serum conditions (Florini *et al.*, 1991b). Myoblasts in which IGF-2 expression was silenced through the transfection with an antisense construct proliferated normally, but died upon transfer to low serum conditions (Stewart and Rotwein, 1996). Taken together, this would indicate that IGF-2 reinforces and amplifies the commitment to the myogenic process, and protects the myoblasts from apoptosis as they exit the cell cycle.

The relationship between the IGFs and their individual contribution to the myogenic process remains unclear. Complicating matters, the effects of both IGF-1 and -2 are mediated through the same surface receptor, insulin-like growth factor-1 receptor (IGF-1R: reviewed in Singleton and Feldman, 2001). The functions of IGF-1R, and the signal transduction pathways leading from it, are discussed in detail in Section 1.3.3.

#### 1.2.3.2.2 Thyroid Hormone

Skeletal muscle is a major target of thyroid hormones. The effect of thyroid signalling would appear to be mediated by T3, produced by de-iodination of thyroxine (T4) in the liver. T3 has been shown to be involved in the induction of differentiation through MyoD activation (Downes *et al.*, 1995). Though thyroid hormones are essential during muscle growth and development, both excess or deficiency in thyroid hormones result in an overall loss of muscle strength (Zurcher *et al.*, 1989). These muscle wasting effects are most commonly seen in muscles rich in slow-oxidative fibres. T3 also has a strong influence on the expression of MyHC isoforms (reviewed in Baldwin and Haddad, 2001). In development, thyroid hormone influences the timing of the switch from embryonic or perinatal MyHC to adult forms, with the repression of developmental isoforms coinciding with the increase in T3 concentration in rat embryos (reviewed in Soukup and Jirmanova, 2000). Hyperthyroidism in developing rats was found to result in an earlier switch, with the converse true for hypothyroid rats. In addition to a role in regulating developmental timing, T3 is also involved in changes in adult fibres. Hyperthyroidism or elevated doses of T3 result in upregulation of MyHC 2x and 2b isoforms, and the repression of the slow/I. Hypothyroidism results in a fast to slow fibre transition (Nwoye *et al.*, 1982), with a shift in MyHC expression along the direction of 2b/2x to 2a to type 1, in all muscle types. But while the influence of thyroid hormone on the fast fibre phenotype seems clear, the mechanism by which the fast MyHCs are induced by T3 remains unclear. Thyroid hormone has been shown to interact with MyoD in mice (Anderson *et al.*, 1998), though the evidence for an effect of MRFs on fibre type remains inconclusive (see Section 1.3.2). While thyroid hormones are necessary for the fast phenotype, it would seem that their role is permissive, rather than instructive (Soukup and Jirmanova, 2000).

#### **1.2.4 PRACTICAL IMPLICATIONS**

The biology and fibre composition in muscle has a number of implications in a wide range of fields from sports and exercise to the breeding of animals for sporting performance or meat quality, to the treatment of myopathies, such as muscular dystrophy or age-related muscle loss. Two such fields are considered here as examples of the interactions between the various intrinsic and extrinsic factors already covered.

The first of these is the physiology of ageing muscle, which could perhaps be viewed as a demonstration of the effects of a breakdown in the regulation and adaptation of muscle. The second is the effect of fibre phenotype on the quality of post-slaughter pork, which shows the biochemical consequences of muscle fibre composition, as well as the necessity for an underpinning knowledge of muscle biology in modern animal husbandry.

#### **1.2.4.1 Ageing**

Ageing muscle undergoes an inexorable decline in strength and speed, in a characteristic process known as sarcopenia (Lauretani *et al.*, 2003). Sarcopenia is associated with a loss of muscle mass (Gallagher *et al.*, 1997), the accumulation of intramuscular lipid content (Poggi *et al.*, 1987) and changes in fibre composition and patterns of MyHC expression (Andersen, 2003). Age-related decline in growth hormone signalling, motor neuron control and satellite cell regenerative capability have all been investigated as contributing factors.

##### 1.2.4.1.1 Deteriorating Signals

The levels of circulating growth hormone/IGF-1 decline with age, as does tissue sensitivity to IGF activation (Renganathan *et al.*, 1997). The key roles played by growth hormone, IGFs and IGF receptor in the proliferation and differentiation of satellite cells (Section 1.2.3.2.1) make their decline a logical candidate for a role in muscle deterioration, and artificially elevated IGF-1 has indeed been shown to delay muscle senescence in mice (Barton-Davis *et al.*, 1998). IGF-1 has also been observed to increase the replicative life-span of satellite cells *in vitro* (Chakravarthy *et al.*, 2000). Treatment with growth hormones in humans has reversed sarcopenia to some extent (Hennessey *et al.*, 2001), although this is associated with severe side effects, such as diabetes, high blood pressure, fluid retention and joint and muscle pain (reviewed by Hintz, 2004).

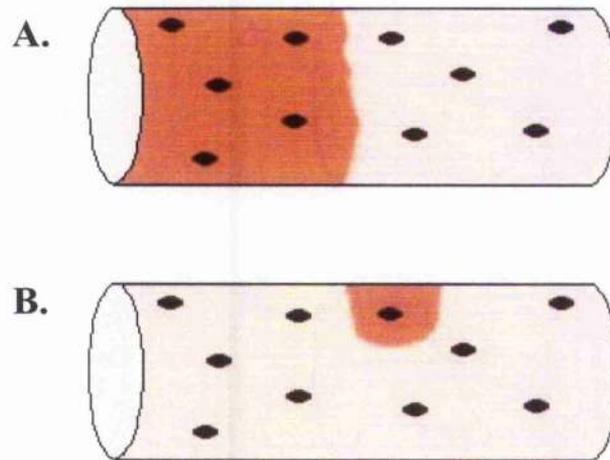
The loss of motor neurons is also associated with ageing (reviewed in DeLbono, 2003). The interactions between nerve and muscle are vital to the survival and function of either tissue, so it is likely that an element of age-related muscle wasting is related to the loss of neural tissue. Ageing fibres may be denervated or re-innervated, with corresponding switches in fibre gene-expression or complete atrophy (reviewed in

Larsson and Ansved, 1995). As with muscle fibres, survival of motor neurons is influenced by IGF-1 (Neff *et al.*, 1993). While the majority of studies on IGF-1 in neurogenesis focus on the developmental role, IGF-1 and its receptor show similar expression patterns in the adult central nervous system and neurons to that in embryonic nervous tissue (Bondy *et al.*, 1992). The extent to which IGF-1 signalling maintains muscle mass through the survival of innervating neurons as opposed to the direct stimulation of muscle growth and regeneration is as yet unknown.

#### 1.2.4.1.2 Ageing and Fibre Type

The adaptive (or maladaptive) response of ageing muscle fibre to these factors, in terms of composition of fibre type and MyHC expression, has been a source of controversy. In humans, the loss of strength associated with sarcopenia was first attributed to the selective loss of fast, type II fibres (Grimby *et al.*, 1982). This condition is well studied in rodents, and seems to tie in well with the decrease in physical activity and muscle loading late in life (Tseng *et al.*, 1995). However, it appears the situation in humans is a little more complex (reviewed in Porter *et al.*, 1995). Andersen *et al.* (1999) found that while there was an increase in the number of type I fibres at ages 60 to 70 relative to young adults, their observation was that this trend had disappeared in subjects over the age of 80.

By examining longitudinal sections, Andersen (2003) also demonstrated a greatly increased number of hybrid fibres in aged muscle, including a greater preponderance of hybrid types that are rare or even entirely absent in younger muscles. In ATPase staining of longitudinal sections of muscle fibres, changes in fibre-type specific expression were observed along the length of the muscle section, and small, localised regions corresponding to a single myonuclear domain were observed to have distinctly different fibre-specific expression to the remainder of the fibre (Figure 1.7). The mechanism proposed for this particular type of change in fibre composition is a loss in co-ordination between myonuclear domains, in that the communications between myonuclei breaks down in older tissue. The mechanism for the co-ordination between myonuclei, and how this might be compromised by the ageing process, is currently unclear.



**Figure 1.7.** Correlation of patterns of ATPase staining with myonuclear domains in the longitudinal sections of hybrid fibres taken from very old subjects (over 80 years). Mechanism proposed by Anderson (2003) associates myonuclei (shown in black) with regions of different ATPase staining (dark and light regions). Dark shaded areas correspond to ATPase staining (pH 4.3) for type I fibres. **A.** Apparent fibre phenotype switch along the length axis of the fibre. **B.** Isolated myonuclear domain.

#### 1.2.4.2 Porcine Meat Quality and Quantity

In the breeding of animals raised for meat, such as the pig, the composition of muscle fibres can have a marked effect on the quality and quantity of the final meat product (Chang *et al.*, 2003b). For example, intensive pig breeding for increased muscle growth resulted in the inadvertent selection for a condition known as malignant hyperthermia (MH), caused by a mutation in the ryanodine receptor (RyR) (MacLennan and Phillips, 1992). RyR is a calcium release channel, releasing  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum during muscle contraction. In the MH susceptible animals, the RyR channel is 'leaky', resulting in excessive  $\text{Ca}^{2+}$  release during stress. This leads to lactic acid build-up, hypermetabolism and high temperature, and can result in the death of the animals. However, a side effect of the condition is an increase in the rate of increase of muscle mass and so the MH condition has been unwittingly selected for in the drive for increased meat production.

Similarly, pig breeding has also had a marked effect on the fibre composition of porcine muscle (Essen-Gustavsson and Lindholm, 1984; Karlsson *et al.*, 1999). Modern breeds have a far greater predominance of fast-glycolytic fibres, which while lower in lipid content, and hence 'leaner', are associated with a number of undesirable meat traits. In studies carried out by Crouse *et al.* (1991) the 'tenderness' of the final product was found to be lower from muscles with a high content of fast-glycolytic fibres, while a high percentage of slow-oxidative fibres correlated to high tenderness. Other negative qualities such as low pH, high drip loss, poor texture and colour are also associated with a high percentage of fast fibres (Maltin *et al.*, 1997; Chang *et al.*, 2003b). This post-slaughter condition is known as the pale soft exudative pork (PSE) syndrome (Schmidt *et al.*, 1972; Gregory, 1998). The association with PSE and fast fibres makes it perhaps unsurprising that this trait is associated with the pig, rather than other animals raised for food such as cattle, given that the pig is the only large animal to express 2b MyHC (Pette and Staron, 2000).

Also caused by the RyR mutation in MH, PSE is a result of the switch to glycolytic metabolism following the cessation of blood-flow and resulting anaerobic conditions. Lack of blood-flow also causes a rapid build-up the end product of glycolysis, lactic acid, causing protein denaturation and a resulting, characteristic loss of water and coloration and a distinctly unsightly final product. Since fast fibres have greater stores of glycogen, it follows that the incidence of PSE will be higher in muscles with a higher percentage of these fibres. Such considerations make a rational understanding of the mechanisms of fibre type switching of considerable economic importance to the pig breeding industry.

## **1.3 POST-NATAL MUSCLE TRANSCRIPTION FACTORS**

### **1.3.1 TRANSCRIPTIONAL CONTROL**

The previous sections have dealt with the properties of different muscle fibre types, and the factors extrinsic to muscle that result in fibre adaptation in response to biological changes. The main focus of this study, however, is centred on transcription factors, intrinsic factors whose activities transduce external stimuli into changes in gene

expression. These intrinsic factors are the end-points in signal transduction pathways. In skeletal muscle, one of the most important regulators of fibre-phenotype is in the control of fibre-specific gene transcription. The following section will introduce the three better studied muscle-specific transcription factors/signal transduction pathways; these being the myogenic regulatory factors (MRFs), the insulin-like growth factor-1 receptor (IGF-1R) pathways and myocyte enhancer factor 2 (MEF-2), to review what is known of their roles in the control of muscle gene expression. The other major signal transduction pathway, the calcineurin/NFAT pathway, is a focus of the thesis and is covered in greater detail in Section 1.4.

### 1.3.2 MRF'S AND FIBRE TYPE

As discussed in Section 1.2.2, members of the MRF family play a vital role in the differentiation of myoblasts and the formation of mature muscle fibre. But while the role of the MRFs is well documented both in embryonic development and in the activation of satellite cells, their role in post-natal specification of fibre type is incompletely understood. Loss of function experiments with *myoD*<sup>-/-</sup> mice (Hughes *et al.*, 1997) have been less conclusive in determining a role in fibre specification, showing only a slight trend towards the fast phenotype. This is perhaps unsurprising given that MyoD and other MRFs are essential for many steps in myogenesis. It is difficult to conclusively demonstrate a muscle-plasticity effect independent of the defects in myoblast determination, fibre development and maturation.

There have been several studies on the relative levels of different MRFs in fast and slow muscle (Hughes *et al.*, 1997; Walters *et al.*, 2000). Hughes *et al.* (1997), in studies in the rat, demonstrated a higher level of myogenin in muscle regions with predominantly slow fibre types, with MyoD higher in predominantly fast muscles. Walters *et al.* also demonstrated a higher level of MRF-4 in slow fibres of the rat *gastrocnemius* muscle, but found no such results in the *soleus*, a postural muscle. Overexpression studies have similarly failed to demonstrate a master role for the MRFs in fibre specification. Overexpression of myogenin in the fast muscles of transgenic mice showed a switch towards oxidative metabolism, but no increase in the levels of slow MyHC expression (Hughes *et al.*, 1998). So while it would seem there is

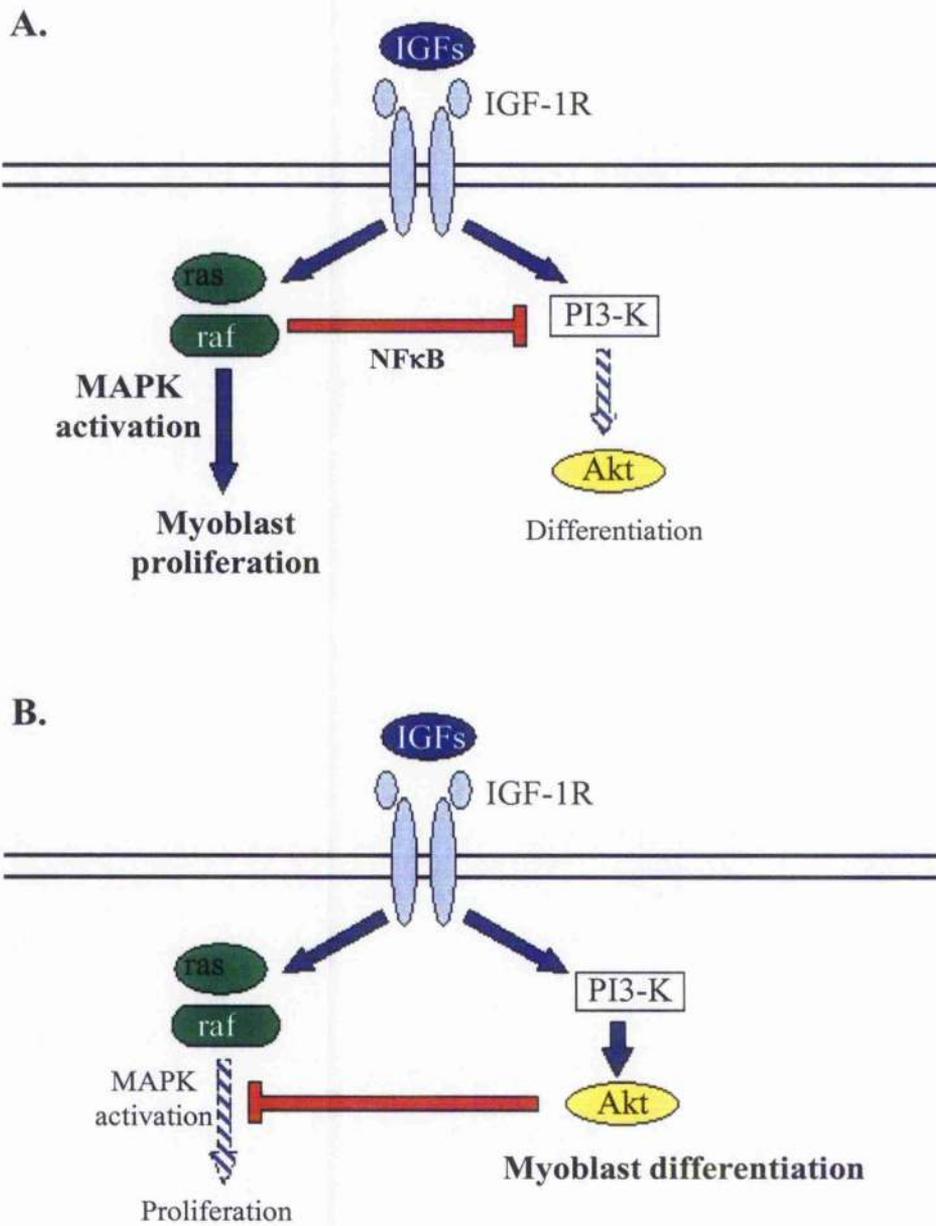
something of a correlation between different MRFs and fibre type, a direct, causal relationship has yet to be conclusively demonstrated.

### **1.3.3 IGF-1R SIGNAL TRANSDUCTION**

As discussed in Section 1.2.3.2, IGF-1 signalling is involved in both myocyte proliferation, and in differentiation/fibre hypertrophy. These two pathways, reviewed in Singleton & Feldman, (2001), both have their origins in a chain of phosphorylation events beginning with the IGF-1 receptor (IGF-1R). The start of the cascade is the conformational change in the tyrosine kinase of IGF-1R following the binding its ligand. This results in autophosphorylation and the binding of cytosolic substrate proteins including, among others, insulin receptor substrate (IRS). Once bound, IRS is phosphorylated and subsequently acts as a docking platform for a number of further signalling intermediaries containing the Src homology-2 (SH2) domain. This is a protein motif that recognises and binds tyrosine-phosphorylated sequences. SH2-mediated signalling pathways include two factors of particular importance to muscle signal-transduction (Moelling *et al.*, 2002). The first is growth factor receptor bound protein 2 (Grb2), which activates the small G protein, Ras, in conjunction with son of sevenless (SoS). The second is phosphatidylinositol 3-kinase (PI3-K), activated by its SH2-domain containing p85 regulatory sub-unit. A slightly simplified way at looking at these two pathways is to divide them between Ras-mediated myoblast proliferation, versus PI3-K mediated hypertrophy, as summarised in Figure 1.8. Also indicated is the degree of cross-talk that exists between the two pathways, which serves as an additional level of control (Rommel *et al.*, 1999). This section will briefly review the divergent pathways following from Ras and PI3-K activation.

#### **1.3.3.1 Ras and Proliferation**

The Ras/Raf/MEK/ERK pathway is a well-studied signal transduction pathway, one of a number of mitogen-activated protein kinase (MAPK) signalling systems used for a wide variety of roles in different cell types (reviewed by Kolch, 2000). The Ras signalling pathway is known more for its role in regulating haemopoietic cell growth in response to cytokine signalling and consequent role in leukaemia when dysfunctional (reviewed in Chang *et al.*, 2003a). Ras proteins belong to an extended family of small (21 kDa) proteins that cycle between activated, GTP-bound states, and inactive, GDP-



**Figure 1.8.** Summary of IGF-1R mediated pathways in myoblast proliferation and differentiation (from Singleton and Feldman, 2001). **A.** PI3 kinase inactivation and Ras/Raf activation of MAPK during myoblast proliferation. **B.** PI3 kinase/Akt activation during muscle hypertrophy.

bound states. As with other MAPK pathways, Ras signalling continues along a series of serine/threonine phosphorylation events, in this case via Raf, mitogen-activated protein kinase/ERK kinase (MEK) and extracellular signal regulated kinase (ERK). Ras signalling is complex, and a full account of the regulation of this pathway can be found in the review by Kolch (2000). In myoblasts, the Ras MAPK pathway is primarily associated with proliferation and the prevention of differentiation (Olson *et al.*, 1987). It has been suggested that Ras-mediated inhibition of muscle differentiation operates via NF $\kappa$ B, a member of the Rel family of transcription factors (Mitin *et al.*, 2001).

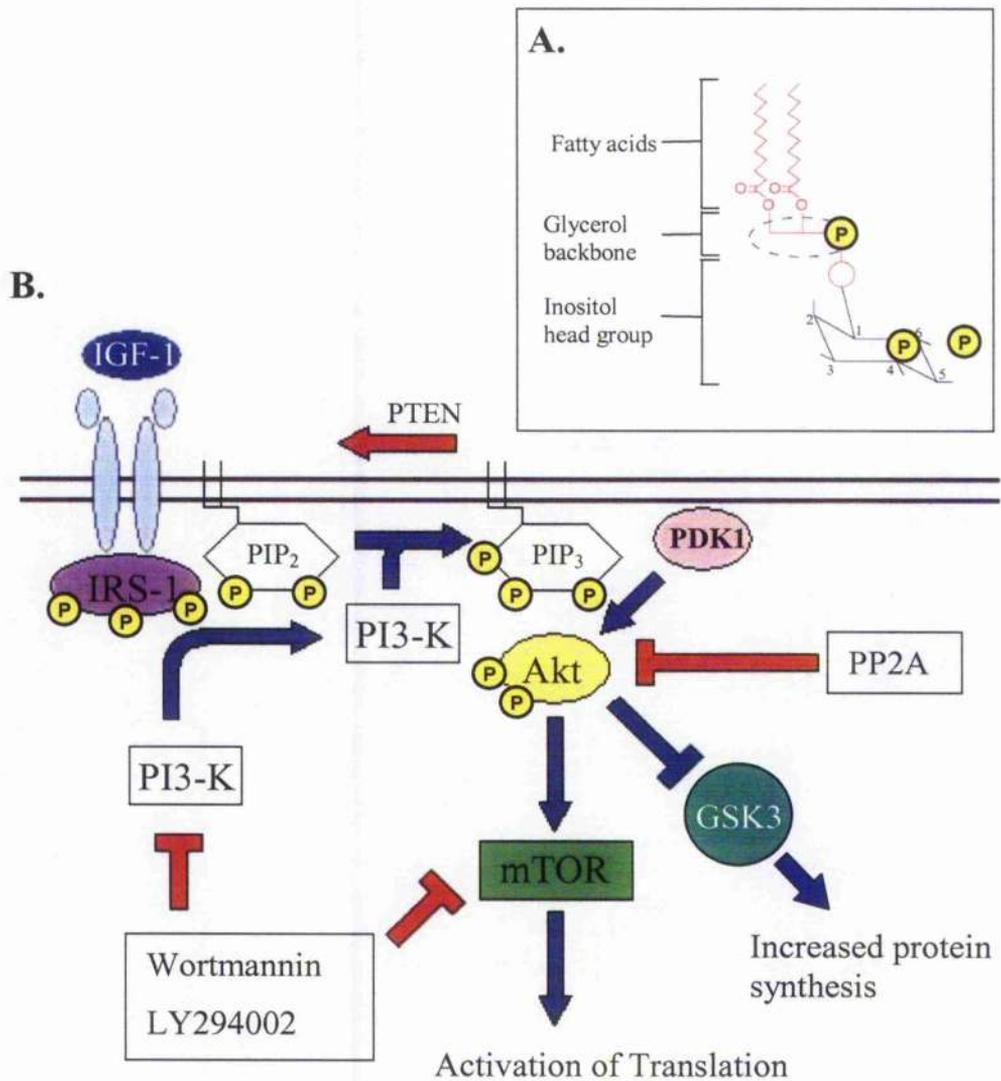
Though involved in myoblast proliferation, Ras-MAPK signalling has also been implicated in later stages of muscle development and differentiation (Bennett and Tonks, 1997; Gredinger *et al.*, 1998). Ras also involved in slow fibre specification (Murgia *et al.*, 2000), and muscle fibre hypertrophy (Haddad and Adams, 2003). In their investigation in the control of fibre type, Murgia *et al.* (2000) employed two forms of constitutively active Ras mutants, using *in vivo* transfections in regenerating muscle. A Ras mutant that specifically activated the MAPK pathway was found to activate a switch to slow-fibre gene expression, while a dominant-negative Ras repressed the effect of slow-neuron firing patterns. By contrast, a second mutant that activated the PI3-K pathway (see below) resulted in increased muscle hypertrophy, but had no effect on fibre phenotype. While this would seem to indicate that the two separate pathways play distinct roles, it would appear that the situation is a little more complex. The study by Haddad and Adams (2003), involving specific inhibition of ERK, blocked IGF-1 related hypertrophy, indicating that the Ras-MAPK pathway is important in both the slow fibre phenotype and hypertrophic muscle growth.

The manner by which Ras can play a role in such a variety of sometimes contradictory pathways remains incompletely understood. One theory is that the outcome of the Ras pathway may be influenced by the timing of the extracellular signals, with a transient activation having a different effect to a sustained one (Marshall, 1995; Gredinger *et al.*, 1998).

### 1.3.3.2 PI3-K and Hypertrophy

Phosphatidylinositol 3-kinase (PI3-K) consists of two sub-units, the SH2 domain containing regulatory unit, also known as p85, and a catalytic sub-unit, p110 (reviewed in Vivanco and Sawyers, 2002). The PI3-K signalling pathway is shown in Figure 1.9. PI3-K acts through a class of membrane phospholipids, the inositol phospholipids. PI3-K produces phosphatidylinositol (3,4,5)-trisphosphate (PIP<sub>3</sub>) through the addition of a phosphate group to the inositol sugar ring of phosphatidylinositol (4,5)-bisphosphate (PIP<sub>2</sub>). PIP<sub>3</sub> subsequently serves as a docking station for protein kinase B (PKB), also known as Akt, and phosphoinositide-dependent protein kinase 1 (PDK1), permitting Akt phosphorylation and activation (Downward, 1998). The activation of Akt is inhibited by phosphatase and tensin homologue on chromosome 10 (PTEN), which dephosphorylates PIP<sub>3</sub> (Stambolic *et al.*, 1998), and by protein phosphatase 2A (PP2A), which dephosphorylates Akt (Andjelkovic *et al.*, 1996; Pankov *et al.*, 2003). PP2A is a trimeric complex consisting of catalytic, regulatory and scaffold sub-units (Sontag, 2001). While the catalytic sub-unit of PP2A is ubiquitous, there is evidence for a muscle-specific regulatory sub-unit, indicating tissue specific regulation of Akt (Tehrani *et al.*, 1996).

Activated Akt is translocated into the nucleus, where it acts upon a large number of phosphorylation targets containing the common motif RXXXX(S/T) (Zimmermann and Moelling, 1999; reviewed in Coffey *et al.*, 1998). Well documented targets of Akt in muscle include mammalian target of rapamycin (mTOR), a protein kinase and an activator of protein translation (Gingras *et al.*, 2001). Akt also represses the activity of glycogen synthase kinase 3 (GSK3), which regulates a variety of physiological properties including glycogen and protein synthesis (Cross *et al.*, 1995). Inhibition of GSK3 leads to an upregulation of protein synthesis (Welsh *et al.*, 1997; Jefferson *et al.*, 1999), and targeted inhibition of GSK3 leads to marked muscle hypertrophy (Rommel *et al.*, 2001). Akt also represses Raf, thus inhibiting the Ras-MAPK pathway (Zimmermann and Moelling, 1999), as part of the Ras/PI3-K pathway cross-talk already discussed (Figure 1.8). In addition to activation via IGF-1R, PI3-K has also been known to act downstream of Ras in some settings, such as regulation of the actin cytoskeleton (Rodriguez-Viciana *et al.*, 1997).



**Figure 1.9.** Details of the PI3-K/Akt pathway. **A.** The structure of phosphatidylinositol (4,5)-bisphosphate (PIP<sub>2</sub>), the substrate of PI3-K. PIP<sub>2</sub> is phosphorylated to form phosphatidylinositol (3,4,5)-trisphosphate (PIP<sub>3</sub>), as part of the PI3-K signalling pathway (adapted from Vivanco and Sawyers, 2002). **B.** Activation of PI3-K by phosphorylated IRS-1 (adapted from Glass, 2003a). **GSK3**, glycogen synthase kinase 3, **IRS-1**, insulin receptor substrate-1, **mTOR**, mammalian target of rapamycin, **PDK1**, phosphoinositide-dependent protein kinase 1, **PP2A**, protein phosphatase 2A, **PTEN**, phosphatase and tensin homologue on chromosome 10.

PI3-K is sensitive to inhibition by the small-molecule inhibitors Wortmannin and LY294002 (Powis *et al.*, 1994; Vlahos *et al.*, 1994). Though these inhibitors are commonly used to study PI3-K function, this sensitivity is shared with the downstream signalling element, mTOR (Brunn *et al.*, 1996). Use of PI3-K and mTOR inhibitors has highlighted the pivotal role played by the PI3-K pathway in IGF-1 mediated hypertrophy (Bodine *et al.*, 2001; Pallafacchina *et al.*, 2002). Genetic studies using Akt mutants also confirmed these findings, dispelling doubts arising from the relative uncertainty of the chemical inhibitors used (Rommel *et al.*, 2001; Bodine *et al.*, 2001). Expression of a dominant-negative form of Akt blocked hypertrophy in cultured myotubes, while a constitutively active form, conversely, was sufficient to cause hypertrophy. This would suggest that the PI3-K/Akt pathway is the primary trigger for IGF-1 mediated muscle hypertrophy. The findings of Haddad and Adams (2003), that hypertrophy can be inhibited *in vivo* through inhibition of the Ras-MAPK pathway, indicates that this the response is regulated by cross-talk from Ras-MAPK.

Pallafacchina *et al.* (2002) found inhibition of muscle hypertrophy through chemical inhibition of the PI3-K/Akt/mTOR pathway by rapamycin, but this treatment had no effect on fast-slow switching of fibre type. Their conclusion was that PI3-K did not mediate fibre-specific gene expression. Instead, it was found that fibre switching was blocked by the inhibition of another signalling pathway, mediated by calcineurin (Section 1.4).

#### 1.3.4 THE MEF-2 FAMILY

The myocyte enhancer family-2 (MEF-2) of transcription factors consists of four genes in vertebrates, *mef-2a*, *b*, *c* and *d* respectively, further subdivided by alternative splicing (reviewed in Black and Olson, 1998). In skeletal muscle, the *mef-2* genes are expressed in distinct, temporal-spatial patterns. MEF-2A, C and D have been characterised in cultured myoblasts, with MEF-2D expressed before the onset of differentiation (Breitbart *et al.*, 1993), and MEF-2A and MEF-2C are expressed early and late, respectively, in the differentiation process (Yu *et al.*, 1992; McDermott *et al.*, 1993).

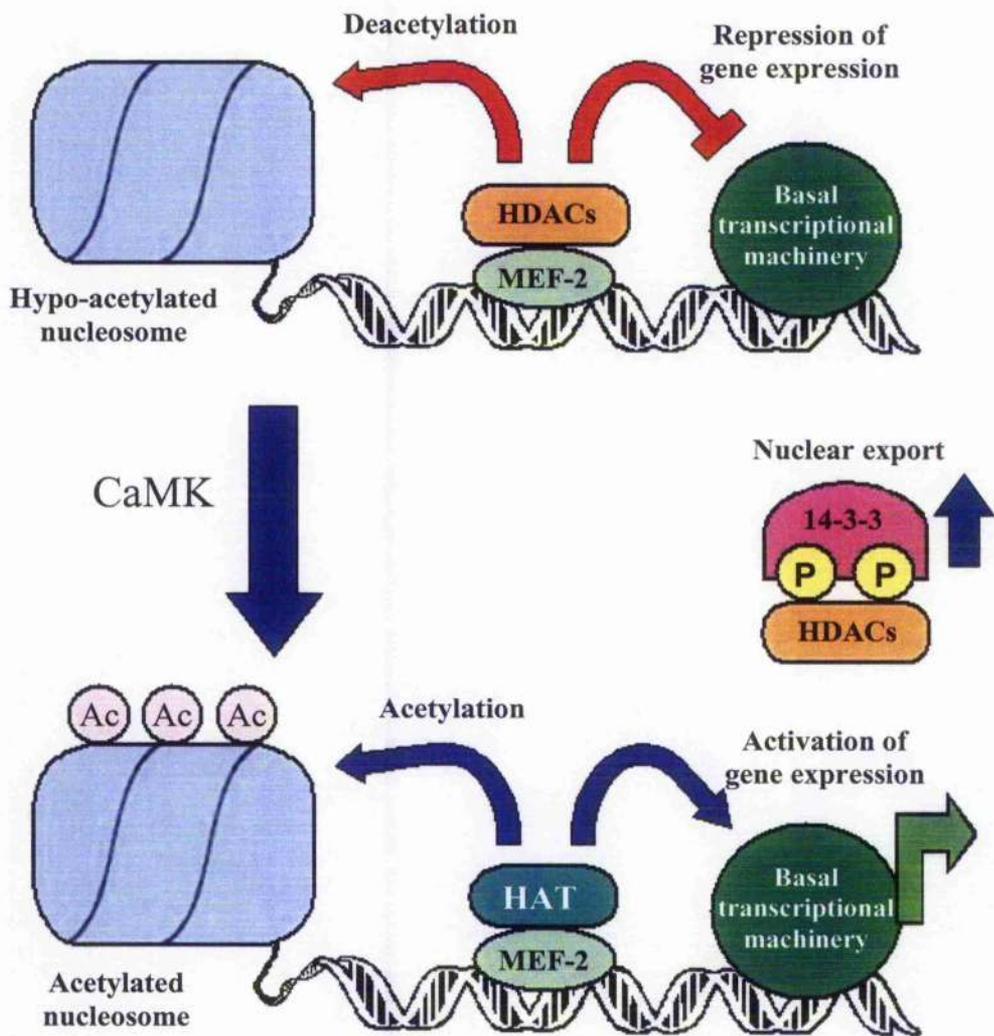
The MEF-2 family belongs to the larger family of MCM1, Agamous, Deficiens and Serum response factor (MADS) box proteins, and possess the characteristic MADS-box

domain. This is an N-terminal 57-amino-acid region responsible for DNA binding. An adjacent, 29 amino-acid region, the MEF-2 domain, mediates high affinity binding to the target transactivation site, and interactions with other co-factors (Molkentin *et al.*, 1996). The MEF-2s are best known for their role in skeletal, cardiac and smooth muscles and as co-activators in the myogenic program (Olson *et al.*, 1995). However, MEF-2 members are also found in a number of other tissues as part of a wide variety of processes, some of which would seem diametrically opposed (reviewed in McKinsey *et al.*, 2002). For instance, in neurons MEF-2 mediates protection from apoptosis, (Okamoto *et al.*, 2000), yet promotes apoptosis in negatively selected thymocytes (Woronicz *et al.*, 1995).

While MEF-2 has been described as having a 'paradoxical' array of different functions (McKinsey *et al.*, 2002), it is worth remembering the different MEF-2 members and splice variants are not necessarily identical in function. Many studies postulating a role for MEF-2 proteins in cellular processes have as their evidence the presence of a MEF-2 consensus binding sites in the relevant gene promoter regions or reporter constructs (e.g. Chin *et al.*, 1998; Wu *et al.*, 2000; Friday *et al.*, 2003). While good evidence of MEF-2 involvement, this does not specify the exact MEF-2 isoform involved. This section will review the regulatory mechanisms controlling MEF-2 activation that link these proteins into a number of other signalling pathways, and the roles played by MEF-2 family members in skeletal muscle.

#### **1.3.4.1 The Regulation of MEF-2 Activity**

The MEF-2 proteins are controlled by a number of major signalling pathways, with perhaps the most important being intracellular calcium. MEF-2 activity is up-regulated by a number of upstream, calcium-responsive signalling factors (Passier *et al.*, 2000; Wu *et al.*, 2000). These include two signalling proteins dependent on calmodulin (CaM), calcium/calmodulin-dependent kinase (CaMK) and the serine-threonine phosphatase, calcineurin (covered in more detail in Section 1.4). CaMK and calcineurin have different mechanisms of action: CaMK acts by relieving MEF-2 repression, while calcineurin interacts directly with MEF-2 proteins.



**Figure 1.10.** The activation of MEF-2 transcription factors by calcium/calmodulin-dependent protein kinase (CaMK) (from McKinsey *et al.*, 2002). **HAT**, histone acetyltransferases, **HDACs**, histone deacetylases.

As shown in Figure 1.10, the activation of MEF-2 involves the removal of an inhibitory complex with histone deacetylases (HDACs), bound to the MEF-2 sites on the promoter regions of target genes (Lu *et al.*, 2000). HDACs inhibit transcription through the deacetylation of core histone proteins and subsequent chromatin condensation. There are three classes of HDAC (I, II and III), of which a number of class II members are able to interact with all MEF-2 family members through direct association with the MADS/MEF-2 regulatory domains (reviewed by McKinsey *et al.*, 2001b; Han *et al.*, 2003). The removal of the inhibitory complex is mediated by CaMK (Lu *et al.*, 2000; Corcoran and Means, 2001). CaMK mediated phosphorylation of the HDAC proteins creates docking sites for the 14-3-3 chaperone protein (McKinsey *et al.*, 2000), facilitating their export from the nucleus. Activation is further enhanced by the association of histone acetyltransferases (HATs), such as the co-activator protein p300. These directly antagonise HDAC activity, acetylating core histones and relieving transcriptional repression (Strahl and Allis, 2000). HAT proteins bind directly to MEF-2, in a manner competitive with HDAC binding (McKinsey *et al.*, 2001a).

Calcineurin enhances MEF-2 activity by different mechanisms, the first of these being via its main downstream target, nuclear factor of activated T-cells (NFAT). Covered in greater detail in Section 1.4, the NFAT family consists of a number of isoforms, of which NFAT-1 has been shown to interact directly with MEF-2A and D (Blaeser *et al.*, 2000). NFAT-1 enhances MEF-2D activity through the recruitment of the HAT, p300 (Youn *et al.*, 2000). Calcineurin also directly dephosphorylates MEF-2A and D, enhancing MEF-2 DNA binding (Mao and Wiedmann, 1999; Wu *et al.*, 2001). As a final note on calcineurin/MEF-2 interactions, the inhibitor of calcineurin, cain (also known as cabin), is also an inhibitor of MEF-2 (Youn and Liu, 2000). Cain has been shown to bind directly to MEF-2B, where it is thought to recruit the inhibitory HDAC complex (Youn and Liu, 2000; Han *et al.*, 2003). The same MEF-2 binding site on cain is also the binding site for CaM.

MEF-2 is also a signal integrator for a number of different pathways, including IGF-1 receptor signalling via PI3-K (Xu and Wu, 2000). Xu and Wu (2000) found that MEF-2 sites in the myogenin enhancer were required for PI3-K mediated activation of this gene, though the mechanism is unclear. While direct phosphorylation of MEF-2 has yet to be demonstrated, loss of MEF-2A and C activity was observed following inhibition

of PI3-K (Tamir and Bengal, 2000). This inhibition of MEF-2 was rescued by p38 MAPK, another parallel pathway responsible for MEF-2 activation in skeletal muscle differentiation and cardiac hypertrophy (Kolodziejczyk *et al.*, 1999; Zetser *et al.*, 1999; reviewed in Han and Molkenin, 2000). MEF-2C and A can be activated by p38 MAPK, which involves direct phosphorylation of the DNA binding domain (Han *et al.*, 1997). This is in marked contrast to a second MAPK activator of MEF-2, external receptor kinase-5 (ERK-5), which phosphorylates the MADS-box regulatory domain (Yang *et al.*, 1998). Unique among MAPKs, ERK-5 possesses a domain for the transactivation of expression (Kasler *et al.*, 2000), and may function as a co-activator of MEF-2 activity. Finally, MEF-2 activity is regulated by Notch-1, the promoter of proliferation covered in Section 1.2.2.6. Constitutively active Notch-1 blocks the DNA binding of MEF-2C (Wilson-Rawls *et al.*, 1999). This inhibition was found to be MEF-2C specific, requiring a 12 amino acid stretch adjacent to the DNA binding domain not found in other MEF-2 proteins.

#### 1.3.4.2 MEF-2 and Muscle Development

Unlike the MRFs, MEF-2 proteins cannot induce myogenesis on their own, but are important co-factors for the MRFs and other transcription factors. It was the interaction with MyoD that led to the discovery of the MEF-2 family, first isolated through binding with the basic domain of MyoD (Davis *et al.*, 1990). MEF-2 proteins form transcriptional complexes with MRF/E-protein heterodimers (Molkenin *et al.*, 1995), and MEF-2 binding sites are found in the enhancer and promoter regions of the majority of muscle-specific genes (Cserjesi and Olson, 1991). The critical importance of MEF-2 in myogenesis was demonstrated by Ornatsky *et al.* (1997) using a dominant-negative form of MEF-2A, which completely ablated MyoD-mediated transformation of cultured fibroblasts into myotubes.

Several of the signalling factors discussed above affect MEF-2 activity in an isoform specific manner. Together with the stage-specific pattern of expression of MEF-2 members, it would seem likely that different MEF-2s perform different functions. MEF-2D is the clearest example, an early marker of the myogenic lineage (Breitbart *et al.*, 1993). MEF-2D is most likely involved in proliferation, through the activation *c-Jun* (Han and Prywes, 1995). A MEF-2 binding site in the promoter region of *c-Jun* was found to be essential for transcriptional activation in HeLa cells. C-Jun is

one of the Jun sub-family of basic-region leucine zipper proteins, that dimerise to form the activator protein 1 (AP-1) complex with leucine zippers from the Jun, Fos, Maf and ATF sub-families (reviewed in Shaulian and Karin, 2002). C-Jun is known to be a major factor in the activation of cell proliferation (Schreiber *et al.*, 1999).

#### 1.3.4.3 MEF-2 and Fibre-phenotype

In addition to skeletal muscle growth and differentiation, MEF-2 members are involved in calcium-dependent, muscle fibre-type specification (Chin *et al.*, 1998; Wu *et al.*, 2000). Wu *et al.* (2000) reported that MEF-2 and NFAT binding sites were required in the enhancer region of the slow-fibre-specific isoform of troponin I, and that a MEF-2 reporter construct in transgenic mice was selectively active in the *soleus* muscles. One of the factors known to interact with MEF-2, and likely to mediate a role in the slow fibre phenotype, is PPAR- $\gamma$ -coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) (Michael *et al.*, 2001). PGC-1 $\alpha$  is a transcriptional co-activator first isolated through its interaction with peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) (Puigserver *et al.* 1998). PGC-1 $\alpha$  is known to upregulate mitochondrial biogenesis in a wide range of tissues (Wu *et al.*, 1999). Muscle-promoter driven transgenic expression of PGC-1 $\alpha$  results in a fast to slow fibre phenotype switch and the co-activation of MEF-2C and D, but not MEF-2A (Lin *et al.*, 2002). Handschin *et al.* (2003) also found that PGC-1 $\alpha$  forms part of an auto-regulatory loop, mediating its own transcription in conjunction with MEF-2. This feature helps to reinforce slow fibre-specification once triggered.

#### 1.3.4.4 MEF-2 Summary

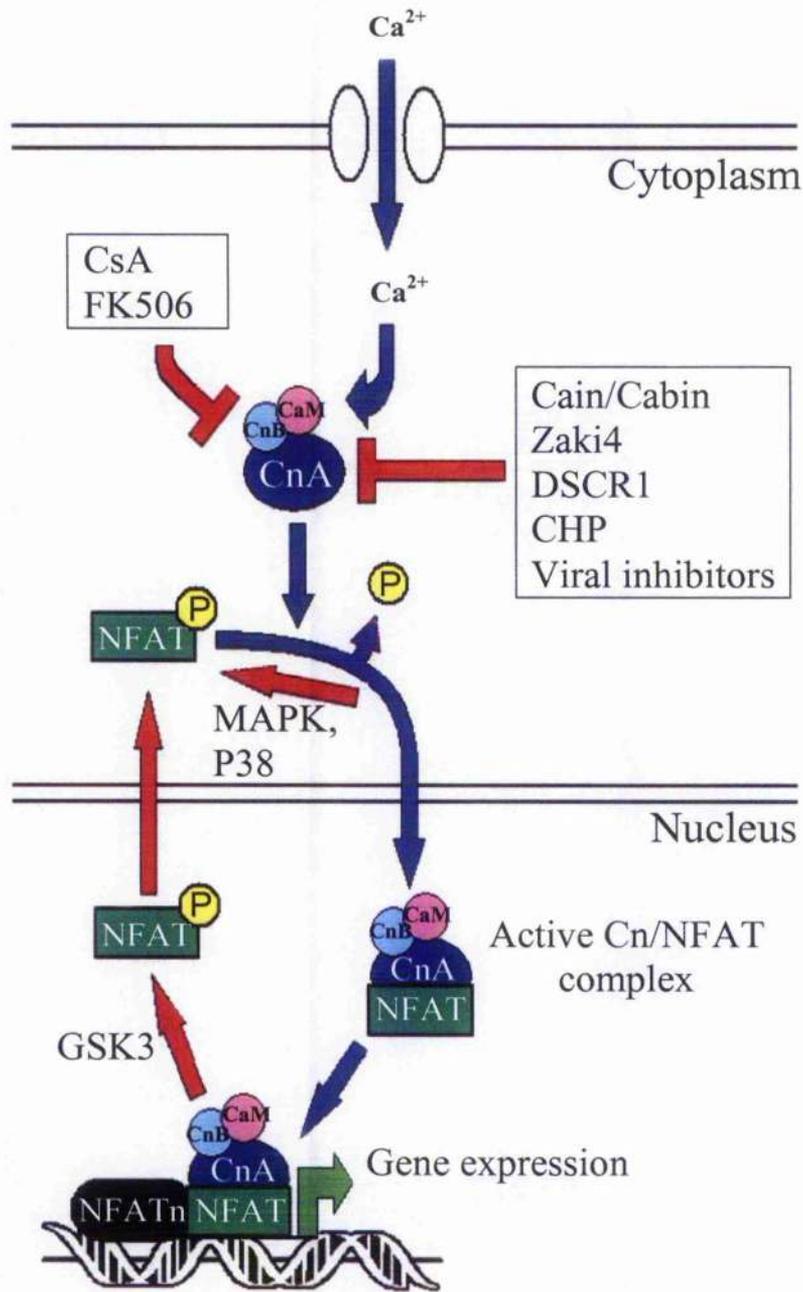
In conclusion, the members of the MEF-2 family are involved in the activation of almost all muscle-specific genes and as such serve to integrate a wide variety of signalling pathways in muscle. Though the diversity of MEF-2 regulated processes seems at first to be almost mutually exclusive, the specificity is conferred not only by the MEF-2 isoform involved, but also in interactions with specific co-factors (McKinsey *et al.*, 2002). In this respect, the MEF-2 family is similar to NFAT, the family of transcription factors downstream of calcineurin. It is the calcineurin/NFAT pathway that is the last major signalling pathway dealt with in this review.

## 1.4 CALCINEURIN AND NFAT

The calcineurin/NFAT pathway is involved in the conversion of a generic, widely distributed signal, the influx of calcium, into specific patterns of gene expression (reviewed in Crabtree and Olson, 2002). This pathway was first described in cells of the immune system (Flanagan *et al.*, 1991; Liu *et al.*, 1991), where it governs the expression of cytokines. Since then, calcineurin signalling has been found to play a role in signal transduction in a wide range of different tissue types (reviewed in Rusnak and Mertz, 2000). An overview of calcineurin/NFAT signal transduction is shown in Figure 1.11. To summarise, a sustained increase in the levels of intracellular calcium results in the sequestration of  $\text{Ca}^{2+}$  ions by calmodulin. This leads to a conformation change exposing two hydrophobic patches on calmodulin that bind to the calmodulin-binding domains on target proteins, including the protein phosphatase calcineurin. Once activated, calcineurin interacts with and dephosphorylates NFAT transcription factors present in the cytoplasm. The calcineurin/NFAT complex is translocated to the nucleus where it forms further interactions with one of a wide range of other factors collectively termed NFAT-n (Flanagan *et al.*, 1991). NFAT-n factors include Fos/Jun (AP-1) complexes and members of the MEF-2 and GATA families of transcription factors, the end-points of different signal transduction pathways. It is this convergence of the calcineurin pathway with other signalling mechanisms that confers transcriptional specificity from the generic signal of calcium influx.

### 1.4.1 STRUCTURE AND FUNCTION OF CALCINEURIN

Calcineurin consists of two subunits: a catalytic- and calmodulin-binding subunit, calcineurin A (CnA), and a regulatory,  $\text{Ca}^{2+}$  binding subunit, calcineurin B (CnB) (Crabtree, 1999; Rusnak and Mertz, 2000). There are three isoforms of calcineurin A:  $\alpha$ ,  $\beta$  and  $\gamma$ , ranging from 58-64 kDa in size, and two isoforms of calcineurin B, both 19 kDa in size. Calcineurin is, in general, widely distributed throughout different tissue types, with the greatest abundance in brain tissue. Different sub-unit isoforms, however, can exhibit distinct patterns of expression. For calcineurin A, the  $A\gamma$  isoform is restricted to brain and testis while  $A\alpha$  and  $A\beta$  are more ubiquitously expressed (Klee *et al.*, 1998; Rusnak and Mertz, 2000). Similarly, CnB1 is widely expressed, in association with calcineurin  $A\alpha$  and  $\beta$ , but CnB2 is expressed only in testis. While both



**Fig. 1.11.** Summary of the calcineurin/NFAT signalling pathway, as is present in a number of cell types (from Crabtree and Olson, 2002). Activation pathway is shown in blue, inhibition and NFAT re-phosphorylation and nuclear export shown in red. **CaM**, calmodulin. **CnA** and **CnB**, calcineurin subunits A and B respectively. **MAPK**, mitogen activated protein kinase. **GSK3**, glycogen synthase kinase 3.

subunits are common to many tissues, the relative abundance of A $\alpha$  and A $\beta$  isoforms can vary, in a tissue specific manner. For example, CnA $\alpha$  is more abundant in rat brain and heart than CnA $\beta$ , a pattern reversed in spleen, thymus and lymphocytes (Kuno *et al.*, 1992; Jiang *et al.*, 1997). A study by Parsons *et al.* (2003) in the muscles of the mouse showed CnA $\alpha$  to be the predominant isoform in almost all the muscle types analysed.

#### **1.4.1.1 Calcineurin Inhibitors**

Calcineurin activity is controlled by a number of endogenous regulatory proteins. Foremost of these is the calcineurin inhibitor protein (cain, or cabin) which non-competitively blocks calcineurin activity (Lai *et al.*, 1998). Another specific inhibitor is the calcineurin B homologous protein (CIIP), a competitive inhibitor that binds to CnA without activating catalytic activity (Lin *et al.*, 1999). Calcineurin is also the target of the modulatory calcineurin interacting proteins (MCIPs). There are three MCIP genes, MCIP-1, -2 and -3 (reviewed in Rothermel *et al.*, 2003). They are well studied in humans where they are encoded on different chromosomes MCIP-1 is encoded within the Down syndrome critical region (DSCR) on chromosome 21, and is also known as DSCR1. MCIP-1 is highly expressed in brain, heart and skeletal muscle, and over-expressed in Down syndrome foetuses (Fuentes *et al.*, 2000). MCIP-2, also known as ZAKI-4, is similarly highly expressed in brain, heart and skeletal muscle, and is known to be upregulated by thyroid hormone (Miyazaki *et al.*, 1996). As discussed in Section 1.2.3.2.2, thyroid hormone is recognised as a major modulator of cardiac and muscle-fibre specific expression. MCIP-1 has been shown to be activated by calcineurin signalling (Yang *et al.*, 2000), which would suggest some form of negative feedback mechanism.

#### **1.4.1.2 Immunosuppression through Calcineurin**

Given the role of calcineurin/NFAT signalling in the immune system, it is perhaps unsurprising that it is a target for a number of viral pathogens that can dampen the immune response by disabling calcineurin activity. For example, the A238L protein of the African swine fever virus has been shown to interact with calcineurin (Miskin *et al.*, 1998). Two pharmacological immunosuppressive agents that target calcineurin activity are cyclosporin A (CsA) and FK506, originally developed to suppress tissue rejection

in organ transplant patients. Both act by blocking substrate access (Griffith *et al.*, 1995; Kissinger *et al.*, 1995), forming complexes that mask the CnA catalytic site, together with cyclophilins and FK506 binding proteins respectively (Klee *et al.*, 1998; Rusnak and Mertz, 2000). As shall be seen, the ability of these drugs to inactivate calcineurin activity has played a major part in many studies into the role of calcineurin/NFAT in skeletal muscle.

#### 1.4.2 THE NFAT FAMILY

NFAT stands for Nuclear Factor of Activated Thymocytes, and it was in cells of the immune system that members of this multi-gene family were first isolated (Shaw *et al.*, 1988; Rao, 1994). There are currently four NFAT family members known to interact with calcineurin (a fifth NFAT has been characterised, but is not a substrate for calcineurin; Lopez-Rodriguez *et al.*, 1999). NFAT 1, 2 and 4 are known to exist as a number of different isoforms, derived from alternative splicing. NFAT-2, for example, has been characterised as having three isoforms, referred to as 2a, 2b and 2c (Serfling *et al.*, 2000). These variants differ primarily in the length of their C-terminal domains (Park *et al.*, 1996; Chuvpilo *et al.*, 1999). There are a number of nomenclature schemes describing NFAT-family members. A single nomenclature system is shown in Table 1.4, correlating the various alternative naming systems (Rao *et al.*, 1997; Serfling *et al.*, 2000). Splice variants of the four isoforms are lettered alphabetically, as for the example of NFAT-2 above. This nomenclature scheme is used throughout this thesis.

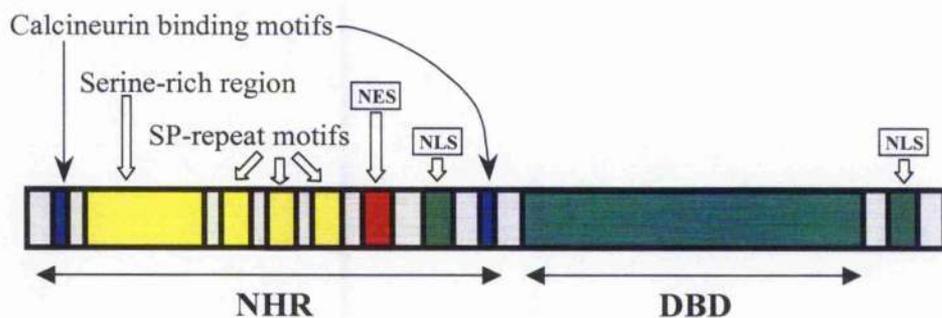
Since their isolation from cells of the immune system, NFAT family members have been found in a range of different tissue types (reviewed in Rao *et al.*, 1997), as summarised in Table 1.5. The structural features of an NFAT protein are shown in Figure 1.12. The DNA binding domain is the region of highest homology among NFAT proteins, and also bears similarity with the Rel homology region of Rel-family transcription factors (Wolfe *et al.*, 1996). Also shown in Figure 1.12 are the SP-repeat motifs and serine-rich regions of the NHR/CAT domain, which are dephosphorylated by calcineurin during NFAT activation (Beals *et al.*, 1997a; Zhu *et al.*, 1998).

	Alternative Nomenclature (if any)
NFAT-1	
NFAT-1a	NFATc2, NFATp
NFAT-1b	
NFAT-1c	
NFAT-2	
NFAT-2a	NFATc/A, NFATc.α, NFATc1, NFATc
NFAT-2b	NFATc/B, NFATc.β,
NFAT-2c	NFATc/C,
NFAT-3	NFATc4
NFAT-4	
NFAT-4x	NFATc3, NFATx
NFAT-4a, b & c	

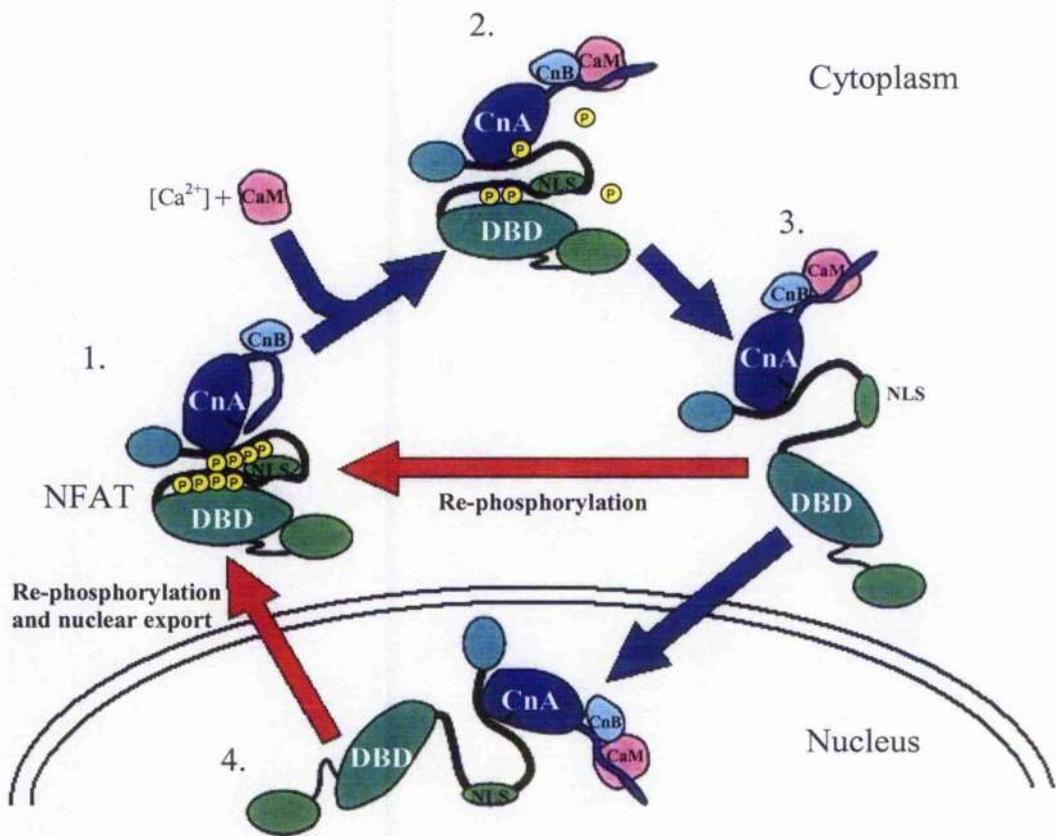
**Table 1.4.** Nomenclature for NFAT family members.

Family member	Tissue	References
NFAT-1	Thymus, spleen, T cells, B cells, NK cells, monocytes, macrophages	(Aramburu <i>et al.</i> , 1995; Ruff and Leach, 1995; Wang <i>et al.</i> , 1995; Weiss <i>et al.</i> , 1996)
	Olfactory bulb and endothelium	(Ho <i>et al.</i> , 1994)
	Endothelial cells	(Cockerill <i>et al.</i> , 1995)
	Myoblasts	(Abbot <i>et al.</i> , 1998)
NFAT-2	Muscle, thymus, spleen, testis, PBL (peripheral blood lymphocytes), testis, ovary, colon	(Hoey <i>et al.</i> , 1995; Masuda <i>et al.</i> , 1995)
NFAT-3	Placenta, lung, kidney, testis, ovary, heart, colon	(Hoey <i>et al.</i> , 1995)
NFAT-4	Heart, thymus, leukocytes	(Ho <i>et al.</i> , 1995)

**Table 1.5.** Tissue distribution of NFAT family members.



**Figure 1.12.** Structural features of NFAT proteins (from Crabtree and Olson, 2002). SP-repeat motif: SpxxSPxxSPxxxxx(E/D)(E/D). **NHR**, NFAT homology region (also known as the calcineurin dependent translocation, or CAT, domain). **DBD**, DNA binding domain. **NES**, nuclear export signal. **NLS**, nuclear localisation signal (nuclear import signal).



**Figure 1.13.** Model for the activation of NFAT proteins and the formation of a transcriptional complex with calmodulin/calcineurin, emphasising the conformational changes involved (from Rao *et al.*, 1997). **1.** Inactive calcineurin/NFAT complex in the cytoplasm. **2.** The binding of calmodulin in response to an increase in intracellular calcium results in a conformational change in calcineurin, unmasking the catalytic site. **3.** The dephosphorylation of NFAT by active calcineurin results in a conformational change in NFAT in turn, which exposes the NLS and results in nuclear import. **4.** Active calcineurin/NFAT in the nucleus, which binds with other co-activators of gene expression.

In the resting form of NFAT, the nuclear localisation signal is masked by the phosphorylated SP-repeat motif and serine-rich region. Dephosphorylation by calcineurin is thought to result in the dissolution of intra-protein interactions and a possible conformational change in the protein. The latter is supported by the observation of (Loh *et al.*, 1996), that the phosphorylated form of NFAT binds DNA less efficiently than the de-phosphorylated. The mechanism of NFAT-activation by calcineurin, the formation of the transitionally active complex and the conformational change in NFAT is represented in Figure 1.13. In addition to being blocked upstream by inhibitors of calcineurin, NFAT activity is also regulated by re-phosphorylation and nuclear export mediated by protein kinases such as p38, mitogen activated protein kinase (p38-MAPK) and glycogen synthase kinase 3 (GSK3) (Beals *et al.*, 1997b).

### 1.4.3 THE GATA FAMILY

The GATA family of zinc-finger transcription factors are named after their consensus DNA binding site, (A/T)GATA(A/G) (reviewed in Patient and McGhee, 2002). There are six family members, which have often been grouped as two separate sub-families. GATA 1, 2 and 3 are predominantly associated with the haematopoietic system, and regulate the expression of T-lymphocytes, erythroid cells and megakaryocytes (reviewed in Orkin, 1995). GATA 4, 5 and 6 are associated with various mesoderm- and endoderm-derived tissues, and with development and tissue-specific expression. GATA-4 and GATA-6 are co-factors with NFAT family members in cardiac hypertrophy (Charron *et al.*, 2001; Liang *et al.*, 2001). Recently, GATA-2 has also been associated with hypertrophy in skeletal muscle (Musaro *et al.*, 1999; Paul and Rosenthal, 2002). This would suggest that there is some degree of overlap between the two GATA sub-families, at least with regard to muscle function. The known functions of the different GATA factors are summarised in table 1.7.

Family member	Role	References
GATA1	Erythropoiesis	(Mead <i>et al.</i> , 2001)
GATA2	Platelet development	(Shivdasani <i>et al.</i> , 1997)
	Erythropoiesis	(Kumano <i>et al.</i> , 2001)
GATA3	Skeletal muscle hypertrophy	(Musaro <i>et al.</i> , 1999; Paul and Rosenthal, 2002)
	T helper cell differentiation	(Mullen <i>et al.</i> , 2001)
GATA4	Cytokine production	(Kimura <i>et al.</i> , 2001)
	TGF- $\beta$ signalling	(Blokzijl <i>et al.</i> , 2002)
	Ectoderm development	(Van Esch <i>et al.</i> , 2000)
GATA5	Cardiac hypertrophy	(Charron <i>et al.</i> , 2001; Liang <i>et al.</i> , 2001; Yanazume <i>et al.</i> , 2002)
	Endoderm development	(Fujikura <i>et al.</i> , 2002)
GATA6	Cardiogenesis	(Reiter <i>et al.</i> , 2001)
	Endoderm development	(Stainier, 2002)
GATA6	Cardiac hypertrophy	(Liang <i>et al.</i> , 2001)
	Smooth muscle transcription	(Wada <i>et al.</i> , 2002)
	Endoderm development	(Fujikura <i>et al.</i> , 2002)

**Table 1.6.** Multiple, possibly overlapping functions of GATA family transcription factors.

The mechanisms by which GATA proteins are activated are not as well studied as NFAT. However it is apparent that GATA is also controlled to some extent by compartmentalisation, being cytoplasmic in its resting state and translocated to the nucleus when activated (Briegel *et al.*, 1996). As with NFAT, the activity of GATA family members is regulated by phosphorylation, as shown by (Morisco *et al.*, 2001) in a study demonstrating re-phosphorylation of GATA4 by GSK3 $\beta$  in cardiac myocytes.

#### 1.4.4 ROLES FOR CALCINEURIN/NFAT SIGNALLING IN MUSCLE

##### 1.4.4.1 Muscle Growth and Development

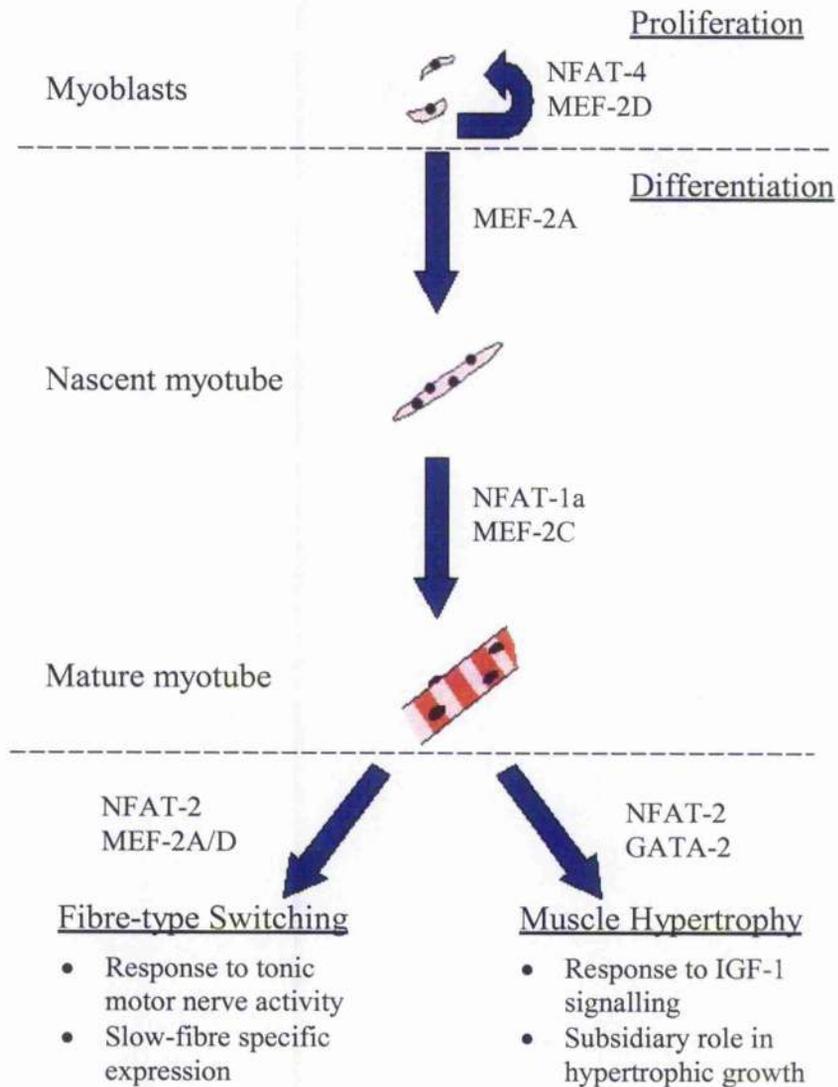
The calcineurin signalling pathway has been identified as playing a major role in the differentiation of myoblasts in a number of studies, the first of these by (Abbott *et al.*, 1998) using cyclosporin A (CsA). In cultured human myoblasts, CsA inhibited differentiation, while permitting normal proliferation. In mouse regenerating muscle, CsA administration blocked the process of fibre repair from satellite cells. The conclusion was that calcineurin was required for myoblast differentiation, and this was supported by further studies in cells transfected with a constitutively active calcineurin

(Delling *et al.*, 2000; Friday *et al.*, 2000). Friday *et al.* (2000) also demonstrated inhibition of differentiation through the physiological inhibitor of calcineurin, Cain.

The role of NFAT in the process of muscle development is more complex. The study by Abbot *et al.* (1998) identified three NFAT isoforms active in muscle, NFAT-1, -2 and -4. The study showed no evidence of NFAT-3 activity in skeletal muscle, and at the time of writing, none has been reported since. The three isoforms observed were found to be activated and nuclear localised in a stage-specific manner at different points of muscle development. NFAT-4 was found to be activated only in myoblasts, NFAT-1 in nascent, immature myotubes and NFAT-2 only in mature myotubes. Since then, (Sakuma *et al.*, 2003) have also found evidence of NFAT-2 activity in differentiating satellite cells in regenerating muscle, in conjunction with GATA-2. The role of NFAT-2 in mature myotubes is discussed below (see Section 1.4.4.3), while activity of the other NFAT isoforms in muscle growth and regeneration is discussed in detail here. A summary of the stage-specific involvement of NFAT isoforms in muscle is shown in Figure 1.14.

NFAT1 has been shown to be a requirement for myotube growth (Horsley *et al.*, 2001). In *nfat1*<sup>-/-</sup> mice, muscles develop normally but with a greatly reduced cross-sectional area and myonuclear number. Such mice also show reduced myotube formation during recovery from local freeze damage. Both of these defects were rescued by the retroviral expression of NFAT1 in *nfat1*<sup>-/-</sup> myoblasts *in vitro*. Since myonuclear number and fibre growth is dependent on the addition and fusion of myoblasts to the growing myotubes, the implication is that NFAT1 mediates myoblast fusion.

Sakuma *et al.* (2003) found increased levels of NFAT-2 protein in regenerating, adult rat and mouse muscle following tissue damage by injection with bupivacaine. This was accompanied by an increase both in co-precipitating calcineurin and NFAT-2, and in co-precipitating NFAT-2 and GATA-2, in the regenerating tissue. CsA was found to inhibit the regeneration of bupivacaine-damaged muscle, further supporting a role for calcineurin in the process. This study is interesting for the relatively short time scale at which NFAT-2 and GATA-2 co-precipitate following bupivacaine-induced damage (8



**Figure 1.14.** A summary of the different myogenic processes mediated by calcineurin (from Schulz and Yutzey, 2004). Acting through different isoforms of NFAT and MEF-2 (see also Section 1.3.4), calcineurin plays different roles at different stages of myotube formation and maturation.

to 12 hours), which would suggest a role for NFAT-2 far earlier than suggested in the previous study by Abbot *et al* (1998).

The role of NFAT-4 in myoblasts is even less clear. Delling *et al.* (2000) proposed a role for NFAT-4 in differentiation, after their finding that NFAT-4 co-transfection greatly enhanced MyoD-directed myogenesis. However, Friday *et al.* (2000) found that specific inhibitors of NFAT failed to abolish myoblast differentiation in the same manner as calcineurin inhibition, and suggest that the calcineurin-mediated control of myoblast differentiation is via different downstream intermediates. Further work by Friday *et al.* (2003) indeed pointed to MEF-2 as a likely candidate, and the indirect activation of MyoD through the inhibition of Egr-1, a transcriptional activator of Id proteins. NFAT-4 was instead found to be involved in the upregulation of myf-5 by activated calcineurin (Friday and Pavlath, 2001). As discussed Section 1.2.2.6, myf-5 is a marker of proliferating satellite cells, which points to NFAT-4 directly antagonising differentiation, by maintaining myoblasts in a proliferative state.

This would point to calcineurin signalling playing a role in two pathways, proliferation and differentiation, generally held to be mutually exclusive. Such an arrangement could allow a single signal of calcium influx in response, for example, to muscle injury to simultaneously generate a rapidly proliferating pool of satellite cells and use cells from this pool to differentiate and fuse with the injured/hypertrophying muscle. The pathways that modulate this generic signal into the specific outcomes of proliferation or differentiation are, at the time of writing, largely unknown. One possible upstream regulator, already mentioned in Section 1.2.2.6, is the asymmetric accumulation of Numb, antagonist of the Notch-1 receptor in the daughter cells of proliferating satellite myoblasts destined for terminal differentiation. In haematopoietic cells, Notch-1 over-expression was found to sustain the expression of GATA-2 (Kumano *et al.*, 2001). It may be that by upregulating GATA-2, Notch-1 enhances the action of NFAT-4 in promoting the proliferation pathway. To summarise, calcineurin has been shown to regulate a number of processes in the proliferation of myocytes and their differentiation and maturation into muscle fibres (Schulz and Yutzey, 2004). While calcineurin is common to all of these processes, different downstream mediators are involved in each. While NFAT-4 is involved in proliferation and NFAT-1a in fibre maturation (Figure 1.14), the initial stages of differentiation are calcineurin dependent, but NFAT-

independent. The role of NFAT-2 in muscle development and regeneration is presently unclear, and NFAT-2 is better studied in fibre type switching and in contributing to muscle hypertrophy.

#### **1.4.4.2 Calcineurin and NFAT in Muscle Hypertrophy**

The calcineurin/NFAT signalling pathway has long been associated with cardiomyocyte hypertrophy. As with skeletal muscle hypertrophy, this is an adaptive response to a variety of stimuli that impose increased biomechanical stress (reviewed in Frey and Olson, 2003). However, if such stimuli are prolonged, cardiac hypertrophy can lead to heart failure. Administration of CsA has been shown to block cardiac hypertrophy, and the expression of a constitutively active form of calcineurin in heart muscle induces a massive hypertrophic response (Molkentin *et al.*, 1998). GATA factors are also involved, with GATA-4 having been shown to induce cardiac hypertrophy (Liang *et al.*, 2001).

Naturally, the discovery of this role of calcineurin signalling in cardiac muscle hypertrophy leads to the hypothesis that a similar pathway might be involved in skeletal muscle hypertrophy, such as that mediated by IGF-1. Indeed several groups have demonstrated the inhibition of skeletal muscle hypertrophy through the administration of CsA, pointing to a key role of calcineurin in this process (Dunn *et al.*, 1999; Musaro *et al.*, 1999; Semsarian *et al.*, 1999b; Mitchell *et al.*, 2002). The case for calcineurin in muscle hypertrophy was furthered by the observation of Musaro *et al.* (1999), of nuclear translocation of NFAT-2 during hypertrophy. It should also be noted that GATA-2, the GATA family member found in skeletal muscle, is also expressed in muscles undergoing hypertrophy (Musaro *et al.*, 2001). GATA-2 has previously been reported as being expressed at no other time in adult muscle (Paul and Rosenthal, 2002).

However, as already stated, proposed roles for calcineurin/NFAT in skeletal muscle, and skeletal muscle hypertrophy in particular, have proven to be controversial. Other groups found no inhibition of hypertrophy by administering CsA *in vivo*, despite delivering a sufficient dose to inhibit other markers of calcineurin activity (Serrano *et al.*, 2001; Dupont-Versteegden *et al.*, 2002), and indeed Rommel *et al.* (2001) demonstrated inactivation of NFAT in response to the hypertrophic trigger of IGF-1.

Some of these differences may be attributed to differences in experimental design, such as choice of target muscle. For instance, positive evidence for a role for calcineurin/NFAT in muscle hypertrophy discovered by Mitchell *et al.* (2002) was more evident in fast-twitch muscle than slow muscle, such as the *soleus*. The work of Dupont-Versteegden and others (2002) was done in the *soleus* muscle alone, and may have missed the effect observed by other groups.

Other lines of evidence, however, are harder to discount. For instance, it has been shown that transgenic animals expressing a constitutively active form of calcineurin do not exhibit elevated skeletal muscle hypertrophy, despite an increase in calcineurin phosphatase activity detected in the transgenic muscles (Dunn *et al.*, 2000; Naya *et al.*, 2000). This is in marked contrast to the evidence supporting the involvement of calcineurin cardiomyocyte hypertrophy. Secondly, calcineurin activity is not increased in muscles undergoing IGF-1 induced hypertrophy, as might be expected if it were to play a key role (Bodine, 2001).

It is clear then that the role of calcineurin in skeletal muscle hypertrophy is not as clear-cut as either its proponents or detractors would maintain. This is similar to the situation in cardiac hypertrophy, a condition caused by a number of different factors (such as hypertension, cardiac arrhythmias, endocrine disorders and genetic mutation in cardiac contractile proteins), some of which have been shown to occur independently of calcineurin signalling (Zhang *et al.*, 1999). But perhaps a more satisfactory explanation would be that calcineurin/NFAT/GATA pathways are involved in a subsidiary role of hypertrophy, such as the proliferation and differentiation of satellite cells discussed above which could contribute towards a hypertrophic change.

#### **1.4.4.3 Fast to Slow Fibre Switching**

A role for calcineurin signalling in the switch from fast to slow fibre phenotype was first suggested by (Chin *et al.*, 1998), in a further investigation into the well-known association between motor neuron activity and fibre type specification. Chin *et al.* (1998) observed that CsA administration in whole animals resulted in a slow to fast fibre transition. Chin *et al.* also observed a difference between fast- and slow-fibre specific genes in their responsiveness to constitutively active calcineurin. Reporter constructs based on the promoter regions of the slow specific genes, troponin 1 slow

(Tn1s) and myoglobin, showed elevated expression. This was found to be dependent on a combination of NFAT and MEF-2 upstream response elements. Similar constructs for muscle creatine kinase (MCK), a gene predominantly found in fast fibres, showed no similar response to active calcineurin. These initial findings have since been supported by further studies demonstrating a calcineurin-mediated activation of slow-fibre genes, as summarised in Table 1.7. Furthermore, the slow upstream regulatory element (SURE) of the Tn1s gene, which contains the MEF-2 and NFAT binding sites, has also been found in another fibre-type specific gene, the slow splice-variant of collagenous protein (ColQ) (Lee *et al.*, 2004). The response of the *ColQ* SURE element was found to be inhibited by CsA.

Experimental Model	Effect on fibre phenotype	Reference
Calcineurin over-expression in C2C12 culture	Increased 1/β MyHC in differentiated myotubes.	(Delling <i>et al.</i> , 2000)
Transgenic mice expressing constitutively active calcineurin	Increased number of slow-staining fibres.	(Naya <i>et al.</i> , 2000)
CsA-treated rats	Decreased percentage of slow fibres in <i>soleus</i> muscle.	(Bigard <i>et al.</i> , 2000)
Calcineurin A and B gene-targeted mice	Reduction in slow fibres	(Parsons <i>et al.</i> , 2003)

**Table 1.7.** Studies supporting the role of calcineurin signalling in the acquisition of the slow fibre phenotype.

However, as with other proposed roles for the calcineurin/NFAT pathway in skeletal muscle, a role in slow-fibre switching has also been the subject of some debate. Swoap *et al.* (2000), in studies using activated calcineurin and NFAT, found no specific upregulation of slow-gene reporter constructs, and no upregulation at all in transfected muscle in whole animals. However, this study was reliant on direct injection of plasmid DNA into the whole muscle, and a co-transfection of both reporter construct and activated calcineurin, so this anomaly may be explained by poor or variable efficiency of DNA uptake using this method (Mitchell-Felton and Kandarian, 1999).

Even in studies supporting the role of calcineurin in the slow fibre phenotype, there seems doubt as to the role of NFAT. Parsons *et al.* (2003) argued that an NFAT-independent pathway, though still involving MEF-2, is involved. Supporting their argument was the finding by Delling *et al.* (2000) that NFAT over-expression in

cultured cells did not stimulate the slow programme, in contrast to calcineurin over-expression. However, the study by Delling *et al.* (2000) was focussed on NFAT-4 in immature myocytes, and in no way ruled out a role for NFAT-1 and -2 in mature myotubes. Even the study by Parsons *et al.* (2003) demonstrated that greater NFAT activity was found in slow fibres than fast fibres, all of which points to a role for calcineurin signalling via NFAT.

Harder to account for is the observation by Calvo *et al.* (1999), in studies on transgenic mice. They demonstrated, in direct contradiction to the work by Chin *et al.* (1998), that an NFAT binding site in the SURE region of Tn1s was not required for fibre-type specific expression. Rather, a different transcription factor, muscle TFII-I repeat domain-containing protein 1/general transcription factor 3 (MusTRD1/GTF3) was found to be required for Tn1s activation. One explanation proposed by (Schiaffino and Serrano, 2002) was that MusTRD1/GTF3 is required for nerve-independent activation of the Tn1s gene in the embryo, while NFAT is required in the mature muscle-fibre.

The activity of NFAT-2 in fibre switching has been best demonstrated in studies clearly showing NFAT-2 nuclear translocation during fast to slow fibre-switching (Liu *et al.*, 2001; Kubis *et al.*, 2002). Liu *et al.* (2001) created a transgenic adult muscle-fibre culture with an adenovirus-delivered GFP-labelled NFAT2 construct. During patterns of electrical stimulation specific to slow-muscle (continuously at 10 Hz or 5 second trains at 10 Hz every 50 seconds), the GFP signal was found to be localised in the nucleus. This nuclear transport was found to be abolished by treatment with CsA, and was absent in electrical stimulation patterns consistent with fast motor neuron firing (0.1 second trains at 50 Hz every 50 seconds) or continuous 1 Hz stimulation. One possible mechanism for the differences observed in NFAT activity is the rapid nuclear export of NFAT. This has been put forward as a mechanism by which the cell can distinguish between different types of calcium signalling (Klemm *et al.*, 1997). Depending on the stimulus, changes in the levels of intracellular calcium can occur as sustained elevation, transient spikes or oscillations (Berridge, 1993). Nuclear localisation of NFAT requires a sustained calcium elevation, while a brief 'spike' results in only temporary nuclear translocation (Limmerman *et al.*, 1996).

To summarise, there is a large body of evidence suggesting a role for calcineurin/NFAT in the control of fibre type, specifically in the promotion of the slow fibre phenotype in response to physiological signalling. This has been demonstrated through the inhibition of the calcineurin/NFAT pathway and through direct observation of labelled NFAT during the switch to the slow fibre type.

## 1.5 SUMMARY

The manner by which physiological signals, such as patterns of motor neuron activity or hormone signalling, can induce a change in muscle phenotype is well studied. It is less clear, however, how such external stimuli are converted into the specific patterns of gene expression required for the associated adaptive response. The possible signalling pathways have been the subject of numerous recent studies, as reviewed in this chapter.

It is clear that the signal transduction from external stimulus to gene involves several inter-linking pathways. Many of the individual pathways involved play a part in a range of different or even mutually exclusive muscle responses. A common theme that emerges is that it is the interactions between the the different signal transduction pathways that generate specific outcomes from generalised signals. For example, the single event of calcineurin activation and NFAT dephosphorylation can induce the muscle to proliferate, differentiate or activate the slow fibre phenotype, depending on which downstream NFAT isoform is involved (see Figure 1.14).

While the role of the calcineurin/NFAT pathway in muscle has been under considerable, recent debate, several aspects remain relatively unknown at the time of writing. In particular, the transcriptional targets of effectors downstream from calcineurin remain largely uncharacterised. NFAT-binding sites have been identified in some isoform-specific regulatory regions, such as the SURE regions (Chin *et al.*, 1998; Lee *et al.*, 2004), but not in other isoform markers such as the MyHC family. The long-term goal of the work described in this thesis was to investigate this area further, and determine what direct effects the calcineurin pathway and associated transcription factors might have in the regulation of stage- and isoform-specific gene expression.

## 1.6 PROJECT AIMS

The aim of the project work described herein was to focus on two of the downstream effectors of calcineurin, the NFAT and GATA families of transcription factors. The pig was chosen as the target species for this investigation given its importance both as a large animal model for muscle development and as a commercially important meat animal. The NFAT and GATA family members important in porcine muscle were to be cloned, in order to determine their effect on fibre-type specific gene expression. This work formed part of a wider collaboration between the Molecular Medicine Laboratory and pig-breeding company, Sygen International, intended to generate an underpinning knowledge of muscle development.

These overall aims were broken down into a number of more immediate objectives, summarised as follows:

1. To isolate cDNA sequences from NFAT- and GATA-family transcription factors present in porcine skeletal muscle, through PCR homology cloning.
2. To use the PCR fragments obtained from such an approach to screen for full-length cDNA sequences, and to then fully characterise and sequence any novel clones isolated.
3. To identify patterns of expression through quantitative real-time RT PCR both across different tissue types and stages of pre-natal development.
4. To conduct *in vitro* studies into the downstream targets of GATA and NFAT in skeletal muscle.

This final goal was, in the first instance, to be accomplished by over-expression of the newly isolated muscle GATA or NFAT in primary myoblast culture, using a real-time RT-PCR approach to quantify any resulting changes in the expression of different MyHC isoforms. The following chapters will describe in further detail the approaches used to meet these aims, and the results obtained.

## **CHAPTER TWO - GENERAL MATERIALS AND METHODS**

## **2.1 INTRODUCTION**

The general methods in use throughout the project are detailed in this chapter. More specialised protocols are listed in the relevant methods section. For the most part, the general methods described in this chapter were derived from standard methods that could be found in common laboratory manuals (Maniatis *et al.*, 1989). Where commercial kits were used, the manufacturer's protocol is presented in summary though it should be noted that some of the reagents and buffers are proprietary and their constituents cannot be disclosed.

## **2.2 METHODS**

### **2.2.1 RECOMBINANT DNA TECHNIQUES**

#### **2.2.1.1 Growth of Bacteria**

Plasmids were maintained in *Escherichia coli* commercially available, competent strains supplied by Stratagene. Specifically, XI.2-blue MRF<sup>+</sup> strains were used for most plasmids, with the XL10-Gold ultracompetent cells used for cloning strategies where the transformation efficiency was expected to be low. Some PCR products, cloned into the Topo-TA cloning system (Invitrogen), were maintained in Invitrogen's 'One-shot' DH5- $\alpha$  cells.

Cells were cultured on LB-agar supplemented with the appropriate antibiotics to maintain the selection of plasmid-containing cells (100  $\mu$ g/ml ampicillin, 12.5  $\mu$ g/ml chloramphenicol, 50  $\mu$ g/ml kanamycin or 15  $\mu$ g/ml tetracycline).

#### **2.2.1.2 Extraction and Purification of Plasmid DNA**

Isolation of plasmid DNA was carried out in all instances by a modified version of the alkaline lysis technique (Birnboim and Doly, 1979).

#### 2.2.1.2.1 Small Scale Plasmid Preparations

Cells picked from single colonies were grown in 5 ml LB, overnight at 37 °C in an orbital shaker. For each overnight culture, 1.5 ml of cells was spun down in a microcentrifuge tube, 10,000 × g for 1 min and the supernatant discarded. A further 1.5 ml from the same culture was added to the pellet and the centrifugation step repeated, for a total of 3 ml of culture processed per overnight culture. The final pellet was spun again briefly, and as much of the remaining LB medium removed as possible, using a pipette.

Plasmid DNA minipreps were prepared using the Spin Miniprep Kit (Qiagen). The manufacturer's protocol was used, re-stated briefly as follows: Each cell pellet was thoroughly resuspended, by pipetting, in 250 µl Buffer P1. Cell suspensions were lysed by the addition of 250 µl Buffer P2, mixed by inversion. Protein was precipitating out of solution with the further addition of 350 µl Buffer N3, the solution mixed thoroughly to prevent localised precipitation. The resulting precipitate was centrifuged for 10 min at 10,000 × g, and the supernatant from each tube applied to a Qiaprep spin columns. DNA was bound to the column by spinning down at 10,000 × g for 1 min, and discarding the flow through. To remove trace endonuclease activity, each column was washed with 500 µl Buffer PB, and the centrifugation step repeated. Columns were further washed by adding 750 µl Buffer PE and spinning the columns down again. To remove residual ethanol from this wash step, the centrifugation step was repeated a final time and the columns transferred to a clean 1.5 ml microfuge tube. The plasmid DNA was then eluted from the column by the addition of 50 µl of 10 mM Tris-Cl, pH 8.0. The columns were left to stand for 1 min, before centrifuging 1 min, 10,000 × g. Plasmid DNA was stored at -20 °C until required.

#### 2.2.1.2.2 Large Scale Plasmid Preparations

Cells picked from overnight colonies were grown in 5 ml LB at 37 °C, overnight, in an orbital shaker. From this overnight culture, 2 ml was used to inoculate 400 ml LB medium, supplemented with the relevant antibiotics, in a 1 L conical flask, cultured overnight at 37 °C, shaking at 200 rpm. At this level of aeration, the cells were approximately mid-log phase when harvested the following morning. Cells were pelleted by centrifugation, 6,000 × g for 15 min at 4 °C.

Cell pellets were processed using the Qiagen Maxiprep kit. The Qiagen protocol is summarised as follows: First the cell pellets were resuspended in 10 ml buffer P1, resuspending by pipetting until no cell clumps were visible. The cells were then lysed with the addition of 10 ml buffer P2, mixing vigorously by inversion. Ten ml of pre-chilled buffer P3 was added and the resulting suspension spun down at  $20,400 \times g$  for 40 min at 4 °C. During the spin a Qiagen maxiprep column was prepared, equilibrated with 10 ml buffer QBT. The supernatant from the centrifugation step was applied to the column, poured through a 0.2 µm cell strainer (Falcon) to remove residual particulate matter. The columns were washed through twice with 30 ml buffer QC, the flow-through discarded.

Plasmid DNA was eluted with 15 ml buffer QF, into a clear, polycarbonate, 30 ml centrifuge tube. DNA was precipitated with the addition of 10.5 ml isopropanol, mixed by inversion and centrifuged at  $20,400 \times g$  for 30 min at 4 °C, carefully pipetting off the supernatant. The pellet was then carefully washed with 5 ml of 70% ethanol, pre-chilled to -20 °C, and spun again at  $20,400 \times g$  for 15 min, 4 °C. The supernatant was pipetted off, and the pellet air-dried. DNA was resuspended in 200 µl 10 mM Tris HCl, pH 8.0 and stored at -20 °C.

#### 2.2.1.2.3 Determination of DNA Concentration by Spectrophotometry

The concentration of DNA preparations, principally from large scale plasmid preparations, was determined spectrophotometrically with optical density measurements at 260 and 280 nm against a distilled-water blank. Plasmid samples were diluted 1:200 in Millipore-pure water, total volume 800 µl, and transferred to a quartz, optical cuvette. An  $OD_{260}$  measurement of 1.0 was taken to indicate a double-stranded, plasmid DNA concentration of 10 µg/µl. The ratio of OD readings at 260:280 nm was used to estimate the purity of the DNA preparation, with a ratio approaching 2.0 being the ideal. Lower ratios were indicative of protein contamination.

#### **2.2.1.3 Restriction Endonuclease Digestion**

For most restriction digests, between 1 and 2 µg of DNA was digested in 20 µl of reaction mix containing 5 to 10 units of appropriate restriction enzyme (Invitrogen,

Promega) and the required enzyme buffer. The reactions were incubated for a minimum of two hours.

#### **2.2.1.4 DNA Electrophoresis**

##### 2.2.1.4.1 Agarose Gel Electrophoresis

Size fractionation of DNA samples was typically carried out in 0.8% agarose gels, made up in TAE buffer (40 mM Tris acetate, 1 mM EDTA). The agarose was melted in a microwave, mixed thoroughly and allowed to cool to around 55 °C. Ethidium bromide was then added to a concentration of 75 µg/ml, and the whole gel mix poured into the casting plate and a gel comb appropriate to the volume of sample added. Once the gel had been allowed to set the casting comb was carefully removed and the solidified gel immersed in TAE buffer. Gel surplus to the requirements of the run was pared away and stored at 4 °C until needed.

DNA samples were prepared for loading by adding the appropriate volume of 10 × gel loading buffer, together with a molecular size standard DNA, typically a 1 kb ladder (Invitrogen). After loading, gels were run for 60 to 120 minutes at 70 volts, and visualised on a UV transilluminator.

##### 2.2.1.4.2 Excision of DNA Bands

Where a particular DNA fragment was required for a particular cloning strategy, the relevant restriction digest was performed, and size fractionated on an agarose gel. The agarose containing the desired band was visualised on a UV transilluminator and quickly excised from the gel using a scalpel, keeping excess agarose to a minimum.

DNA was recovered from the gel slice using the QIAquick Gel Extraction Kit provided by Qiagen. The kit protocol was followed, as summarised: Each gel slice was transferred to a 1.5 ml microcentrifuge tube and weighed. For each gel slice, 3 µl Buffer QG was added for every mg of sample (3 gel volumes). Samples were then heated for 10 min in a 50 °C waterbath. Once the gel slice was completely dissolved, one gel volume of absolute isopropanol was added, and the mixture transferred to a Qiaquick spin column/collection tube assembly. Column assemblies were spun at 10,000 × g in a Beckman microcentrifuge for 1 min. Flow throw was discarded, and

750  $\mu$ l of PE wash buffer added to the column. The columns were spun at 10,000  $\times$  g for another 1 min, and the flow through discarded. To remove all traces of ethanol present in Buffer PE, the centrifugation step was repeated, and the columns transferred to clean, 1.5 ml microcentrifuge tubes. To elute the DNA from the column 30  $\mu$ l 10 mM Tris HCl, pH 8.5, was added and the column left to stand for 1 min, before being spun again at 10,000  $\times$  g for 1 min. The extracted DNA was either used immediately or stored at 20  $^{\circ}$ C

### **2.2.1.5 DNA Ligation**

T4 DNA ligase (Promega) was used for most of the cloning strategies. The multiple cloning site of the relevant plasmid vector was cut with the appropriate restriction enzymes (2.2.1.3) and treated with calf alkaline phosphatase (Roche) for 1 hour at 37  $^{\circ}$ C. Between 50 to 100 ng of vector DNA was mixed with the target insert, at a molar ratio of 1:5, together with an appropriate volume of ligation buffer and 4.5 units of T4 DNA ligase (Promega), in a final volume of between 10 to 20  $\mu$ l. Reactions were left overnight at 14  $^{\circ}$ C, before proceeding to the transformation step, described below (2.2.1.6).

### **2.2.1.6 Transformation of Bacteria With Plasmid DNA**

#### 2.2.1.6.1 Transformation of Commercially Available Ultracompetent Cells

As previously stated, most transformations utilised the XL2-Blue MRF' and XL10-Gold ultracompetent cells supplied by Stratagene. Plasmid DNA transformation of these cells was carried out according to the supplier's guidelines, summarised as follows: Competent cells were thawed on ice, and  $\beta$ -mercaptoethanol added to a final concentration of 25 mM, mixed gently and incubated for 10 min on ice. Plasmid DNA was then added to the cells, at a concentration of between 0.1 to 50 ng of DNA per 100  $\mu$ l of competent cells. The mixtures were incubated on ice for 30 min, then the cells heat-shocked for 30 s at 42  $^{\circ}$ C. Bacteria were put back on ice for 2 min, before diluting ten-fold in pre-heated (42  $^{\circ}$ C) NZY broth, and incubated in an orbital shaker for 1 hr at 37  $^{\circ}$ C. Following the incubation, 200  $\mu$ l of the transformation reaction was plated on LB agar plates containing appropriate antibiotics.

#### 2.2.1.6.2 Topo-TA cloning

The Topo-TA system was a specialised cloning kit (Invitrogen) specifically designed for the cloning of PCR products (section 2.2.2), taking advantage of non-template terminal transferase activity of Taq polymerase that leaves a single deoxyadenosine residue overhang on PCR products. The linearised vector in the kit possesses a single deoxythymidine residue overhang, allowing any PCR product to be ligated into the vector.

As per protocol, 4  $\mu$ l PCR product was added to 1  $\mu$ l Topo-TA vector, mixed gently and left for 5 min. This was added to an aliquot of One Shot cells, mixed gently and incubated on ice for 30 min. The cells were heat-shocked for 30 s at 42 °C, put back on ice for 2 min and 250  $\mu$ l room-temperature SOC medium added. Cells were incubated in an orbital shaker for 30 min at 37 °C. At the end of the incubation, 50  $\mu$ l was plated out onto LB agar supplemented with ampicillin, and incubated overnight at 37 °C.

### **2.2.2 POLYMERASE CHAIN REACTION**

The polymerase chain reaction (PCR), a means of amplifying small amounts of specific DNA template from a complex mixture is reviewed extensively by (Innis and Gelfand, 1990). A general overview of how this method was applied to DNA amplification throughout the project is given below. Where appropriate, the details of more specific PCR experiments will be given in the relevant chapter.

#### **2.2.2.1 Primer Design**

Custom oligonucleotide synthesis was by MWG Biotech. Most of the oligonucleotide primers were between 20 to 30 deoxynucleotide residues in length, with a base composition of roughly 50 to 60 % guanine or cytosine where possible. Primer pairs with complementary sequences were avoided, and both primers designed so as to have matching annealing temperatures ( $T_m$ ).

#### **2.2.2.2 Reaction Conditions**

PCR reagents were supplied by Promega. Amplification reactions were typically set up in a reaction mix of the following: 200  $\mu$ M of dNTP, PCR buffer (50mM KCl, 10mM Tris-HCl, pH 9.0, 0.1% Triton<sup>®</sup> X-100 and 1.5mM MgCl<sub>2</sub>) and 2.5 units of *Taq* DNA

polymerase. The appropriate amount of DNA or cDNA template varied between PCR reactions, but was generally between 10 to 100 ng. Thermal cycling was carried out on an Applied Biosystems GeneAmp 2400. For standard PCR, the template was denatured for 3 min at 95 °C, followed by 30 to 35 cycles of a 1 min, 95 °C denaturation step, 1 min at 55 to 60 °C annealing (the annealing step being greatly dependent on the primer sets used) and an extension step at 72 °C. This was followed by a final 5 min extension step, before holding at 4 °C. PCR amplification products were stored at -20 °C if not used immediately.

Amplification products were visualised by agarose gel electrophoresis, as described in section 2.2.1.4.1. Up to 10 µl of the PCR reaction was loaded onto each well, more if the product was to be used in TopoTA cloning.

### **2.2.3 DNA SEQUENCE ANALYSIS**

The majority of clone and plasmid sequence data was obtained using the ABI sequencing kits (Applied Biosystems), based on the chain termination method (Sanger *et al.*, 1977) with cycle sequencing modifications described by Innis *et al.* (1988). Early in the project, the LI-COR system was used for emMyHC regulatory region sequencing. This is covered in the specific methods section (3.2.5.1). Two versions of the ABI thermal cycle sequencing system were used in the course of the project work, Versions 1.0 and 3.0 respectively, with the latter being an improved version available later in the project.

#### **2.2.3.1 Primer Design**

As for PCR, custom oligonucleotides for cycle sequencing were supplied by MWG biotech. Sequencing primers were typically 20 to 25 oligonucleotides in length, with at least 50% G/C content, roughly 50 bp upstream (5') of the region to be sequenced. A last (3') base of either adenosine or thymidine was avoided, with a 3' stretch of four or five guanidine or cytosine residues preferred.

#### **2.2.3.2 Preparation of DNA samples for Cycle Sequencing**

Plasmid DNA was used as the template for all of the sequencing done in the course of the project work, whether prepared by small (2.2.1.2.1) or large scale (2.2.1.2.2)

preparations. Manufacturer's protocols (Applied Biosystems) were followed in both versions of ABI cycle sequencing. They are summarised as follows:

#### 2.2.3.2.1 Version 1.0 Chemistry

The cycle-sequencing reaction mixture consisted of ABI PRISM Big Dye reagent mixture (Applied Biosystems) and appropriate buffer, together with 200 to 500 ng of DNA template and 3.2 pmol sequencing primer, made up to 20  $\mu$ l with Millipore-pure water. Cycle sequencing was carried out on an Applied Biosystems GeneAmp 2400, using the following program: one denaturing step at 96 °C for 2 min, followed by 25 cycles of 10 s denaturation at 96 °C, 5 s annealing at 50 °C and 4 min extension at 60 °C, with a final hold at 4 °C. Reaction products were kept at -20 °C if not immediately processed for loading.

The reaction mixture was purified by ethanol precipitation to remove unincorporated dye-labelled nucleotides. The samples were transferred to 1.5 ml microcentrifuge tubes, and ethanol added to a final concentration of 96%. The tubes were vortex mixed briefly and then left to stand at room temperature for fifteen minutes. The tubes were spun at 10,000  $\times$  g in a Beckman microcentrifuge for twenty minutes at room temperature. After the centrifugation step, the supernatant was immediately aspirated and the DNA pellet washed with 250  $\mu$ l 70% ethanol. Samples were spun again at 10,000  $\times$  g for ten minutes, and the supernatant carefully aspirated as before. Samples were then dried by placing them in a heat block at 90 °C for one minute.

The dried samples were resuspended in 25  $\mu$ l Template Suppression Reagent (TSR), mixed by vortexing and briefly spun down in the Beckman microcentrifuge. The samples were then denatured by heating in a heat block at 95 °C for 2½ minutes, and then chilled on ice. The samples were vortexed again, briefly spun down and transferred to ABI sample vials. The samples were stored at -20 °C until ready to load.

Visualisation of the cycle sequencing products was carried out on an ABI Prism 310 Genetic Analyser (Applied Biosystems). Loading was largely automated and the set-up carried out according to manufacturer's instructions.

#### 2.2.3.2.2 Version 3.0 Chemistry

The reaction mixture comprised of the version 3.0 Big Dye reagent mixture and appropriate buffer, together with 200 to 500 ng DNA template and 3.2 pmol sequencing primer as before. Cycle sequencing was carried out on an Applied Biosystems GeneAmp 2400, using the following programme: one denaturing step at 96 °C for 2 min, followed by 25 cycles of 10 s denaturation at 96 °C, 10 s annealing at 50 °C and 4 min extension at 60 °C, with a final hold at 4 °C. Reaction products were kept at -20 °C if not immediately processed for loading.

For the ABI version 3.0 chemistry, DNA purification was carried out by column filtration, in filter cartridges provided by EDGE Biosystems (Performa™ DTR gel filtration cartridges). Before loading the samples, each cartridge was spun at 750 × g in the microcentrifuge for two minutes, to remove the storage buffer. The cartridge was removed to a fresh tube (provided in the kit), and the sample applied to the gel matrix. The cartridge was spun for another two minutes at 750 × g, and the cartridge discarded. The samples were dried by vacuum desiccation. The dried samples were stored at -20 °C until loading, at which point they were resuspended in formamide.

Visualisation of the cycle sequencing products was carried out on an ABI Prism 3100 Genetic Analyser. As for the ABI Prism 310, loading was largely automated and the set-up carried out according to manufacturer's instructions.

#### **2.2.3.3 Data analysis**

Data analysis was done by GCG and Chromas software packages. Sequence pileups and comparisons were done using either Blast (NCBI), AliBee Multiple Alignment (GeneBee) or ALIGN (Pearson *et al.*, 1997) software.

### **2.2.4 SOUTHERN AND NORTHERN BLOT HYBRIDISATION**

#### **2.2.4.1 Preparation of Radiolabelled DNA Probes**

All legal requirements and local procedures governing the safe use of radioisotopes were adhered to in the course of the project work.

Typically, the radioisotopes used were Redivue preparations provided by Amersham Bioscience. These contain a red dye for ease of handling and a stabilising solution, allowing storage in liquid form, at 4 °C. Early in the project, these Redivue preparations were not available and the standard solutions (Amersham Bioscience) were used instead. Since these were stored at -20 °C it was necessary to thaw the pot containing the isotope for one hour prior to labelling.

#### 2.2.4.1.1 Random Hexamer Labelling

Large, double stranded templates were labelled by the random hexamer method of Feinberg and Vogelstein, (1983). Using random hexanucleotide sequences as primers, and denatured DNA as a template, a complementary strand incorporating the radiolabel, <sup>32</sup>P-dCTP, can be generated with the Klenow fragment of DNA polymerase I.

This random hexamer labelling was carried out using the Rediprime II DNA labelling kit (Amersham Pharmacia). The procedure is summarised as follows: DNA was diluted to a suitable concentration (between 2.5 to 25 ng) in 45 µl TE buffer. The sample was denatured by heating to 100 °C for 5 min in a heating block, snap cooled on ice for 5 min and spun down briefly. The DNA was added to a Rediprime-kit reaction tube containing a pre-aliquoted reaction mix of nucleotides (minus dCTP), random primers and exonuclease-free Klenow enzyme in the appropriate buffer. Finally, 5 µl of α<sup>32</sup>P-dCTP (50 µCi) was added and the tube mixed thoroughly, and the reaction was incubated for 10 to 30 min at 37 °C, and stopped by the addition of EDTA to a final concentration of 20 µM.

Unincorporated <sup>32</sup>P-labelled oligonucleotides were separated from the reaction mix by use of NICK columns (Pharmacia Biotech) containing Sephadex G-50 gel. The column was equilibrated with distilled water during the incubation, before applying the reaction mix. The column was washed once with 400 µl distilled water, and the radiolabelled probe eluted by the addition of a further 400 µl distilled water. A 2 µl aliquot was taken off for liquid scintillation counting. Probes were typically used at a concentration of 10<sup>6</sup> cpm/ml of hybridisation buffer. The remaining probe aliquot was either used directly or stored at -20 °C until required. Probes were denatured by boiling for 5 min before use.

#### 2.2.4.1.2 End Labelling of Oligonucleotides

Small oligonucleotide probes were made by the addition of a  $^{32}\text{P}$ -labelled phosphate group to the unphosphorylated 5' end found, by nature of their manufacture, on all oligonucleotides. The addition of the labelled phosphate group is mediated by T4 polynucleotide kinase.

The reaction mixture was made up with 0.8 pmol/ $\mu\text{l}$  oligonucleotide template, together with 20 units of T4 polynucleotide kinase, forward reaction buffer and 50  $\mu\text{Ci}$  of  $\gamma^{32}\text{P}$ -ATP, in a final reaction volume of 50  $\mu\text{l}$ . The reaction mixture was incubated at 37  $^{\circ}\text{C}$  for 1 hour.

Unincorporated labelled oligonucleotides were removed by passing the reaction mix through NAP-5 columns (Pharmacia Biotech) containing Sephadex G-25. These were equilibrated three times with distilled water before use. The 50  $\mu\text{l}$  reaction volume was mixed thoroughly and applied to the column. The column was washed with 450  $\mu\text{l}$  of distilled water, and the labelled probe eluted with a further 1 ml distilled water collected in a screw-cap vial. An aliquot of 2  $\mu\text{l}$  was removed for liquid scintillation counting, with the probe typically used at a concentration of  $10^6$  cpm/ml hybridisation buffer. End-labelled probes were heated to around 90  $^{\circ}\text{C}$  for 1 min, to remove any secondary structures.

#### **2.2.4.2 Southern Blot Hybridisation**

The Southern blot transfer method of detecting specific DNA sequences among fragments separated by gel electrophoresis was used as previously described (Southern, 1975). The protocol is summarised here, including the more general variations employed during the project work.

##### 2.2.4.2.1 Blotting

DNA samples were run out on agarose gel as described in section 2.2.1.4.1. The gel was briefly visualised on a UV transilluminator. The gel was then incubated in three buffers in turn: 15 min in depurination buffer (0.25 M HCl), 30 min in denaturation buffer (1.5 M NaCl, 10.5 M NaOH), and 15 min equilibrating in 0.4 M NaOH. The gel

was then 'blotted' to a Hybond-N nylon membrane (Amersham) overnight, wicking the 0.4 M NaOH solution up through Whatman filter paper, gel and filter by paper towels. After the incubation, the filter was briefly washed, DNA side up, in  $2 \times$  SSC and left to dry, before cross-linking the blotted DNA to the nylon filter by UV. Filters were stored in Saran wrap at room temperature until use.

#### 2.2.4.2.2 Hybridisation and Autoradiography

The filters were first pre-washed to remove any remaining pieces of agarose that might interfere with hybridisation in  $5 \times$  SSC, 0.5% SDS for 30 min. The wash buffer was then replaced with hybridisation solution. For routine applications, a commercially available buffer was used, the Quickhyb solution from Stratagene. Filters were incubated in Quickhyb at  $50^\circ\text{C}$  for 1 hr for pre-hybridisation before the addition of the radiolabelled probe. After the addition of probe, the hybridisation incubation was continued for 60 to 90 min, temperature dependant on the annealing temperature ( $T_m$ ) of the particular probe sequence.

For more sensitive applications requiring a lower level of background caused by non-specific probe-binding, a 'home-made' buffer was used. This comprised of 50% formamide,  $5 \times$  SSC,  $5 \times$  Denhardt's solution, 1 mM EDTA, 1% SDS, 50 mM sodium phosphate solution, pH 7.0, and 100 mg of sheared salmon sperm ssDNA. Pre-hybridisation using this buffer was carried out overnight, at  $42^\circ\text{C}$ , as was the subsequent hybridisation.

Following hybridisation, filters were subjected to a series of washes of increasing stringency. Stringency was increased by decreasing salt concentration and increasing temperature. Again, the wash temperature depended on the  $T_m$  of the probes involved, typically between  $50$  to  $65^\circ\text{C}$ . After the last wash, the filters were rinsed, placed in saran wrap, and exposed to autoradiography film (Amersham Pharmacia) in an intensifier cassette. The films were left at  $-70^\circ\text{C}$  for a period of typically 3 days to a week before developing.

#### 2.2.4.2.3 Genomic DNA Preparation

The above procedure was used for DNA plasmid preparations. Some alterations were required for identifying specific DNA sequences in genomic DNA preparations, the biggest of these being the preparation of the DNA samples from porcine tissue. Pigs were slaughtered by captive bolt, and tissue samples quickly dissected from various tissues (section 2.2.4.4 for a full list). These tissue samples were placed in cryotubes and immediately snap-frozen in liquid nitrogen, and kept in liquid nitrogen storage until required.

Tissues were removed from liquid nitrogen storage and kept on dry ice as much as possible. Approximately 100 mg tissue was weighed out, and ground into fragments using a mortar and pestle chilled in liquid nitrogen. The fragments were collected in a microcentrifuge tube, and to them was added ten volumes of extraction buffer (10 mM Tris, pH 8.0, 100mM EDTA, 0.1 % SDS and 20 µg/ml Pancreatic RNase). This mixture was incubated for 1 hour in a 37 °C waterbath, after which proteinase K was added to final concentration of 100 µg/ml. The tubes were incubated for a further 3 hours at 50 °C. Phenol chloroform extraction was used to purify the DNA. The supernatant was removed to a clean, 2 ml screw-cap microcentrifuge tube, and a half-volume of 1:1 phenol/chloroform added. Tubes were vortex mixed, and centrifuged for 15 min at 10,000 × g, room temperature. The aqueous phase was removed to a fresh tube, taking care not to disturb the interface. An equal volume of 1:1 phenol/chloroform was added, and the tube vortex mixed and centrifuged as for the first round of extraction. This was repeated again for a third extraction step.

The DNA was precipitated from the final aqueous phase by the addition of 0.2 volumes of 3M sodium acetate, pH 8.0, and 1 volume of isopropanol. This mixture was left at room temperature for a few minutes until the formation of a white DNA precipitate. Samples were then spun at 10,000 × g for 5 min, and the supernatant removed. Pellets were left to air-dry, and then resuspended in 100 µl Millipore-pure water. For genomic Southern blots, a 20 µl aliquot of genomic DNA (typically 10 to 20 µg) was digested by restriction endonuclease (section 2.2.1.3) in a larger reaction volume of 50 µl for several hours, with the amount of reaction mixture components adjusted appropriately. The entire restriction digest was run out on a large-comb gel, the subsequent blot-

transfer to Hybond-N nylon membranes and autoradiography steps carried out as described for normal Southern blots above (section 2.2.4.2.2).

#### **2.2.4.3 Colony Lift Hybridisation**

Colony lifts were used to detect specific DNA sequences (often cloned PCR products) within a mixture of *E. coli* colonies. Hybond-C nitro-cellulose membranes were placed over the surface of the agar plates containing the colonies of interest. The filter was marked asymmetrically with India ink to allow subsequent location of positive colonies, and the filter lifted with care so as to preserve the relative positions of colonies on the plate. The nitro-cellulose filters were then incubated on Whatman filter paper soaked in the following three buffers, for 5 min each: denaturing buffer (0.5 M NaOH, 1.5 M NaCl), neutralising buffer (0.5 M Tris, pH 7.4, 1.5 M NaCl) and finally equilibrating in  $2 \times$  SSC, 1.5 M NaOH. Since nitro-cellulose is inflammable, the filters were not cross-linked by UV, but baked at 80 °C for 2 hours. Hybridisation and autoradiography were carried out as for standard Southern blotting (section 2.2.4.2.2).

#### **2.2.4.4 Northern Blot Hybridisation**

##### 2.2.4.4.1 Extraction of Total RNA from Porcine Tissues

Pigs were slaughtered by captive bolt, and tissue samples quickly dissected from liver, kidney, brain, spleen, heart, uterus, and from the *psaos* and *longissimus dorsi* (LD) skeletal muscles. These tissue samples were placed in cryotubes and immediately snap-frozen in liquid nitrogen.

As required, tissue samples were removed from liquid nitrogen storage and kept on dry ice. Keeping the tissue frozen at all times was essential for preventing RNA degradation, and where possible all manipulation was carried out over dry ice or liquid nitrogen. Tissue fragments were ground using a pre-chilled mortar and pestle, and around 50 mg of the pieces quickly and carefully weighed into a sterile 2 ml screw-cap tube. To the tissue fragments was added 800  $\mu$ l Denaturing Solution, containing guanidine thiocyanate, 0.75 M sodium citrate, pH 7.0, and 10% N-lauroyl sarcosine, and the mixture mechanically homogenised using an Ultra-Turrax<sup>®</sup> T8 homogeniser (IKA Labortechnik). Once the tissue was thoroughly lysed, 50  $\mu$ l of 3 M sodium acetate was added to the preparation, along with 800  $\mu$ l phenol and 160  $\mu$ l chloroform

(5:1 phenol to chloroform). This mixture was vortexed thoroughly, and spun down. The aqueous phase was removed to a fresh tube, and the extraction repeated two or three times more with the addition of 1.6 ml 1:1 phenol chloroform, until a clean interface was observed indicating minimal protein contamination.

The RNA in the final aqueous phase was precipitated with the addition of one volume of  $-20^{\circ}\text{C}$  isopropanol, leaving the mixture at  $-20^{\circ}\text{C}$  for 20 min. This was spun down at  $10,000 \times g$  in a pre-chilled rotor,  $4^{\circ}\text{C}$  for 30 min. Supernatant was removed, and the pellet carefully washed with 500  $\mu\text{l}$  of 70% ethanol, spinning again for 5 min. The final pellet was resuspended in 30  $\mu\text{l}$  of RNase-free distilled water.

#### 2.2.4.4.2 Running Formaldehyde Gels

Size fractionation of total RNA was performed essentially as described by Lehrach *et al.* (1977). Gels were made up of 0.8% agarose in MOPS-EDTA-sodium acetate (MESA) buffer, melting the agarose as normal and allowing to cool, and then adding formaldehyde to the mixture and pouring into the gel casting plate. Since formaldehyde is volatile, formaldehyde-containing gels were used immediately, or stored for no longer than overnight at  $4^{\circ}\text{C}$ . For each sample, 15  $\mu\text{g}$  RNA was mixed with RNA sample buffer (consisting of 1 ml 10X MOPS, 3.5 ml 37% formaldehyde and 10 ml formamide, per sample), made up to 20  $\mu\text{l}$  with DEPC treated water. Samples were heated at  $68^{\circ}\text{C}$  for 5 min, chilled on ice for 5 min, then 2  $\mu\text{l}$  loading buffer (50% v/v Glycerol, 1 mM EDTA, 0.25% Bromophenol blue, 0.25% Xylene cyanol in DEPC water) and 1  $\mu\text{l}$  0.4 mg/ml ethidium bromide added. Samples were loaded onto the gel, and run overnight at 30 volts in  $1 \times$  MESA buffer.

#### 2.2.4.4.3 Blotting and Hybridisation

The transfer of RNA to Hybond-N nylon filter was similar to the method used for Southern Blotting, with the transfer buffer drawn up through gel and filter by means of paper towels and Whatman filter paper. In this case,  $10 \times$  SSC was used as the transfer buffer. The following morning, the filter was dismantled from the transfer apparatus and washed for 10 seconds in  $6 \times$  SSC. Filters were left to dry, covered in Saran wrap and then UV cross-linked. For the most part, hybridisation with the appropriate

radiolabelled probes and subsequent autoradiography were carried out as detailed in section 2.2.4.2.2.

## **2.2.5 PRIMARY CELL CULTURE**

### **2.2.5.1 Isolation and Maintenance of Porcine Muscle Satellite Cells**

#### 2.2.5.1.1 Satellite Cell Isolation

Pigs were killed by captive bolt, followed by severance of major neck blood vessels. Sections of LD and *psoas* were dissected from the muscle, taking care to remove obvious fatty or connective tissues. Muscle samples were stored in ice-cold tissue culture medium until proceeding to the next stage. Muscle pieces were finely dissected using a scalpel blade. The finely cut muscle pieces were incubated in 1.4 U/ml dispase in PBS for two hours at 37 °C, resulting in a tissue 'slurry'. This was spun down at 400 × g, and the single-cell suspension in the supernatant removed to a fresh tube and kept. The remaining cell slurry pellet was washed with a further 10 ml of culture medium, and the supernatant again removed and transferred to a fresh tube. Both of these tubes were spun at 1300 × g, 5 min, to pellet the cells and debris, removing the dispase-containing media and replaced with fresh culture media.

To remove the last remaining tissue clumps, the resuspended cell pellets were passed through a 100 µm cell strainer, pelleted by centrifugation again and resuspended in fresh culture medium. Cells were seeded on 75 cm<sup>2</sup> or 175 cm<sup>2</sup> flasks and grown at 37 °C for 3 to 5 day before changing the media. Typically, depending on the growth rate of the particular muscle isolate, the cells were passaged at this point into several flasks until a sufficient number of cells were reached for cell freezing and storage.

#### 2.2.5.1.2 Culture Media for Satellite Cell Growth and Differentiation

The first satellite cell cultures were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 15% foetal calf serum (FCS), 1 U/ml penicillin, 1 µg/ml streptomycin and 10 µg/ml cyproxin. Subsequent cultures were grown in PromoCell Skeletal Muscle Cell Growth Medium (cat. N<sup>o</sup>. C-39360). The basal, serum-free medium was supplemented before use with the following growth factors and hormones: 5% foetal calf serum, 0.4 µg/ml dexamethasone, 10 ng/ml hEGF, 1 ng/ml hbFGF, 50

$\mu\text{g/ml}$  Fetuin and  $10 \mu\text{g/ml}$  bovine insulin, together with antibiotics,  $50 \mu\text{g/ml}$  gentamicin and  $50\text{ng/ml}$  amphotericin B.

Cells were induced to undergo differentiation by changing the medium to DMEM with 4% horse serum, in place of the FCS, with the usual antibiotic supplementation of  $1 \text{ U/ml}$  penicillin,  $1 \mu\text{g/ml}$  streptomycin and  $10 \mu\text{g/ml}$  cyproxin. As for growth medium, a new PromoCell differentiation medium was made available later in the project. The PromoCell basal medium was supplemented with  $10 \mu\text{g/ml}$  bovine insulin and antibiotics,  $50 \mu\text{g/ml}$  gentamicin and  $50\text{ng/ml}$  amphotericin B.

#### 2.2.5.1.3 Cell Passaging

Satellite cell cultures were routinely passaged once they had reached around 80% confluency. Culture medium was carefully removed, and the cells washed with PBS. The cells were detached from the culture substrate by the addition of 0.05% trypsin,  $0.53 \text{ mM}$  EDTA, incubating for 3 min at  $37^\circ\text{C}$ , and tapping the flask to dislodge the cell monolayer. Cells were resuspended in serum-containing medium, sufficient for a ten-fold dilution of the trypsin for serum inactivation of the enzyme, transferred to 50 ml falcon tubes and spun down. The supernatant was discarded and the cells resuspended in fresh growth medium. The suspension was split between fresh flasks, chambered slides or culture wells as required.

For maintaining the cells in culture, the cells were typically passaged one in three, depending on the observed rate of growth for a particular cell isolate. Care was taken to ensure an optimal cell density following cell splitting. Too sparse a coverage of cells would deprive the cells of growth factors normally secreted by their neighbours. Too dense and the cells could overgrow, which in myoblast culture could result in the initiation of differentiation and terminal withdrawal from the cell cycle.

#### 2.2.5.1.4 Cell Storage

Primary cells cannot be maintained indefinitely in culture without undergoing senescence and death, and so isolates were periodically taken from the porcine satellite cell cultures for storage in liquid nitrogen. Cells were trypsinised, as described for cell passaging above, but resuspended in foetal calf serum containing 10% DMSO instead

of growth medium and transferred to cryovials (Invitrogen). The cryovials were frozen at a controlled rate (1 °C/min) to -70 °C overnight. Controlled freezing was obtained by using an isopropanol-filled freezing container placed in a -70 °C freezer ('Mr Frosty', supplied by Nalgene). Once frozen, the cells were transferred to liquid nitrogen storage.

When removing cells from cryostorage, the vials were quickly thawed in a 37 °C waterbath, and added to 10 ml growth medium. The cells were spun down, supernatant removed and the cells resuspended in fresh culture medium, to remove the cyto-toxic DMSO, before transferring to a culture flask. Cells were typically grown overnight before splitting, but this could vary up to 48 hours depending on the number of viable cells surviving cryo-preservation and thawing.

#### 2.2.5.1.5 Surface coating

In order to improve the culture of primary myocytes, culture flasks, multi-well plates and chambered slides were coated with gelatin or collagen.

To coat with gelatin, 0.1% gelatin solution in PBS was added sufficient to cover the surface of the culture vessel. This was left to set for 20 min at room temperature, before removing the excess with a pipette. After leaving to dry for 1 hr, the coated flasks, plates or chambers were ready to use. Gelatin-coated culture materials were stored at room temperature, for a maximum of six months.

For collagen coating, 1 ml glacial acetic acid was made up to 180 ml with distilled water, sterile filtered (0.2 µm) and added to 20 ml 0.1% calf skin collagen (Sigma) in 1 N acetic acid. This was added sufficient to coat the surface of the culture vessels, which were incubated overnight at room temperature. Excess collagen was removed with a pipette and stored at 4 °C for re-use, and the culture vessels washed with PBS and allowed to dry. Collagen coated flasks, plates or slides were stored at room temperature, up to six months.

### **2.2.5.2 Lipofectamine Transfections**

The introduction of heterologous DNA into mammalian cell culture was typically done through liposome-mediated transfections, specifically using Lipofectamine (Invitrogen) as described by Hawley-Nelson *et al.* (1993).

Cells were transfected in six-well plates, after reaching 60 to 80 % confluency. For each transfection experiment, a master mix was made up, sufficient for 2 µg plasmid DNA and 10.5 µl Lipofectamine in 200 µl Optimem solution (Invitrogen), per well of cells. This mixture was incubated at room temperature for 15 min for the formation of DNA-liposome complexes. In the meantime, the growth medium was removed from the wells, and the cells washed twice with Optimem. After the incubation period, the master mixes were made up to a final volume of 1 ml per well, and this was overlaid on the cell monolayer. The transfections were incubated for two hours at 37 °C.

Where stable transfections were required, the transfected cells were selected in media containing 2 mg/ml Geneticin (G418). To gauge the effectiveness of the selection process, a control population of untransfected cells was maintained in identical conditions. Once these control cells were completely killed, the surviving transfected cells were switched to media containing only 1 mg/ml Geneticin, to maintain selection pressure.

To culture populations of transfected cells, the transfection wells were passaged into 75 cm<sup>2</sup> flasks. These cell populations were generally maintained only for a short time, at most three or four passages, and were usually stored in liquid nitrogen to preserve stocks.

### **2.2.5.3 C2C12 myoblast culture**

Though porcine cells were regarded a better *in vitro* model for investigating the function of porcine transcription factors, for some cell culture applications in the course of the project the use of porcine primary culture was unfeasible. In such instances, the C2C12 mouse myoblast cell-line (Yaffe and Saxel, 1977) was used in place of the primary cultures.

C2C12 cells have several advantages over the usage of primary culture. Firstly, the cells are effectively a homogenous population of myoblasts without the risk of contaminating populations of other cell types. Secondly, the C2C12 cells are more robust and amenable to laboratory manipulation than those isolated from muscle, and have a faster growth rate. There were some differences, therefore, between the culture conditions used for this cell line and those described for primary cultures above.

All C2C12 cells were cultured in DMEM supplemented with lower concentrations of serum: 10% foetal calf serum (FCS) or 2% horse serum for growth and differentiation media, respectively, together with the standard antibiotic supplementation of 1 U/ml penicillin, 1 µg/ml streptomycin and 10 µg/ml cyproxin. Also the C2C12s were able to withstand a longer transfection period, for improved transfection efficiency (see section 2.2.5.2). The incubation step with the liposomes was increased from two to four hours. The faster growth rate meant shorter intervals waiting for the cells to reach a certain degree of confluency. Care was taken not to allow the mouse myoblasts to reach full confluency, as this triggered differentiation.

In all other respects (passaging, cell storage, etc) the C2C12 cells were cultured as described for the porcine primary cells. Though theoretically capable of being grown for many more passages than the primary culture, in practice the number of passages was kept low with isolates taken frequently for storage in liquid nitrogen.

#### **2.2.5.4 Immunocytochemistry**

Staining cell samples using cell-lineage or differentiation-specific antibodies was used several times during the project. This involved the use of two antibodies: a primary antibody raised against the epitope under investigation, and a secondary antibody raised against the animal used in creating the primary.

Cells taken for antibody staining were grown in six-well plates or chambered slides. The medium was removed and the cells washed with PBS. Cells were then fixed with the addition of 1 ml of 1:1 acetone/methanol, for 20 min, at room temperature. Once the fixative was removed, the cells were either used immediately for antibody staining, or else could be stored at -20 °C.

Before incubation with antibody, the slides/wells were rinsed twice in 1 × tris buffered saline (TBS). Primary antibody was added, diluted to the appropriate concentration (determined experimentally) in Tris containing 0.1% BSA and 0.01% sodium azide. This incubation was carried out overnight at 4 °C. The cells were washed, three times in TBS-tween, and incubated with 1% serum (from the animal species used in raising the secondary antibody) in TBS for 30 min at room temperature. The secondary antibody was added and incubated a further 40 min to an hour at 37 °C. Cells were rinsed as before, and endogenous peroxidases blocked with the addition of 0.5% hydrogen peroxide in methanol for 30 min. The hydrogen peroxide was rinsed off in water, and the TBS-tween wash steps repeated. Strep-ABC reagent was added to the cells and incubated for 45 min at room temperature. Cells were washed in TBS, DAB chromagen was prepared and added to the cells. The slides/wells were monitored under the microscope for colour development, typically 2 to 3 min. Following this, the slides/wells were washed in water, and the cells counter-stained in Gill's Haemotoxylin (Gill *et al.*, 1974).

## **2.2.6 QUANTITATIVE REAL-TIME RT-PCR**

Real-time RT-PCR is a combination of two techniques. The first of these is reverse transcriptase-PCR (RT-PCR), the conversion of mRNA to cDNA that then acts as a template for gene-specific amplification by PCR. This is a sensitive technique allowing for the detection of low-level gene expression that could be missed by traditional methods such as Northern Blot hybridisation. The real-time element comes from the monitoring of the progress of the PCR reaction as it occurs, by means of a fluorogenic probe. The probes used in this study were based on TaqMan, or fluorogenic 5' nuclease, chemistry (Roche). In this method, the probe is labelled with a 5' fluorochrome and 3' quencher. As the DNA polymerase encounters the annealed probe, its 5' nuclease activity cleaves it and separates fluorochrome from quencher. This results in an increase in fluorescence that serves as an indicator of the PCR reaction progress. The reaction is characterised by the point at which the level of fluorescence, and hence the PCR amplification, crosses a set threshold. The higher the target's copy number, the sooner this threshold will be reached. The target is quantified relative to an endogenous control, typically a 'house-keeping' gene such as glyceraldehyde-3-

phosphate dehydrogenase (GAPDH) or, as was used in the project work,  $\beta$ -actin. Combined, then, these two techniques allow for the sensitive detection and relative quantification of gene expression.

### **2.2.6.1 Messenger RNA isolation**

#### **2.2.6.1.1 Extraction from Tissues**

The isolation of purified messenger RNA from tissue lysate was carried out using Dynabeads<sup>®</sup> Oligo (dT)<sub>25</sub> (DynaL Biotech). This method relies on the base pairing between the poly-adenylated tails of mRNA transcripts and the oligo deoxythymidine sequences bound to the magnetic Dynabeads<sup>®</sup> beads. The magnetic beads and attached mRNA may then be separated out of the tissue lysate, and subsequent wash steps, using a magnetic particle collector (MPC).

Tissue samples were taken from  $-70^{\circ}\text{C}$  or liquid nitrogen storage. Tissue was ground into smaller fragments in a mortar chilled with liquid nitrogen. About 50 mg of tissue fragments were added to 1ml lysis-binding buffer, consisting of 100mM Tris HCl, pH 7.4, 500mM lithium chloride (LiCl), 10mM EDTA, 1% lithium dodecyl sulphate (LiDS), 5mM DTT in diethylpyrocarbonate (DEPC) treated water. Samples were rapidly lysed using an Ultra-Turrax<sup>®</sup> T8 mechanical homogeniser (IKA Labor Technik). Complete and rapid lysis of the samples was essential to prevent RNA degradation. After lysis for 1 min the samples were placed on ice. To remove cell debris, the lysate was spun for 1 min at  $13,000 \times g$  in a microcentrifuge and the supernatant transferred to a clean tube.

Dynabeads were preconditioned in lysis-binding buffer, and kept on ice until required. These preconditioned beads were applied to the MPC, the supernatant removed and the sample lysate added. The beads were thoroughly resuspended by pipetting, then incubated at room temperature for 12 min. To prevent the beads settling out during the incubation period, the tubes were incubated on a rocker. Once the mRNA had been bound to the beads, the beads were collected. Tubes were placed in a Dynal MPC for 5 min, and the supernatant carefully pipetted off. The beads were washed twice in 1 ml of Wash Buffer A (10mM Tris HCl, pH 7.4, 150mM LiCl, 1mM EDTA, 0.1% LiDS, 5mM DTT in DEPC treated water), and once in 1 ml Wash Buffer B (10mM Tris HCl,

pH 7.4, 150mM LiCl, 1mM EDTA, 5mM DTT in DEPC treated water). For each wash, the beads were collected using the Dynal MPC as above. The supernatant was carefully removed and the beads completely resuspended in the next wash buffer.

To prevent enzymatic inhibition during the subsequent first-strand cDNA synthesis, the beads were washed a further three times in 1 × RT buffer (50mM Tris HCl, pH 8.3, 8mM MgCl<sub>2</sub>, 10mM DTT, 30mM KCl in DEPC treated water). Care was taken to completely remove the supernatant from the last wash, before the mRNA was eluted from the beads. To elute, 21 µl of 10 mM Tris HCl, pH 8.0, was added to the beads and the samples incubating at 65 °C for 2 min. The beads were briefly magnetised in the MPC, and the supernatant, containing the mRNA, removed to a new, clean 1.5 ml microfuge tube. The eluted mRNA was either stored at -70 °C or used immediately for first-strand cDNA synthesis.

#### 2.2.6.1.2 Extraction from Cell Pellets

For the most part, cells harvested for mRNA were grown in six-well plates. Typically, each individual mRNA extraction came from the pooled cell populations of two, paired wells. Where larger cell populations were harvested, the procedure described below was scaled up as appropriate.

Growth medium was removed, the cells washed in ice-cold PBS and 1 ml of fresh, pre-chilled PBS was added per well. The cells were scraped free into the PBS, and the each pair of cell suspensions pooled into a clean, 2 ml microcentrifuge tube. The cells were spun down at 10,000 × g for 5 min at 4 °C in a pre-chilled rotor. The supernatant was removed, and the pellet then snap-frozen in dry ice. Cell pellets could be stored at -70 °C, if not used immediately.

As with mRNA extraction from tissue samples, rapid lysis of the cells was essential for preventing RNA degradation. Cells were kept in liquid nitrogen or on dry ice for as much as possible during manipulations. Each cell pellet was lysed in 1 ml Lysis/binding buffer for 1 min and placed on ice, before being spun at 10,000 × g in a microcentrifuge for 2 min and the supernatant transferred to magnetic beads. The

addition of the lysate to the magnetic beads, and subsequent washes, was carried out exactly as for the extraction of mRNA from tissue.

#### **2.2.6.2 Reverse Transcription**

The first-strand synthesis of cDNA from mRNA template was carried out using the Reverse Transcription System kit (Promega). The procedure is summarised as follows: extracted mRNA was incubated at 70°C for 10min, during which a master mix of the various reaction components was prepared. The final reverse transcription mixture consisted of 5 mM MgCl<sub>2</sub>, 1 × reaction buffer (containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1 mM each dNTP, 25 ng/μl random primers and 1 U/μl recombinant RNasin ribonuclease inhibitor.

Once the required master mix had been added to the mRNA, a 5 μl aliquot was removed as control against possible genomic DNA contamination. To the remainder, AMV reverse transcriptase was added to a final concentration of 0.75 U/μl. Samples and controls were then incubated at room temperature for 10 min, to allow for primer extension, then at 42 °C for one hour. AMV-RT enzyme was inactivated by heating the samples at 95 °C for 5 min, then on ice for a further 5 min. Samples and controls were stored at -20 °C. For most real-time RT-PCR experiments, a 100-fold dilution of the cDNA samples was used.

#### **2.2.6.3 Real-Time PCR Primer and Probe Design**

Probe and primer sequences were designed using Primer Express v2.0 (Applied Biosystems). Typically, probe and primer sequences had a G-C content of between 30-80%, avoiding long nucleotide repeats. Annealing temperature was typically around 70 °C for probes, 60 °C for primers. Probes were designed based on TaqMan chemistry (fluorogenic 5' nuclease chemistry: Roche).

#### **2.2.6.4 Reaction Conditions**

Reactions were set up in 96-well optical plates, with the following reaction mixture in each sample well: 1 × Universal Master Mix (AmpliTaq Gold DNA polymerase, dNTP mix and MgCl<sub>2</sub>: Applied Biosystems), cDNA template, and the appropriate concentrations of primers and probe (determined experimentally), made up to 25 μl per

well. Samples were quantified using an ABI Prism 7700 Sequence Detection System, using version 1.6 software (Applied Biosystems), using the following programme: 50 °C for 2 min, 95 °C for 10 min, followed by forty cycles of 95 °C for 15 s and 60 °C for 1 min.

#### **2.2.6.5 Data Analysis**

Quantification data was normalised against the endogenous reference,  $\beta$ -actin, to account for variation between cDNA samples caused by differences in the efficiency of the mRNA extraction, the reverse transcription step or sample loading. For each set of samples, the target quantity was determined relative to a calibrator sample, typically the lowest quantity. All other quantities were expressed as an n-fold difference relative to this calibrator. Quantitation relative to the calibrator was carried out by the Comparative  $C_T$  method, as described in the manufacturer's protocol (Applied Biosystems). It should be borne in mind that this method assumes that the efficiency of the  $\beta$ -actin amplification is approximately equal to that of the target genes.

**CHAPTER THREE - CLONING PORCINE MUSCLE  
GATA-2 AND NFAT-2**

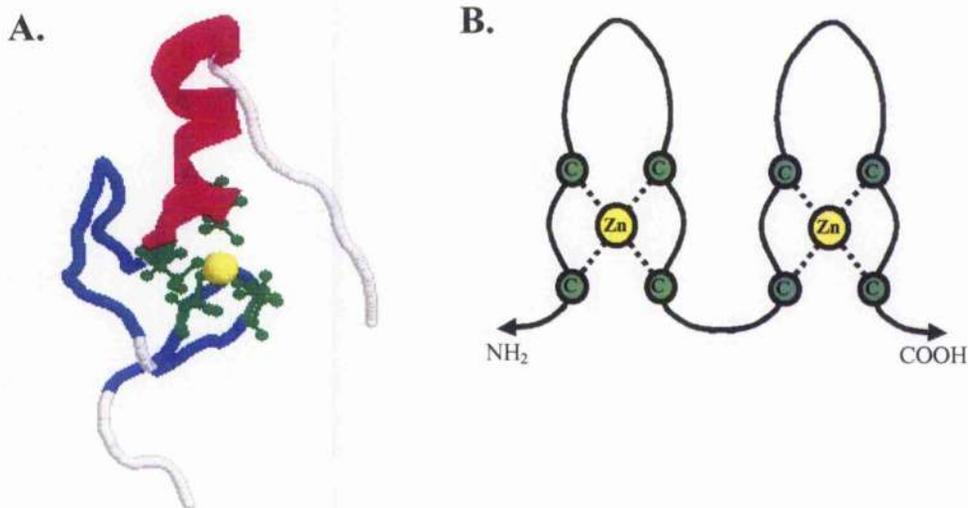
## 3.1 INTRODUCTION

The activation of calcineurin-mediated transcription in response to increases in intracellular calcium levels is a generic signalling pathway that is widely distributed and found in a range of tissues. Its downstream components, GATA and NFAT transcription factors, are therefore widely distributed too and modulate the transcription of a range of calcium-activated genes. However, specific GATA and NFAT family members are distributed in a tissue specific manner (Rao *et al.*, 1997; Patient and McGhee, 2002), and NFAT isoforms are also known to be restricted to specific stages of myogenesis even within muscle (Abbott *et al.*, 1998). The GATA-2 and NFAT-2 isoforms respectively have been previously described as prevalent in skeletal muscle fibres, and as promising candidates for a role in fibre-type specific gene expression (Abbott *et al.*, 1998; Musaro *et al.*, 1999; Paul and Rosenthal, 2002). The study of these two isoforms would therefore form the basis for much of the project work.

A necessary first step in investigating the role of GATA-2 and NFAT-2 in porcine muscle, however, is to obtain porcine homologues of these two transcription factors. As covered in Chapter One, these factors are already well characterised in other organisms. A PCR-homology cloning strategy was therefore employed, using primers designed to amplify regions of high homology across different species or family members. For both GATA and NFAT, these regions are highly conserved DNA binding motifs, the zinc-finger domain and Rel-homology region respectively. It is worth considering these two functional motifs, before detailing the cloning strategy that utilised them.

### 3.1.1 GATA ZINC FINGERS

The zinc-finger DNA binding motif is found in a large number of transcription factors (reviewed in Krempler and Brenig, 1999), and consists of looped projections (fingers) stabilised by a tetrahedrally co-ordinated  $Zn^{2+}$  ion. There are two classes of zinc finger protein, which are distinguishable on the basis of the amino acid residues that interact with the zinc and the number of fingers possessed by the protein.



**Figure 3.1.** Structure of GATA zinc fingers. **A.** GATA N-terminal Zinc finger, showing four cysteine residues (in green) co-ordinating a zinc atom (in yellow) (Kowalski *et al.*, 1999). **B.** Line schematic, highlighting the four zinc co-ordinating cysteine residues (C).

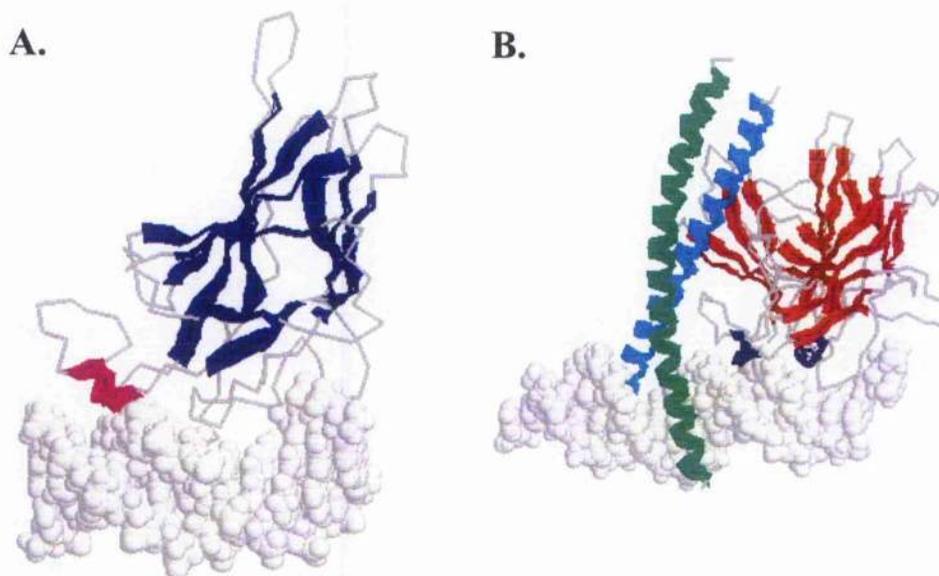
The first class of zinc finger proteins, the classical type, is also known as 'C2H2' zinc fingers. The zinc ion is co-ordinated by two cysteine and two histidine residues. The C2H2 proteins often have large numbers of repeated zinc finger elements for greater binding efficiency. In the second class of zinc finger proteins, to which the GATA family belongs, the zinc ligand is chelated by four cysteine residues. These transcription factors lack the long, repeated zinc finger motifs of the C2H2 group, instead having only two zinc fingers. The structure of this second class of finger is shown in Figure 3.1.

Both types of zinc finger interact with their DNA binding site through an  $\alpha$ -helix, which lies along the major groove when binding to DNA. In GATA, the two zinc fingers have slightly different roles. The N-terminal finger has been shown to interact with protein, an unusual feature for a domain traditionally associated with DNA binding, being necessary for interactions with Friend of GATA (FOG) (Fox *et al.*, 1998). The C-terminal finger, on the other hand, has been shown to be sufficient for

DNA binding (Omichinski *et al.*, 1993). It is thought that the N-finger is involved in GATA activation, and in modulating the binding affinity of the C-finger. These sequences are highly conserved between different GATA members, and make very useful targets for PCR homology cloning.

### 3.1.2 NFAT REL-HOMOLOGY REGION

As with GATA zinc fingers, the NFAT DNA binding domain (DBD) is highly conserved among NFAT family members, across both different family members and species. This region is also known as the Rel homology region (RHR), as it shares a low level of sequence identity with the characteristic of the Rel family of immune response proteins (Jain *et al.*, 1995; Verma *et al.*, 1995). The Rel-binding domain consists of a number of  $\beta$ -strands, connected by loops that interact with the DNA major groove. Additional interactions are formed between the DNA minor groove and an  $\alpha$ -helical domain. This structure is illustrated in Figure 3.2.



**Figure 3.2.** Structure of the highly conserved NFAT binding domain. **A.** Core NFAT binding domain/DNA complex, with the alpha helix shown in purple, beta sheet in blue. Note the looped connectors interacting with the major groove (from Zhou *et al.*, 1998a). **B.** Co-operative DNA-binding complex of NFAT with the basic leucine zipper transcription factors Fos/Jun (from Chen *et al.*, 1998).

### 3.1.3 CLONING STRATEGIES

The PCR homology cloning strategy may be broken down into two sections: the actual PCR-based cloning approach, and the subsequent use of the PCR fragments generated to screen a cDNA library.

#### 3.1.3.1 Homology-based PCR

The technique of PCR homology cloning, reviewed extensively by Pytela *et al.* (1994), is best used as a means of isolating unknown members of a well-conserved gene family of which several sequences are already known. The target sequence must have two regions that are very similar to known sequence, as these are the regions on which the primers for DNA amplification are based. These target regions can be quite small, as little as twelve nucleotides long, but the results of the cloning are more reliable and specific with targets of at least eighteen to twenty-four nucleotides in length. Primers are typically 'degenerate', or made up of a mixture of oligonucleotides with substitutions for nucleotides at different positions. Degenerate primers are required to compensate for the imperfect sequence conservation found between members of even the most highly conserved protein families, particularly on the nucleotide sequence level. Due to the degenerate nature of the triplet code in which several codons can code for the same amino acid, the third nucleotide of a given codon is often poorly conserved.

The use of degenerate primers places constraints on the cloning strategies that can be used. Since there will be mismatches between primer and target, the efficiency of the PCR will be lower. The optimal size for amplification is typically around 500 nucleotides, or 0.5 kb. The minimum separation between the two primer regions is 150 base pairs, since smaller fragments are of scant use in subsequent cloning steps. In any case, a few hundred base pairs of sequence are rarely sufficient for characterising a novel gene or performing functionality studies. But such a short stretch of novel sequence is particularly useful as a labelled probe for screening a library of full-length cDNA inserts.

### 3.1.3.2 Lambda Library Screening

A cDNA library represents the mRNA molecules of a particular tissue sample, converted from its extremely labile form into a stable cDNA duplex by reverse transcription followed by second strand synthesis. Clones isolated from a cDNA library are an extremely useful resource for further characterisation of a gene. Since cDNA is a faithful copy of the mRNA transcript, it contains a continuous coding sequence (cds) uninterrupted by introns and is thus an ideal template for the production of recombinant protein. The lack of intronic sequence also makes cDNA a more useful ideal template for the PCR homology cloning strategy described above. Genomic DNA is seldom suitable due to the possibility of large introns intervening between the PCR primers in certain family members, which prevents cloning. A major hurdle to be overcome in screening cDNA libraries is the presence of incomplete or truncated clones. This arises because the reverse transcription used in most cDNA library construction uses the poly-A tail as a primer site for the initiation of first strand synthesis. If the reverse polymerase is prevented from reaching the extreme 5' end of the mRNA transcript, the result is the loss of 5' sequence data. If parts of the cds are missing, further characterisation of a gene's function can be virtually impossible.

The cDNA libraries used during the project work were constructed using the ZAP Express cDNA synthesis kit (Stratagene) (Short *et al.*, 1988). Neonatal muscle offered the best possibility for obtaining GATA or NFAT cDNA since they are associated with muscle development or hypertrophy. The library used in the study was derived from mRNA taken from the *gastrocnemius* muscle of a 3 day-old neonatal Meishan pig.

### 3.1.4 AIMS

The aim of this part of the study was to obtain full-length, functional GATA and NFAT clones. This chapter covers the specific methods used in the cloning strategy and the sequence data obtained.

## 3.2 MATERIALS AND METHODS

### 3.2.1 PCR-HOMOLOGY CLONING

PCR primers were designed from sequence pile-ups of the conserved GATA and NFAT domains, being the zinc fingers and Rel-homology regions respectively. As well as the primer pair used for PCR amplification, an internal primer was designed for both cloning experiments. This internal primer was used as a radiolabelled probe to identify positive clones in subsequent colony-lift hybridisation used to screen TopoTA clones.

#### 3.2.1.1 GATA Primer Design and Conditions

The sequences used for the selection of PCR primers are shown in Figure 3.3. Degenerate primers were used for the initial runs, but the PCR efficiency was found to be too low, and more specific primers were tried instead. The selected primers are as follows:

Sense Primer

5' GAA GGC CGG GAG TGT GTC AAC TGT 3'  $T_m$  66.1 °C

Anti-sense Primer

5' ATT GTG CAG CTT GTA GTA GAG GCC 3'  $T_m$  62.7 °C

The internal primer used to screen the colony lifts was designed as follows:

5' GGC CAC TAC CTG TGC AAT GCC TG(C/T) 3'  $T_m$  67.0 °C

The PCR amplification conditions were as follows: template was denatured for 3 min at 95 °C, followed by 30 to 35 cycles of a 1 min, 95 °C denaturation step, 1 min at 55 to 60 °C annealing (the annealing step being greatly dependent on the primer sets used) and an extension step of 1 min at 72 °C. This was followed by a final 5 min extension step, before holding at 4 °C. The PCR amplification products were stored at -20 °C if not used immediately.

Mouse	CCTGGGTGGC	CCCGCCTCCA	GCTTCACCCC	TAAGCAGAGA	AGCAAGGCTC	50
Human	-----G--A	--G-----	-----	-----C-C	-----	
Chicken	-----G--	--G-G--	-----	C---CAC--	-----CA	
Mouse	GCTCCTGCTC	AGAAGGCCGG	<u>GAGTGTGTCA</u>	<u>ACTGTGGTGC</u>	<u>CACAGCCACC</u>	100
Human	-T-----T--	-----	-----G--	-----	-----	
Chicken	-A-----T--	-----A-A	-----G-	-----A--	A--C--T--	
Mouse	<u>CCTCTCTGGC</u>	<u>GACGAGATGG</u>	<u>TACGGGCCAC</u>	<u>TACCTGTGCA</u>	<u>ATGCCTGTGG</u>	150
Human	-----	-G-G-C--	C-C--	-----	-----	
Chicken	-----A	--A--GG	C--C--G--T	-----T-	-C--C--	
Mouse	GCTCTACCAC	AAGATGAATG	GACAGAACCG	GCCGCTCATC	AAGCCCAAGC	200
Human	C-----	-----	-G-----	A--A-----	-----	
Chicken	-----	--A--C-	-T-A--	A--T--T--	--A--A-	
Mouse	GGAGGCTGTC	TGCTGCCAGA	AGAGCGGGCA	<u>CCTGTGTGC</u>	<u>AAATTGTACG</u>	250
Human	-A-A--	G-C--	-----C-	-----	-----	
Chicken	-A-----	A-G--G	---A-G-	-G-----	C--C--	
Mouse	<u>ACGACAACCA</u>	<u>CCACCTTATG</u>	<u>GCGCCGGAAC</u>	<u>GCCAACGGGG</u>	<u>ACCCGTGTG</u>	300
Human	-----	-----	-----A-	-----	-----C--	
Chicken	--A-C--	-G-----	---A-C--	-----	---G--T--	
Mouse	<u>CAAGGCCTGT</u>	<u>GGCCTCTACT</u>	<u>ACAAGCTGCA</u>	<u>CAATGTTAAC</u>	<u>AGGCCACTGA</u>	350
Human	-----	-----	-----	-----	-----	
Chicken	T--T--C	--A--	-T-A--	-----G--	-----T--	
Mouse	CCATGAAGAA	GGAAGGGATC	CAGACCCGGA	ATCGGAAGAT	GTCCAGCAAA	400
Human	-----G--	-----	-----T--	-C-----	-----A--G	
Chicken	-----A--	-----A-T	-----A--	-A-----	-----A--	

**Figure 3.3.** Pileup of zinc-finger DNA binding domains from mouse, human and chicken GATA-2 (Accession N<sup>o</sup>s AK004675, M68891 and X56930 respectively), used to generate PCR-homology cloning primers. Dashes denote identical sequence to the mouse GATA-2. Zinc finger sequences are highlighted in blue; the sequences used for primers are underlined.

### 3.2.1.2 NFAT Primer Design and Conditions

The known sequences compiled for the Rel-homology region were more degenerate than those used for designing the GATA primers, as shown in Figure 3.4. The NFAT PCR primers were likewise designed with greater degeneracy, as follows:

Sense Primer

5' CCG CAC GCC TTC TAC CA (AG) GT (AGC) CAC 3'  $T_m$  67.3 °C

Anti-sense Primer

5' GC (AT) GA (GCT) CGC TG (AG) GA (AG) CAC TCG AT 3'  $T_m$  65.4 °C

The internal primer used to screen the colony lifts was designed as follows:

5' CAC CAA (AG)GT CCT GGA GAT CCC 3'  $T_m$  59.8 °C

PCR conditions for the NFAT homology cloning were largely as described for GATA (section 3.2.2.1). The greater degeneracy of the NFAT primers resulted in a significant drop in PCR efficiency, and a degree of optimisation was required. Different concentrations of magnesium were used, with final concentrations of 1.0, 1.5, 2.0 to 3.0 mM respectively, and lower annealing temperatures, 50 °C, from the standard conditions.

### 3.2.1.3 Cloning and selection of PCR products

PCR products obtained were cloned into 'One-shot' DH5- $\alpha$  *E. coli* (Invitrogen) using the TopoTA cloning kit (section 2.2.1.6.2). Colonies were screened for positive fragments by colony lift hybridisation (section 2.2.4.3) using  $\gamma^{32}$ P radiolabelled internal primers, prepared by end-labelling (section 2.2.4.1.2). Positive colonies were picked and sequenced using ABI PRISM (version 1.0) cycle sequencing (section 2.2.3.2.1). For sequencing, primers were used based on the T7 RNA polymerase promoter sequence:

5' TAA TAC GAC TCA CTA TAG GG 3'

```

Mouse2      CCATTGTGCA GGTACACGGT TACTTGGAGA ATGAACCTCT CACGCTACAG 50
Mouse1      -TG-G----- ---C----C ---A----- -CA-G-   GCG T---
Human2      ---C-----  G T C -----   ---G--G-- G-T--G---
Human1      -TG-G--T--- ---C--T--C ---A----A- -CA-G----- GGA--T---

Mouse2      CTGTTCTATG GGACCGCACA CGACCGCCCG CTGAGGCCCC AGCCCTTCTA 100
Mouse1      A-C----- ---A--A-- T--GA-GA-C --T-A--G- -----
Human2      --T----- ---G-- C-----   ---C-C-G- -----
Human1      A-C-----  A ---- T--G GA C --T-A--G- -----

Mouse2      CCAGGTCCAC CGGATCACGG GGAAGACTGT CTCCACCACC AGCCACGAGA 150
Mouse1      ---A--A--- A-----T-   A G-- -A-----G ---T-T---
Human2      ---G--- --C-----A- ---G--C-- G-----   ---G
Human1      --G--- --A-----G- ---A----- -A----- ---T-T---

Mouse2      TCATCCTGTC CAACACAAA GTCCTGGAGA TCCCTTTGCT TCCAGAAAAAT 200
Mouse1      AG--G AGG -----G -----   ---CC--GA G--A-G--C
Human2      CT-----C-----   -----   ---AC-C- G--G--G--C
Human1      AG--AG--GG -----   -----   ---C--GA G--CA-A--C

Mouse2      AACATGCGAG CCATCATCGA CTGTGCTGGG ATCCTGAAGC TCAGAAACTC 250
Mouse1      ---A--- ---C---G- ---A--G ----- ---C-----G-
Human2      -G--- --G---T- ---C--A -----A-----A-
Human1      ---A-G- -A-C----- ---C----- -T----- -T-----G-

Mouse2      TGATATTGAG CTGAGGAAAG CCGAGACAGA CATCGGGAGG AAGAACACCA 300
Mouse1      ---C-C--- ---C---G- -C---G--- ---C-----GC
Human2      C--C---A --TC----- -A--- G ----- ---AC
Human1      C--C--- ---C---G- -C---G--- ---T-A--A -----GC

Mouse2      GGGTGAGGCT GGTCTCCGA GTTCACATCC CACAGCCCA TCCCCGGACG 350
Mouse1      -T---C-C--- ---G---C- -G---G--- --G-----G ---G--C-TC
Human2      ---AC-G--- ---G---C- ---G---G--- -G--A---G C-----C---
Human1      -----A- ---T----- ---G---T---G A A TC

Mouse2      CTGTCTCTCC AGGTGGCCTC GAACCTATC GAGTCTTCCC AGCGTCCAGC 400
Mouse1      G-C--C--G- ---CT--G-- C-----C--- ---C---T- C T--
Human2      ---C--G- ----- C-----C--- --A--C----- ---C--A--
Human1      G-C---T-A- --ACT--A- T-----C--- C----- ---A--T--

Mouse2      CCAGGAGCTG CCGCTCGTGG AGAAGCAGAG CACAGACAGC TACCCAGTCA 450
Mouse1      ---C----- ---A-G--- ---GA ACA TG----- -G--TG---T
Human2      T----- --TC-G--- ----- ---G----- -T--G---G
Human1      T--C----- ---A-G--T- -A-GA--AGA ----- -G--TG---

```

**Figure 3.4.** Pileup of Rel-homology regions from mouse and human NFAT-2 and NFAT-1 isoforms (Accession N<sup>o</sup>s NM\_010899, U02079, U08015 and U43341 respectively), used to generate PCR-homology cloning primers. Dashes denote identical sequence to the murine NFAT-2 sequence. The sequences used for primers are underlined.

Multiple clones with high homology to known GATA and NFAT isoforms (as determined by BLAST search) were selected for use as radiolabelled probes for screening the ZAP-Express™ cDNA library. TopoTA clones were prepared by miniprep (2.2.1.2.1), digested with *EcoRI* (2.2.1.3), separated in agarose gel and the insert fragments purified (2.2.1.4), pooled and prepared as a mixed probe of GATA and NFAT PCR products, using random hexamer labelling (2.2.4.1.1).

### **3.2.2 LAMBDA LIBRARY SCREENING**

Screening for full-length cDNA clones was carried out using a lambda-vector library (Short *et al.*, 1988), constructed using the ZAP Express™ cDNA synthesis kit (Stratagene). The library was from the *gastrocnemius* muscle of a 3 day old Meishan pig, with an estimated one million primary clones.

#### **3.2.2.1 Growth of the Host Strain**

A tetracycline-resistant, XLI-blue mrf<sup>r</sup> strain was used for the titering and screening of the lambda library, kanamycin-resistant XL0LR cells for plaque excisions. Cells were streaked out on LB-agar plates with appropriate antibiotic selection, and grown overnight at 37 °C. A single colony was picked to inoculate a starter culture, 5 ml LB with antibiotic selection, which was incubated overnight at 37 °C in an orbital shaker. The following day, 1 ml of the starter culture was used to inoculate 100 ml LB supplemented with 0.2% (w/v) maltose and 10 mM MgSO<sub>4</sub>. The culture was grown to mid-log phase ( $A_{600}$  readings of between 0.5 and 1) before being spun down in 50 ml Falcon tubes, 500 × g, 10 min at 4 °C. The cell pellets were resuspended by pipetting in 10 mM MgSO<sub>4</sub>, to a final O.D.<sub>600</sub> reading of 0.5. These cell solutions could be stored at 4 °C for a maximum of one week.

#### **3.2.2.2 Plating and Titering**

The phage titre was determined by making serial dilutions of the stock library, combining 1 µl of each dilution with 200 µl of host cells and incubating at 37 °C for 15 min. After incubation, the bacteria/phage mix was added to melted 0.7% NZY top agarose, pre-warmed to 48 °C, and poured onto on NZY agar plates, swirling gently to

ensure an even covering. The plates were incubated for 10 hours at 37 °C to allow the formation of plaques, which were counted for phage titre.

For screening, 50,000 pfu of phage were added to 650 µl of host XL1-blue mrf<sup>r</sup> cells, incubated 15 min at 37 °C, and added to 0.7% NZY top agarose, as described above. Larger, 150 mm NZY agar plates were used for the screening process, these being at least two days old and pre-warmed to 37 °C for two hours. Once poured, the top agarose was left for 10 min at room temperature to set, and then the plates were incubated at 37 °C for 10 hours. For the first round of screening, the plaques were quite confluent, at 50,000 plaques per plate. Twenty plates were used for this first screen. Subsequent rounds of screening were undertaken to ensure monoclonal, discrete plaques.

### **3.2.2.3 Plaque Lift Hybridisation**

Hybond-C nitro-cellulose membranes were used to perform plaque lifts. Plates were chilled to 4 °C beforehand to prevent the top agarose layer peeling away during the lift. Each membrane was overlaid on its respective plate for 1 min, marked asymmetrically using black ink in a needle for subsequent location of positive plaques, and the filter slowly peeled from the plate. Two lifts were taken for each plate. Filters were incubated in denaturing buffer (0.5 M NaOH, 1.5 M NaCl), neutralising buffer (0.5 M Tris, pH 8.0, 1.5 M NaCl) and finally washed in 6 × SSC, 5 min each, on Whatman filter paper soaked in the respective buffer. Filters were dried by baking at 80 °C for two hours, and stored at room temperature until required for hybridisation.

Hybridisation and autoradiography were carried out as described in section 2.2.4.2.2, using the mixed probe for GATA and NFAT made up as described above (3.2.2.3). Positive plaques were selected for further rounds of screening, and plaque cores taken using a Pasteur pipette. Plaque cores were placed in 500 µl SM buffer (0.1M NaCl, 10mM MgSO<sub>4</sub>, 50 mM Tris, pH 7.5, 2% (v/v) gelatin) and 40 µl chloroform, and vortexed to release the phage. Phage stocks not immediately used for subsequent rounds of screening were stored at 4 °C for up to six months. In order to increase the chance of obtaining a monoclonal plaque, the plaque screening process was repeated a further two times using phage obtained from the positive plaque cores.

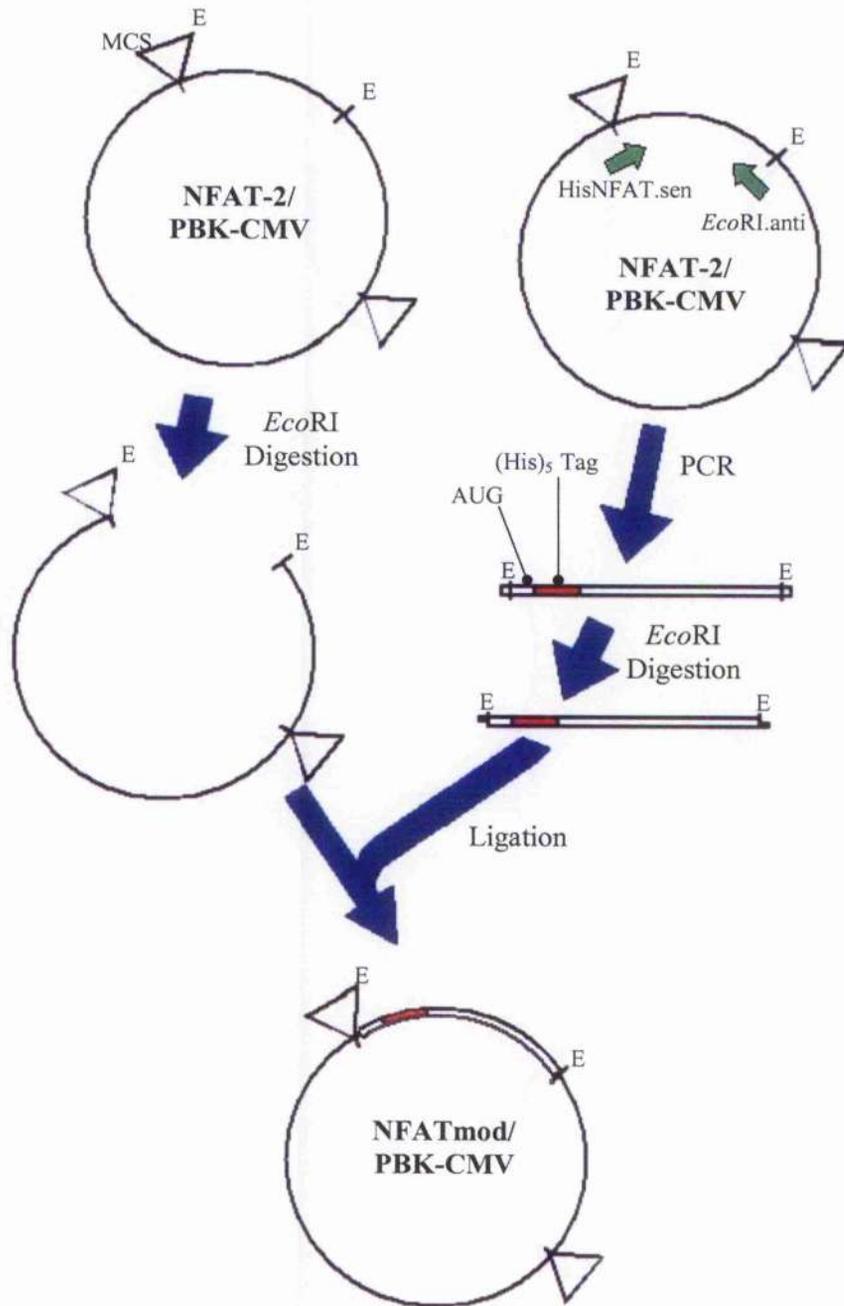
### 3.2.2.3 Phage Excision

In order to excise the bacteriophage for conversion to plasmid, the concentration of phage was increased by performing a phage titre as described in section 3.2.2.2.1 and pooling ten plaque cores into a single stock of 500  $\mu$ l SM buffer, 20  $\mu$ l chloroform and vortexing as described above, storing overnight at 4 °C before use. The following day XL1-blue mrf<sup>r</sup> and XLOLR host cells were grown as described in section 3.2.2.1, spun down at 1,000  $\times$  g for 10 min and resuspended in 10 mM MgSO<sub>4</sub> to a final A<sub>600</sub> reading of 1. For each of the concentrated phage mixtures, 200  $\mu$ l of XL1-blue mrf<sup>r</sup> cells were combined with 250  $\mu$ l phage stock, and 5  $\mu$ l ExAssist helper phage, supplied by Stratagene, in a 1.5 ml microcentrifuge tube. Tubes were incubated at 37 °C for 15 min, before being transferred to 15 ml Falcon tubes containing 3 ml NZY broth. The Falcon tubes were incubated at 37 °C for 3 hours. After this incubation, the tubes were heated at 65 °C for 20 min, and then centrifuged at 1,000  $\times$  g for 15 min. The supernatant containing the filamentous lambda-phage particles was decanted into 20 ml universal tubes, where it could be stored at 4 °C for up to 2 months.

For each excision reaction, a microcentrifuge tube containing 200  $\mu$ l of XLOLR cells was prepared. An equal volume of phage supernatant was added to the cells, and the mixture incubated for 15 min at 37 °C. Following the incubation, 300  $\mu$ l of NZY was added to the cells and incubated a further 45 min, shaking, at 37 °C before plating out 200  $\mu$ l on LB agar plates supplemented with kanamycin. After overnight incubation at 37 °C, bacterial colonies were picked and the excised ZAP Express<sup>TM</sup> plasmid clone prepared by miniprep.

### 3.2.3 NFAT 5' MODIFICATION

A full-length clone of the porcine NFAT was not obtained using the ZAP Express<sup>TM</sup> library. A number of techniques were employed to counter this deficiency, including SMART-RACE PCR (see section 3.2.5 below). In the meantime, a PCR cloning strategy was employed to produce a functional NFAT from the truncated clone as a short-term measure. The cloning strategy, summarised in Figure 3.5, involved the replacement of the 5', 900 bp *EcoRI* fragment of the clone with one modified by a PCR step. Two PCR primers were designed, based on sequences flanking the 5' *EcoRI*



**Figure 3.5.** Summary of the cloning strategy used to modify the 5' end of the truncated NFAT clone. **E.** *EcoRI* site. **MCS.** Multiple cloning site.

fragment of the NFAT clone.

5' HisNFAT.sen

5' AAG AAT TCG CCA CCA TGC ATC ACC ATC ACC ATC AC G AGG AGT  
'TTC CGC CCT TCC AGC 3' T<sub>m</sub> >75 °C

3' EcoRI.anti

5' AAG AAT TCG GCT TGC ACA GGT CTC C 3' T<sub>m</sub> 64.6 °C

The 5' primer contains an *EcoRI* restriction site, Kozak consensus sequence and ATG start site, and codons for six histidine residues for 'tagging' the resulting protein. PCR was carried out as described in section 2.2.2. The PCR product was Topo-TA cloned as described in section 2.2.1.6.2, and sequenced to verify the identity of the insert (2.2.3). The insert was released from the Topo-TA clone by *EcoRI* digestion, and purified by gel extraction. The original NFAT clone was digested with *EcoRI* to remove the original *EcoRI* fragment, treated with calf alkaline phosphatase and ligated with the modified PCR product (2.2.1.5).

### 3.2.4 SMART-RACE PCR

It was subsequently decided that the best option for cloning a full-length NFAT transcript was the use of the 5'-rapid amplification of cDNA ends (RACE) technique (Frohman *et al.*, 1988). In the SMART RACE cDNA amplification kit (Clontech), the RACE technique is combined with Switching Mechanism At 5' end of RNA Transcript (SMART) described by Zhu *et al.* (2001). The actual RACE makes use of a variant of Moloney murine leukaemia virus (MMLV) reverse transcriptase that exhibits terminal transferase activity upon reaching the end of the RNA transcript, adding 3-5 deoxycytosine residues to the 3' end of the first-strand cDNA. A 'SMART' oligonucleotide with a terminal stretch of deoxyguanine residues anneals to this poly-C tail, and serves as a primer once the RT-enzyme switches to using the cDNA strand as a template. Since the poly-C tailing activity is more efficient if the reverse transcriptase has reached the end of the RNA molecule, the SMART oligonucleotide tends to be added only to full-length cDNAs. This can be combined with the use of a gene-specific primer (GSP) during first-strand synthesis, in order to produce a full-length cDNA of a

specific mRNA transcript. For the most part, the procedures were carried out as recommended in the manufacturer's instructions. A summary of the technique is provided here.

#### **3.2.4.1 Isolation of mRNA**

Tissue samples were taken from the *longissimus dorsi* (LD) muscle of porcine embryo at 91 days development. Messenger RNA was isolated using Dynabeads, for the most part as described for the standard procedure (section 2.2.6.1). In order to increase the yield of mRNA obtained, and improve the chances of successful amplification later, the RNA extraction was from a total of six 50 mg samples of LD muscle. The washes in 1 × RT buffer was replaced with only two, and 100 µl TE used in the elution step. After heating the samples at 65 °C as standard, the eluates were pooled into a final 600 µl volume. In this instance, beads were re-used between sets of three samples by adding a further 100 µl of TE, resuspending, and heating at 65 °C for 2 min, and then reconditioning with 1 ml lysis/binding buffer. These recycled beads were used only once, and not stored.

The pooled, 600 µl mRNA sample was taken to an isopropanol precipitation step, by adding 60 µl 3 M Na Acetate and 600 µl isopropanol. The mixture was shaken well and incubated at -20 °C for 1 hour. The precipitated mRNA was spun at 10,000 × g for 1 hour at 4 °C, the supernatant discarded. The pellet was carefully rinsed in 70 % ethanol, spun again at 10,000 × g for 15 min at 4 °C. The final pellet was resuspended in 8 µl of TE buffer. Quality of the mRNA obtained was tested by spotting 1 µl of the final eluate onto agarose, and comparing it to a 50ng/µl DNA standard, under UV transillumination.

#### **3.2.4.2 First Strand Synthesis**

Two sets of cDNA were prepared for subsequent RACE PCR, one for 5' RACE and the other for 3' RACE. Both tubes contained between 0.5 to 1 µg of mRNA, together with the appropriate CDS primer and, for 5' RACE reactions, the SMART II oligonucleotide. Reaction volumes were made up to 5 µl with distilled water and the RNA briefly denatured, heating for 2 min at 70 °C and then on ice a further 2 min. To each tube was added First-Strand buffer, DTT, dNTP mix and reverse transcriptase,

mixing by pipetting. Reactions were then incubated for 90 min at 42 °C. Because of the low volume, the tubes were incubated in an air-incubator to prevent evaporation of the tube contents. Following the incubation, the reaction was diluted with the addition of 250 µl Tricine-EDTA buffer and the tubes heated at 72 °C for 7 min. The cDNA was stored at -20 °C.

### 3.2.4.3 RACE PCR

#### 3.2.4.3.1 Primer Design

Gene-Specific Primers (GSPs) were designed according to the recommendations given in the Clontech protocol. Custom oligonucleotide synthesis was by MWG Biotech. GSPs were designed between 23 and 28 deoxynucleotide residues in length, with a base composition of 50 to 70 % guanine or cytosine. GSPs were designed with a  $T_m$  of greater than 70 °C, allowing for the use of "Touchdown" PCR (Don *et al.*, 1991). This refinement to the standard PCR conditions allows for greater specificity, by using an annealing temperature greater than the universal primer for the initial PCR cycles. This ensures that only gene-specific amplification occurs, through the accumulation of gene-specific product before the annealing temperature is lowered in subsequent cycles to accommodate the universal primers.

The following set of GSPs was designed, based on the previously obtained NFAT clone sequence:

GSP1 (antisense, for 5' RACE)

5' CGG GAT CTC GAC CAA GGA ATT CGG C 3'  $T_m$  71.0 °C

GSP2 (sense, for 3' RACE)

5' GGA GAC GGA CAT TGG GCG GAA GAA C 3'  $T_m$  71.0 °C

#### 3.2.4.3.2 Reaction Conditions

PCR reagents were supplied by BD Biosciences as part of the BD Advantage 2 PCR kit. As such, the PCR conditions differed slightly from the standard procedure detailed in section 2.2.2.2. Manufacturer's protocols were followed, summarised briefly here. PCR reactions consisted of 1 × Advantage 2 PCR buffer and polymerase mix, 200 µM dNTPs, 1 × Universal Primer Mix (UPM), 200 nM of the appropriate GSP and 2.5 µl of

the 5'- or 3'-RACE-ready cDNA, as appropriate, made up to 50 µl with PCR-grade water.

Thermal cycling was carried out on an Applied Biosystems GeneAmp 2400. Hot start and touchdown cycles consisted of 5 cycles of 94 °C for 5 seconds, 72 °C for 3 min, followed by a further 5 cycles of 94 °C for 5 seconds, 70 °C for 10 seconds and 72 °C for 3 min. The subsequent, standard PCR consisted of 30 cycles denaturing at 94 °C for 5 seconds, annealing at 68 °C for 10 seconds and extension at 72 °C for 3 min. PCR amplification products were stored at -20 °C if not immediately visualised by agarose gel electrophoresis.

### 3.2.5 SEQUENCING

Sequencing of the novel GATA and NFAT clones was carried out largely as described in Section 2.2.3. Initial sequencing was performed using sequencing primers derived from T3 and T7 polymerase promoters:

T3

5' ATT AAC CCT CAC TAA AG 3'

T7

5' TAA TAC GAC TCA CTA TAG GG 3'

Internal sequences were obtained using custom-designed primers (MWG Biotech) as described in section 2.2.3.1. There were a few specific techniques required, however, for more problematic stretches of DNA chiefly encountered in the sequencing of the porcine GATA clone. The techniques used were as follows: sequencing using increased concentrations of DMSO, sequencing from single-stranded DNA (ssDNA) template, and the use of the ABI PRISM dGTP Big Dye kit.

#### 3.2.5.1 Sequencing using DMSO

The addition of dimethylsulphoxide (DMSO) can reduce the occurrence of DNA secondary structure. However, the amount that can be added to sequencing reactions is limited by its inhibitory effect on Taq DNA polymerase. Typically, a concentration of

5% was used in attempts to read-through difficult sequence. In all other respects, the sequencing reaction was carried out as for normal ABI version 1.0 chemistry (section 2.2.3.2.1).

### 3.2.5.2 Single Strand Excision

Stretches of DNA that exhibit a high degree of secondary structure can be more easily sequenced using single-stranded template. Because the pBK-CMV vector used in the cloning of the porcine GATA clone contains the bacteriophage  $\phi$ 1 origin, it can be secreted as single stranded DNA template, in the presence of M13 helper phage. In *E. coli* bacteria possessing the F' episome, such as the XL1-blue strain, the pBK-CMV plasmid will be secreted as a single-stranded, packaged phagemid when infected with the helper phage.

XL1-blue cells transformed with the GATA clone were grown at 37 °C overnight in LB supplemented with kanamycin. The following morning,  $A_{600}$  measurements were taken, and an inoculum taken from the starter culture sufficient to give a final  $A_{600}$  reading of 0.1 when added to 50 ml Terrific Broth. For optimal aeration, the 50 ml culture was incubated in a 250 ml conical flask, with shaking, at 37 °C. After 1 hour, with the cells at log phase, helper phage was added to the culture sufficient for a multiplicity of infection of 20 phage particles per host cell. The culture was incubated a further 8 hours, transferred to a 50 ml Falcon tube and heated at 65 °C for 15 min. The cells were spun down at  $17,000 \times g$  for 5 min and the supernatant transferred to a fresh tube. This could be stored at 4 °C for a month.

To extract the ssDNA from the packaged phagemid, 1.2 ml of the supernatant was transferred to a microcentrifuge tube and 300  $\mu$ l of 3.5 M  $\text{NH}_4\text{Ac}$  (pH 7.5) 20% polyethylene glycol (PEG) solution added and mixed by inversion. This was left to stand at room temperature for 15 min, and then spun at  $10,000 \times g$  for 20 min. The supernatant was removed and the tube spun again for 1 min and all remaining supernatant removed. The resulting phage pellet was resuspended in 300  $\mu$ l TE buffer (pH 8.0), and 200  $\mu$ l (1:1) phenol-chloroform added. The extraction was mixed by vortex for 1 min, and spun in a microcentrifuge for 1 min at  $10,000 \times g$ . The aqueous phase was removed to a fresh tube, taking care not to disturb the interface. This phenol-

chloroform extraction was repeated until a clear interface was observed. Finally, 200  $\mu$ l of chloroform was added, mixed by vortexing for 1 min and spun at 10,000  $\times$  g for 2 min, and the aqueous phase again removed to a fresh tube. To precipitate the ssDNA, 200  $\mu$ l of 7.5 M  $\text{NH}_4\text{Ac}$  (pH 7.5) and 800  $\mu$ l of 100% pre-chilled ethanol was added to the recovered aqueous phase. This was left on ice for 15 min and then centrifuged at 10,000  $\times$  g for 20 min, in a rotor chilled to 4  $^\circ\text{C}$ . The supernatant was removed and the pellet washed in 80% ethanol. This was spun again for 5 min, supernatant was removed and the pellet dried completely before resuspending in 20  $\mu$ l of TE buffer. Excised ssDNA was stored at  $-20$   $^\circ\text{C}$ .

### 3.2.5.3 ABI PRISM dGTP BigDye Sequencing

The ABI PRISM sequencing kits use Inosine, an analogue of guanine, in order to reduce band compression. Band compression is usually caused by the formation of secondary structure in the sequencing products, resulting in an altered mobility through the sequencing gel and subsequent misidentification of bands. Inosine incorporation reduces such secondary structure formation due to the lower affinity binding to cytosine. This is of little use, however, when there is already significant secondary structure present in the template sufficient to terminate the Taq polymerase activity. In such situations the re-substitution of dGTP in the reaction mix allows for better incorporation and improves the ability of the Taq polymerase to 'read-through' the problem area. Naturally this re-introduces the problem of band compression, and so this approach should only be used for resolving difficult stretches of sequence, such as those encountered in the GATA clone.

For the most part, the preparation of DNA template, sequencing primers, and purification of extension products were as described in section 2.2.3.2.2 for the version 3.0 ABI PRISM Big-Dye cycle-sequencing. The Big-Dye reagent mixture contained dGTP in place of dITP, and a different AmpliTaq DNA polymerase requiring a much shorter cycle sequencing programme: one denaturing step at 96  $^\circ\text{C}$ , followed by 25 cycles denaturing for 10 s at 96  $^\circ\text{C}$ , followed by 2 min at 68  $^\circ\text{C}$  with a final hold at 4  $^\circ\text{C}$ . As with other versions of ABI sequencing, loading was largely automated and the set-up carried out according to manufacturer's instructions.

### 3.3 RESULTS

#### 3.3.1 PCR CLONING

The PCR homology cloning yielded a number of positive Topo-TA clones, identified by colony-lift hybridisation using the GATA and NFAT internal primers as probes. Of these colonies, six each of the identified GATA and NFAT clones were chosen arbitrarily for sequencing. Identical sequences were obtained for all the GATA and NFAT clones, respectively, as shown in Figure 3.6 below. These sequences showed high homology with previously characterised GATA-2 and NFAT-2 factors from other species, as determined by BLAST search, and were deemed suitable for screening for full-length clones from the porcine ZAP Express<sup>TM</sup> library. One GATA and one NFAT PCR fragment was taken forward for use in a combined, GATA/NFAT probe.

**A.**

```
1      GAAGGCGGGG AGTGTGTCAA CTGTGGGGCC ACAGCCACCC CTCTCTGGCG
51     CCGAGACGGG ACCGGCCACT ACCTGTGCAA CGCCTGGCGG CTGTACACAA
101    AGATGAACCG GCAGAACCGG CCACTCATCA AGCCCAAGCG GAGACTGTCT
151    GUCUCAGGA GAGCAGGCAC CTGTTGTGCA AATTGTGAGA GCAGAACCCAC
201    CACCTTATCG CGCCGAARCG CCAACGGGGG CCGTGTCTGC AACGCCTGTG
251    GCCTCTACCA CAAGCTGCAC AAT
```

**B.**

```
1      TCAGGTGCAC CGGATCACTG GGAAGACCGT GTCCACCACC AGCCACGAGG
51     TCATCTCTTC CAACACCAAG GTCCCTGAAA TCCCGCTTCT TCCGGAAAT
101    AACATGCCCG CAATCATGGA CTGTGCTGGG ATCCTGAAGC TCCGAAACTC
151    TGACATTGAG CTTCGCAAAG GGGAGACGGA CATTTGGCGG AAGAACAACA
201    GAGTGAAGGT GGTCTTCCGC GTCCACATCC CGCAGCCAA CGGCCGGACA
251    CTGTGCTGTC AGTGTGGCTC CAACCCCATC GATGTCTCTC AGCGCTCAGC
```

**Figure 3.6.** Sequences obtained for the products of PCR homology cloning, for **A.** GATA and **B.** NFAT.

#### 3.3.2 LIBRARY SCREENING

A representative sample of the plaque-lift hybridisations is shown in Figure 3.7. This demonstrates the progressively more discreet plaques required for obtaining monoclonal plaques. The first round of library screening resulted in twelve positive plaques, which were carried forward to the next screen. By the time of the third round of screening and the subsequent phage excision, this was narrowed down to ten

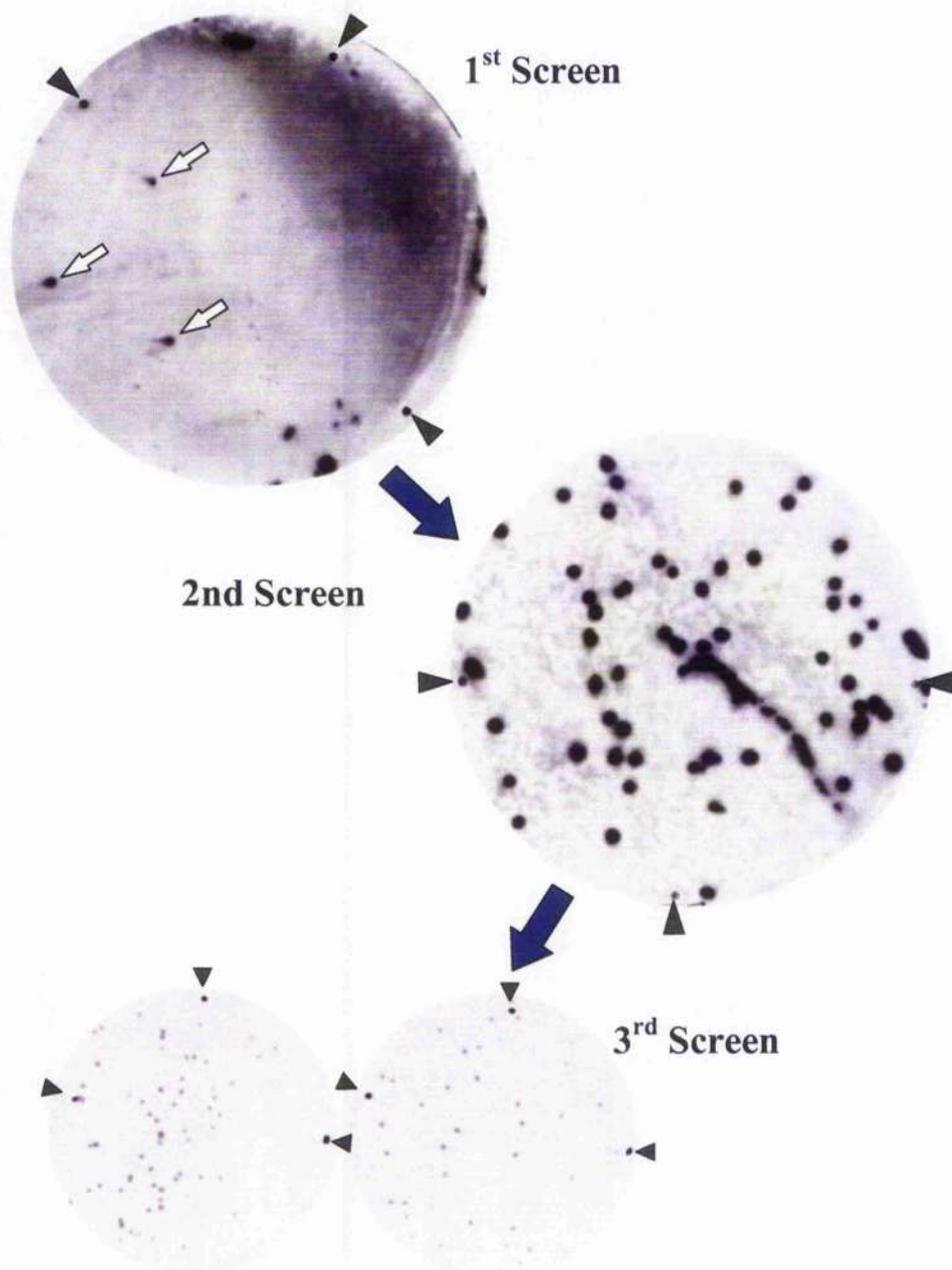
positive, monoclonal plaques. The initial round of sequencing revealed four GATA-homologous clones, and six NFAT-homologous clones. These were termed GATA clone-1, -4, -7 and -10, and NFAT clone-2, -3, -5, -6, -8 and -9 respectively. Comparison of the sequences with known GATAs and NFATs through BLAST homology search indicated that the GATA clones-1, -4 and -10 were truncated at the 5' end. GATA clone-7, however, appeared to represent a full-length cDNA, and it was this clone that was used for all further work. All of the NFAT clones examined, however, appeared to be truncated, each missing 1 kb of sequence from the 5' end. The first of these, NFAT clone 2, was of poor DNA quality, and so NFAT clone 3 was used for further restriction mapping and sequence analysis.

### **3.3.3 GATA**

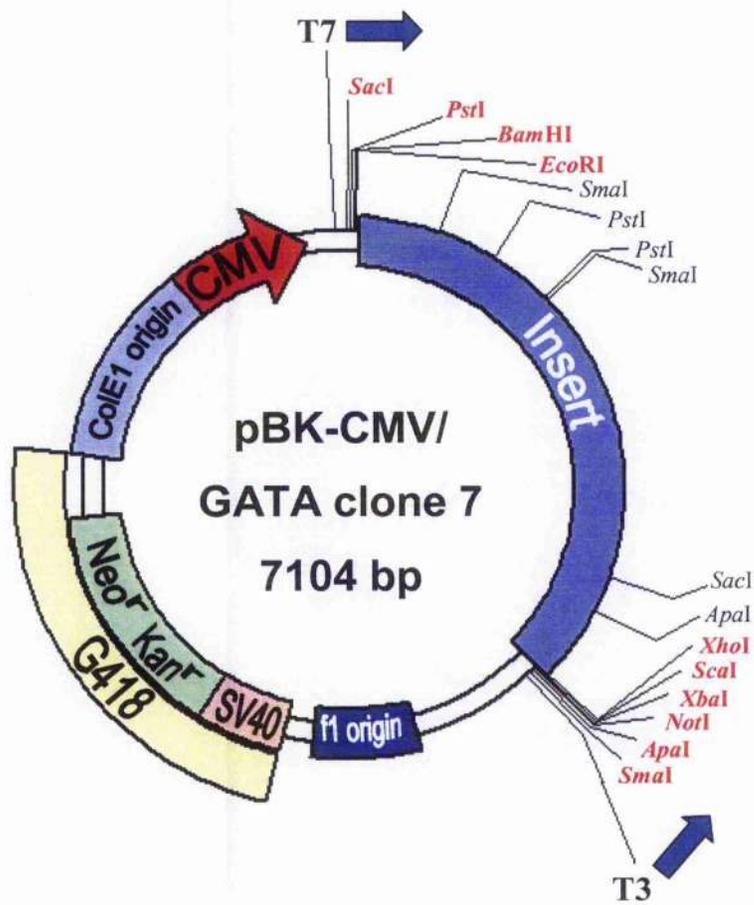
A restriction map of GATA clone-7, showing the enzymes used in generating the map, is given in Figure 3.8. There were few internal restriction sites within the insert, which restricted the number of sub-clones that could be generated for sequencing. A single *Pst*I sub-clone was generated for automated, ABI sequencing, in addition to the original phage excision product.

#### **3.3.3.1 Primary Sequence Data**

Obtaining primary sequence for the GATA clone proved to be problematic, with a short stretch of the 5' region of the coding sequence (bases 696 to 733 of the final sequence) proving to be unobtainable by standard sequencing methods. This problem persisted in both directions of sequencing. This was attributed to the formation of secondary structure in the DNA molecule, likely due to a high G-C content in that particular region. In order to reduce the incidence of such secondary-structure formation and resolve these difficulties, several different techniques were employed.



**Figure 3.7.** Results of successive rounds of library screening. Shown here is a representative sample of the plates used in screening the ZAP-Express<sup>TM</sup> library. The white arrows on the first screen denote positive plaques, which can be harder to distinguish from background save for the characteristic 'tails' caused by lifting the filter from the plate. The black triangles denote marks corresponding to those on the filters, added to the film to align with the plates.



**Figure 3.8.** Restriction map of the full-length GATA clone ligated into the pBK-CMV vector. Sites in the multiple cloning site (MCS) are shown in red. Vector features shown include: origins of replication and antibiotic selection genes for both prokaryote and eukaryote hosts, cytomegalovirus promoter and T3 and T7 primer sites used for initial sequencing.

### 3.3.3.2 High G-C Sequencing

The first attempt to remove secondary structures was the use of DMSO. This approach was unsuccessful, with the sequence terminating at the same region as before. Secondly, a single-stranded form of the plasmid template was prepared in the hope that this would exhibit less secondary structure. The single-stranded DNA synthesis proved successful, but there was no noticeable improvement in sequencing with the change in template. However the third and final method using the ABI PRISM dGTP BigDye Terminator system did solve the problem of secondary structure in the GATA clone. A complete read-through of the problem region was obtained, which completed the primary sequence for the porcine muscle GATA.

### 3.3.3.3 Sequence Characterisation

A number of sequencing runs were made for each set of sequencing primers, with comparisons between the multiple sequences sufficient to resolve any sequence ambiguities encountered. The final, full-length GATA sequence is listed in Figure 3.9. The most likely translation start site is located at nucleotide 256. This ATG codon has a flanking G residue at position +4, but lacks the purine residue at position -3 that is the other element of a strong consensus site or ATG 'context' (Kozak, 1996). Nevertheless, this site remains an adequate context according to the criteria described by Kozak (1996), of YNNatgG (where Y is pyrimidine). The only other, upstream ATG (nucleotide 26) also possesses only one of the two flanking elements, and is followed by an in-frame stop codon (nucleotide 92).

Using the ATG at 256 as the presumptive start site, the in-frame stop codon is located at nucleotide 1696, giving a deduced peptide of 480 amino-acid residues. Calculating the protein size from the deduced amino acid sequence using PeptideMass software (Wilkins *et al.*, 1997) gave a predicted size of 52.98 kD. Also shown in Figure 3.9 are putative functional domains on the porcine GATA-2 clone, based on comparison with other family members. The zinc-finger DNA binding domains are present at amino acid residues 296 - 319 and 349 - 373. The basic region runs from amino acid residues 374 - 397.

1 GAGCTGGCGC CAGGGCGGCC GGAGGATGCC GAGGGGCGCG AGCCGGGCGG GCCTGAGGCC GAGGCGCGTG  
 71 CTGCCCTAG CTCACACCTT GTGAGCCCGC CCAGCCGCCC GACGCACTGT CCCTGAGCCC TCAGTCCCCC  
 141 CCACCCGGCT CCGTAGGTG CTGCCGCTC TGAGGCGCGC CCCCCTCCC TGCGGCGCTG CCGCTGCGGA  
 211 CGCTGCCACC GCCTCCGCC AGATCCCGAT CCTCCGCGC CGGCC  
 256 **ATG** GAG GTG GCG CCC GAG CAG CCG CGC TGG ATG GCG CAC CCC GCC GTG CTG AAT GCG CAG  
 1 M E V A P E Q P R W M A H P A V L N A Q  
 316 CAC CCC GAC TCG CAC CAC CCG GGC CTG GCG CAC AAC TAC ATG GAG CCC GCG CAG CTG CTG  
 21 H P D S H H P G L A H N Y M E P A Q L L  
 376 CCT CCC GAC GAG GTG GAC GTC TTC TTC AAC CAC CTC GAC TCG CAG GGC AAC CCC TAC TAC  
 41 P P D E V D V F F N H L D S Q G N P Y Y  
 436 GCC AAC CCG GCC CAC GCG CGG GCG CGT GTC TCC TAC AGC CCC GCT CAC GCC CGC CTG ACC  
 61 A N P A H A R A R V S Y S P A H A R L T  
 496 GGA GGC CAG ATG TGC GCG CCA CAC TTG TTG CAC AGC CCA GGG CTG CCC TGG CTG GAT GGG  
 81 G G Q M C R P H L L H S P G L P W L D G  
 556 GGT AAA GCA GCC CTC TCG GCT GCT GCA GCA CAC CAT CAC AAC CCC TGG ACC GTC AAC CCC  
 101 G K A A L S A A A A H H H N P W T V N P  
 616 TTC TCC AAG ACG CCA CTG CAC CCT TCA GCT GCT GGA GGC CCT GGA GGC CCC CTC TCC GTG  
 121 F S K T P L H P S A A G G P G G P L S V  
 676 TAC CCA GGG GCA GGG GGT GGG GGT GGG GGA GGC AGT GGC AGC TCT GTG GCC TCC CTC ACC  
 141 Y P G A G G G G G G G G S G S S V A S L T  
 736 CCC ACC GCA GCC CAC TCT GGC TCC CAC CTC TTC GGC TTC CCA CCT ACA CCA CCC AAG GAA  
 161 P T A A H S G S H L F G F P P T P P K E  
 796 GTA TCT CCT GAC CCC AGC ACC ACC GGG GCT GCC TCC CCA GCC TCG TCC TCT GCA GGG GGT  
 181 V S P D P S T T G A A S P A S S S A G G  
 856 AGT GCA GCC CGG GGG GAG GAC AAG GAC GGC GTC AAG TAC CAG GTG TCA CTG ACT GAG AGC  
 201 S A A R G E D K D G V K Y Q V S L T E S  
 916 ATG AAG ATG GAA AGC GGC AGT CCC TTG CGC CCA GGT CTG GCT GCC ATG GGC ACC CAG CCT  
 221 M K M E S G S P L R P G L A A M G T Q P  
 976 GCC ACG CAC CAC CCT ATC CCG ACC TAC CCC TCC TAC GTG CCA GCT GCC GCC CAT GAC TAC  
 241 A T H H P I P T Y P S Y V P A A A H D Y  
 1036 AGC AGC GGG CTC TTC CAC CCC GGA GGC TTC CTG GGT GGC CCT GCC TCC AGC TTC ACC CCT  
 261 S S G L F H P G G F L G G P A S S F T P  
 1096 AAG CAG CGG AGC AAG GCA CGC TCC TGC TCA GAA GGC CGG GAG TGT GTC AAC TGT GGG GCG  
 281 K Q R S K A R S C S E G R E C\* V N C\* G A  
 1156 ACA GCC ACC CCT CTC TGG CGC CGA GAC GGG ACC GGC CAC TAC CTG TGC AAC GCC TGG GGG  
 301 T A T P L W R R D G T G H Y L C\* N A C\* G  
 1216 CTG TAC CAC AAG ATG AAC GGG CAG AAC CGG CCA CTC ATC AAG CCC AAG CGG AGA CTG TCG  
 321 L Y H K M N G Q N R P L I K P K R R L S  
 1276 GCC GCC AGG AGA GCA GGC ACC TGT TGT GCA AAT TGT CAG ACG ACA ACC ACC ACC TTA TGG  
 341 A A R R A G T C C\* A N C\* Q T T T T L W  
 1336 CJC CGA AAC GCC AAC GGG GAC CCT GTC TGC AAC GCC TGT GGC CTC TAC TAC AAG CTG CAG  
 361 R R N A N G D P V C\* N A C\* G L Y Y K L H  
 1396 AAT GTA AAC AGG CCA CTG ACC ATG AAG AAG GAA GGC ATC CAG ACT CGG AAC CGG AAG ATG  
 381 N V N R P L T M K K E G I Q T R N R K M

(Continued overleaf)

```

1456 TCC AAC AAG TCC AAG AAG AAC AAG AAG GGT GCC GAG TGC TTC GAG GAG CTG TCC AAG TGC
401  S  N  K  S  K  K  N  K  K  G  A  E  C  F  E  E  L  S  K  C

1516 ATG CAG GAA AAG GCC TCC CCC TTC AGC GCG GCT GCC CTG GCC GGA CAC ATG GCA CCC GTG
421  M  Q  R  K  A  S  P  F  E  A  A  A  L  A  G  H  M  A  P  V

1576 GGC CAC CTG CCG CCC TTC AGC CAC TCG GGA CAC ATC TTG CCC ACG CCG ACG CCC ATC CAC
441  G  H  L  P  P  F  S  H  S  G  H  I  L  P  T  P  T  P  I  E

1636 CCC TCC TCC AGC CTC TCC TTC GGT CAC CCC CAC CCG TCC AGC ATG GIG ACC GCC ATG GGC
461  P  S  S  S  L  S  F  G  H  P  H  P  S  S  M  V  T  A  M  G

1696 TAG
481  *

1699 GGACTGCCCA CACCAACCGA CAGTCAGATC CCCCCACGA AGCCCCCCC AGGATAGGAG GACCAGACCC
1769 TTAGCTGCCC GTCCTTCCCT GGAGACTGAC ACACCCCTGC CAGCGCAGCC GGAGCCCCAC GCTGCCTCAC
1839 CTGTGGGGCG TCCACTCCAT CGGCCGTCAC TGTAAGATT CACCAGAGGC TGGGAGGCCG CTTGAGCCTG
1909 GCTGCCACCA GGTGGGCTT CCTGCATGGA CACCTGTTT CAGAGCAGAA AGGACAACCT TATGAAGATG
1979 AAAGAGGGGG GCAAGGGACA AAACACCAA ACCATTTTTA GAAGAAGAAG GAGTAGGCAA ACATAATTTA
2049 TTTTGCTCTT GILTCIAACA AGGCTTGGGA GACTCAGGAG GCATGGGGCTG CCGTAGGCTC TCTGGCTCTC
2119 CTCAGAGCCA AGGGCCACTT GGCTGGCCCG CCTGCCAGGC TCTGGGGCCA GGTCTCCAGA GCCCTCAACC
2189 CACATGCTTT CCTCTAGCTC CCTCTCTGAA GCAGCTGGAC CCAAGTTGGC CTGAGCCAAG CCAGAGGGGC
2259 CCCAGCCGGG GAGGCTGAGC CAGTGCAGGC CCTGGGGCCU AAGCCCTGGA GTCCAGAGAC AATCAAGGGC
2329 GGTCTGCGC AGATTCCCAG GCCAGGGCTG GGTACAGGA AGGAACATT TTCTTGAGAG GGGAAACCTC
2399 TCCCAGATCG CTCCTCTGCG TCTGAGGCTG AAGCTGGTGT GACCCACGTC CCCTAACGG GCCTGAGCCA
2469 TAGCCTGGCT CCTCTGCCAG TGGACCCCTG TACATAGATC TTTTTCTTG CTAACTTCTC AACCCCTCC
2539 TCTCCAACTC CGAGAC

```

**Figure 3.9.** Nucleotide sequence and deduced amino acid sequence of porcine GATA-2 transcription factor (1-2554 bp). Underlined sequences denote primers used for cycle sequencing. Blue shaded sequence denotes putative zinc-finger DNA binding domains, with asterisks marking the cysteine residues responsible for generating the zinc finger domain. Green shading denotes basic region (nuclear localisation signal).

Porcine	MEVAPEQPRW	MA..HPAVLN	AQHFDSSHFG	LAKNYMEPAQ	LLPPDEVDVF	50
GATA2	-----G-	-----	-H-----	-----H	V-----	
GATA3	---TAD---	VSIHI---	G---T---	-S-S-DA--	YPL-E---L	
GATA1	--F....PG	LG....SIG	YSH.....	-P.QFVD--L	VGSTP-SG--	
GATA4	-YQS.....	LP..WP....	---TTGRPP	VPTTRAA--P	SCUARARU..	
GATA5	-YQS.....	L.....	.....	---AS-R-	AAAYADSGS..	
GATA6	-YQT.....	L.....ALS	SGG..AAYDG	APGGFVHS-A	AAAAAAAAA..	
Porcine	F...NHLOS	QGN...PYVA	NPABARARVE	YSPAILARLTG	GQMCPRPHLLH	100
GATA2	-----	-----	-----A-	-----	S-----	
GATA3	F....NI-G	---HVP---	-SVR-.CVQR	-PPTH...HG	SQVCRPPLLH	
GATA1	FPSGPEG--A	AASSTA-.S	TXATAAAAAA	.....	.....	
GATA4	.....R	OST..C-ERG	ALLRAGPVL	..PPGR--RG	LCVRRP....	
GATA5	F...L-APG	AGS...MFV	P--RVPSMLS	.....LSG	...CEP....	
GATA6	.....	ASS...VYV	PTTRVGSMLD	GLPYH..LQG	..SGSGPAN..H	
Porcine	SPGLPWLDGG	KAALSAAAAH	KHNPTVTPNF	SKTPLHPSAA	GGPGGPLSVY	150
GATA2	-----	-----	-----S-	-----	-----	
GATA3	G.S-----	--LG-HHTA	..S--NLS--	---SI-H...	-S-----	
GATA1	.....YRD..	.....AEAY	RHSP...V-	QVY--LNCME	GIPG.....	
GATA4	.....S--	SSGGAASGA	.....	.....	..GPG.....	
GATA5	--QP-E-...	.....AA.	..RP...GW	AQTATAD-S-	RGPGSP...H	
GATA6	A.....	.....AGA.	..HP...GW	PQAS..AD-PP	YSGS.....	
Porcine	PGAGGGGGGG	SGSSVASLTP	TAHSGSHLF	GFPPTPKKEV	S.P.DPSTTG	200
GATA2	-----S-	-----	-----	-----	-----	
GATA3	.....PA	-S--LSG...	..G-ASP---	T-----D-	-----	
GATA1	.....	..G-PYAGW..	..YKKTG-	.Y...-AST-	C.....-R	
GATA4	.....TQQ-S	PGW-Q-GADG	..Y.....	...T-P-	...-RF...	
GATA5	.....	.....	P---	.....-GAT	AF.....F	
GATA6	..G-AA--A	A-PGG-GS..	A---VSAR.F	PYSPS--MAN	GAARE-G..-	
Porcine	AASPASSSAG	GSAANGEDKD	GVKIQVSLTE	SMKMBSGSPL	RPGLAAMGTQ	250
GATA2	-----	-----	-----	-----	-----T-	
GATA3	LST-G-...	...QDE-E	CL---P-PD	...L...SS	GSMT-L-GA	
GATA1	EDS-PQ...	...AV--L-	..GTSFSL-	TLKTER...	SPD-LTL-PA	
GATA4	..F-GITGSL	AA--AAAARE	AAAY.....S	-GGGAA-AG-	A..-REQY-RA	
GATA5	H..SGPGS-	..SAG-RD..	-SA--GA...	..LPRKQ...	..FA-IL-RP	
GATA6	Y-AAGSGG--	-VSGG-SSLA	AMGGREPQYS	LSAAR...-	N.GTYHHHHH	
Porcine	P.ATHHFIPT	YPSYV.PAAA	HDY.....S	SGLF.....H	PGGFLLGGPAS	300
GATA2	-----	-----	-----	-----	-----	
GATA3	SSS-----T-	--P-----	PE-----	-----P...	---SSL---SPT	
GATA1	L.PSSL-VP.	NBA-GGP...	-F.....	-ST...FS	-T-SPLNS-A	
GATA4	AP-GSYSSP.	A.M.ADVG	ASWAAAAAA	A-P-DSZVL-	SLPGRAN-GA	
GATA5	V.GTSYSAT.	--A-VS-DV-	QSW.....T	A.P.DGSVL-	...-LPGRRPT	
GATA6	N.HK--SP.	YSP-VGAPLI	PAW.....P	A-P-ETPVL-	SLQSRAGAPI	
Porcine	SPT.PKQRSK	ARSCS.EGRM	CVNCGATATP	LWRRDGTGHY	LCNACGLVHK	350
GATA2	-----	-----	-----	-----	-----	
GATA3	G-G.C-S-P-	---ST.	-----S-	-----	-----	
GATA1	YSS.P-L-GT	LPLEPC-A--	-----	-----R---	-----	
GATA4	RHP.N..IDM	FDDFS.	...-MG-	-----	-----	
GATA5	FVS.....DF	LEEFPG---	-----LS-	-----	-----	
GATA6	FVPRGFSADL	LEDLS--S-	-----SIQ--	-----	-----	
Porcine	MNGQNRPLIK	PKRRLSAARR	AGTCCANQCT	TTTTLWRRNA	NGDFVCNACG	400
GATA2	-----	-----	-----	-----	-----	
GATA3	-----	-----	-----S-	-----	-----	
GATA1	-----R	K.IVSK-	---Q-T---	-----	S-----	
GATA4	---I-----	-Q-----S-	V-LS-	-----	E-I-----	
GATA5	---V---VR	-QK---SS-	-L--T--H-	N-----	E-E-----	
GATA6	---LS-----	-QK-VFSS-	L-LS----H-	-----	E-E-----	

(Continued overleaf)

```

Porcine      LYYKLNHNVR PLTMKKEGIQ TRNRKMSNKS KKNK...KGA ECFEELSK.. 450
GATA2       -----S-----
GATA3       .T.-----S-- --C...-VH DSL-DFP-..
GATA1       -----Q---R-D---A-G-C --KRGSSLGG TGAA-GPAGG
GATA4       --M--G-P--A-R-----K--PK-LK -SK...PAAP SGS-S-PP..
GATA5       --M--G-P--A-----S--K--PKIA -ARG..SSGS TRNASASP..
GATA6       --M--G-P--A-----K--PK-IN -SKT...CSG NSNNSIPM..

Porcine      .CMQEKASPF SAAALAC.HM A...PVGHL PP.PSESGH. ....IL 500
GATA2       -----S-----
GATA3       .....N-S- NP--SR-- S...SLS-I S-----S- .....M-
GATA1       #MNVVAGG-GS GNCGEVASGL T..LG-.PGT AHLYQGL-PV VLSGPVSHIM
GATA4       .ASGASSNSS N-TTSSSEE- R.PIKTEPGL SSKYGHSSSV SQTFSVSAM.
GATA5       .SAVASILLSS A-TSK-KPSL ASPVC-GPSM A...QAS-Q. ....EDDSL
GATA6       .TPTSIS-NS DDCSKNTSPT TQPTASGAG. APVMTGA-E. ....STN

Porcine      PIPTP.I.IIP GSS..LSPGH P...IIP... SSMVTAMG.. .... 550
GATA2 ( 451) -----Y-----
GATA3 ( 445) T----M-- P-----H-----
GATA1 ( 414) -F-G-LLGS- TG-----F -TGPMPTTS TTV-AEPLSS. ....
GATA4 ( 424) SGHG-SI-- VL-.A-KLS. -QGYAS-VSQ -PQTSSKQDS WNSLVLADSH
GATA5 ( 389) -GHLEPKPE- ..E...DFAF -STAPS-.O AGLRG-LRO. ....EAW
GATA6 ( 440) -ENSE.L.KY -GQDG-YIGV S..LAS-... AEVTSSVR.. ....PDSW

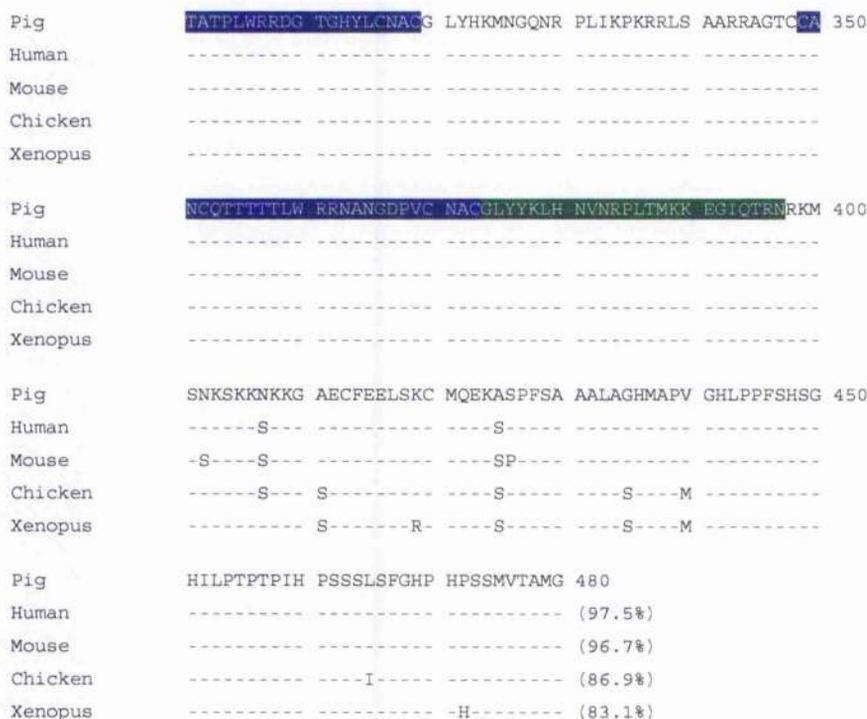
Porcine      ..... 600
GATA2 ( 481) ..... (97.5%)
GATA3 ( 445) ..... (62.8%)
GATA1 ( 414) ..... (36.8%)
GATA4 ( 424) GDIITA (34.3%)
GATA5 ( 389) CALALA (34.9%)
GATA6 ( 440) CALALA (34.0%)

```

**Figure 3.10.** Sequence comparison of the deduced amino acid sequence of the acquired GATA clone with those of different human GATA isoforms (Accession N<sup>o</sup>s NM\_002049, M68891, A39794, I57561, NP\_536721 and Q92908 for human GATA 1, 2, 3, 4, 5 & 6 respectively). A '-' denotes a homologous residue. The percentage amino-acid homology with GATA clone-7, as determined by ALIGN software, is shown at the end of each sequence.

Pig	MEVAPEQPRW	MAHPAVLNAQ	HPDSHHPGLA	HNYMEPAQLL	PPDEVDVFFN	50
Human	-----	-----H	D-----	-----HV-	-----	
Mouse	-----	-----	-----	-----	-----	
Chicken	---TD---	-T-H---	G--E---	-----	-----	
Xenopus	---TD---	--H--LN-	-----	-----T-	-----	
Pig	HLDSQGNPYY	ANPAHARARV	SYSPAARLT	GGQMCRPHLL	HSPGLPWLDG	100
Human	-----	-----A-	-----	-S-----	-----	
Mouse	-----	-----	-----	-----	-----	
Chicken	-----	--S-----	--Q-----	-S-----I	---I---S	
Xenopus	-----	--S-----	--Q-----	-S-----	---L---ES	
Pig	GKAALSAAAA	HHHPWTVNP	FSKTPLHPSA	AGGPGGPLSV	YPGAGGGGGG	150
Human	-----	-----S-	-----	-----	-----S-	
Mouse	-----	-----S-	-----	--S-----	---A-I--	
Chicken	S-----...	-----	-T-----	--A--AI--	---...SST	
Xenopus	--T-----...	-----S-	-G-A--A-	R-...S-	-----	
Pig	SGSSVASLT	PTAAHSGSHL	FGFPPTPPKE	VSPDPSTGA	ASPASSSAGG	200
Human	-----	-----	-----	-----	-----	
Mouse	C-----	-----	-----	-----	-----	
Chicken	S-TA--S---	-A.S-----	-----	-----NS-S-	---...A	
Xenopus	-T-----AC	-SSS--SP--	-----	-----...-P	--P-----	
Pig	SAARGEDKDG	VKYQVSLTES	MMESGSPLR	PGLAAMGTQP	ATHHPIPTY	250
Human	-----	-----	-----	---T-----	-----	
Mouse	-V-----	-----S--	---G-----	---T-----	-----	
Chicken	...-Q---S	I-----S-G	---A---	SS-TS--A--	S-----	
Xenopus	...-L---S	I---M--S-G	---GG---	SS--P---C	S-----	
Pig	SYVPAAAHDY	SSGLFHPGGF	LGGPASSFTP	KQRKARSCS	EGRE <span style="background-color: #ccccff;">VMCGA</span>	300
Human	-----	-----	-----	-----	-----	
Mouse	-----	G-----	-----	-----	-----	
Chicken	-----	--S-----S-	-----	-P-----	-----	
Xenopus	-----	-----SL	-----	-----S-	-----	

(Continued overleaf)



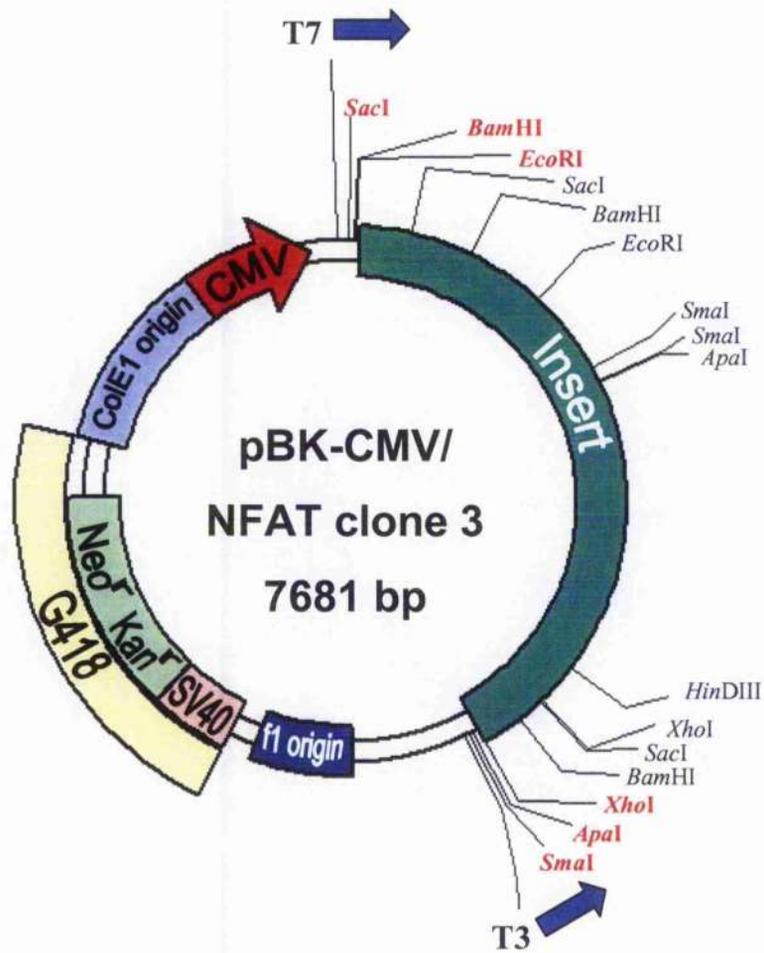
**Figure 3.11.** Pileup of amino-acid sequences for GATA-2 transcription factors across a range of species (Accession N<sup>os</sup>: human P23769, murine AK004675, chicken P23824, *Xenopus* P23770). A '-' denotes a homologous residue. Blue shaded sequence denotes putative zinc-finger DNA binding domains. Green shading denotes basic region (nuclear localisation signal). The percentage homology with GATA clone-7, as determined by ALIGN software, is shown at the end of each sequence.

The GATA clone-7 sequence was submitted to GenBank (Accession N<sup>o</sup> AY251012). Comparison with other, previously described family members by ALIGN software (Pearson *et al.*, 1997) showed highest homology with human GATA-2 (Accession N<sup>o</sup> M68891), with an amino acid homology of 97.5%. A sequence comparison with other GATA isoforms is shown in Figure 3.10, showing GATA-2 to be the most likely isoform. A summary of the sequence homologies with GATA-2 molecules from other species is given in Figure 3.11. As might be expected, given the homology PCR-based strategy used in its isolation, GATA clone-7 showed high homology with previously characterised family members, particularly in the zinc-finger DNA-binding domains.

### 3.3.4 NFAT

NFAT cloning and sequencing presented entirely different problems to those encountered in obtaining the GATA-2 clone. Restriction digest analysis and initial sequencing of the six putative NFAT clones seemed to indicate little, if any, difference in their sequences. A restriction map of NFAT clone-3 is shown in Figure 3.12. This map was common to all the NFAT clones analysed. As stated in Section 3.3.2, initial sequencing showed that all of the clones were missing an estimated 1 kb of sequence from the 5' end, in comparison with known NFAT sequences. Based on BLAST homology searching, the section of transcript isolated started in the middle of a coding sequence, and was thus unlikely to represent an alternate splice variant. A repeat of the library screening process was no more successful. A PCR modification was attempted, in order to generate a useful clone from the incomplete material as detailed in Section 3.3.4.3.

The truncated sequence, obtained from NFAT clone-3, is shown in Figure 3.13. Also shown is the deduced amino acid sequence from the available coding sequence. Comparisons of this sequence with previously published data, through ALIGN software (Pearson *et al.*, 1997), showed a high homology (96.8%) with a recently published porcine NFAT-2 (Accession N<sup>o</sup> AF069996) isolated from macrophages termed NFAT<sub>mac</sub> (Miskin *et al.*, 1998). A sequence comparison with other NFAT isoforms is shown in Figure 3.14, showing that the NFAT clone is likely to be an NFAT-2 isoform (with a sequence homology of 82.6% with human NFAT-2).



**Figure 3.12.** Restriction map of the truncated NFAT clone ligated into the pBK-CMV vector. Sites in the multiple cloning site (MCS) are shown in red. Vector features shown include: origins of replication and antibiotic selection genes for both prokaryote and eukaryote hosts, cytomegalovirus promoter and T3 and T7 primer sites used for initial sequencing.

1 .CC GAC TTC CCA CCT GAG GAG TTT CCG CCC TTC CAG CAC ATC CGC AAG GGC GCC TTC TGC  
1 D F F P E E F P P F Q H I R K G A F C  
60 GAC CAG TAC CTG TCG GTG CCG CAG CAC CCA TAC CCG TGG GCC AGG CCA CGG TCC CCG ACG  
20 D Q Y L S V P Q H P Y P W A R P R S P T  
120 CCC TAT GCC AGC CCG TCT CTC CCT GCC CTG GAC TGG CAG CTG CCG TCA CAC TCA GGA CCC  
40 P Y A S P S L P A L D W Q L P S H S G F  
180 TAC GAG CTG AGG ATC GAG GTG CAG CCC AAG TCC CAC CAC AGA GCT CAT TAC GAG ACA GAG  
60 Y E L R I E V Q P K S E H R A H Y E T E  
240 GGG AGC CGT GGG GCT GTG AAG GCA TCG CCC GGA GGA CAC CCC AGC GTG CAG CTG CAC GGC  
80 G S R G A V K A S A G G H P S V Q L H G  
300 TAC GTG GAG AGC GAG CCG CTC ACC CTG CAG TGG TGC ATC AGG ACG GCA GAC GAC CCG CTA  
100 Y V E S E P L T L Q L F I R T A D D R L  
360 CTG AGG CCG CAC GCC TTC TAC CAA GTG CAC CCG ATC ACT GGG AAG ACC GTG TCC ACC ACC  
120 L R P H A F Y Q V H R I T G K T V S T T  
420 AGC CAC GAG GCC GTC CTC TCC AAC ACC AAG GTC CTG GAA ATC CCG CTT CTG CCG GAA AAT  
140 S H E A V L S N T K V L E I P L L P E N  
480 AAC ATG CGC GCA ATC ATT GAC TGT GCT GGG ATC CTG AAG CTC CGA AAC TCT GAC ATT GAG  
160 K M R A I I D C A G I L K L R N S D I E  
540 CTT CGC AAA GGG GAG ACG GAC ATT GGG CCG AAC AAC ACC AGA CTG AGG CTG GTC TTC CGC  
180 L R K G E T D I G R K N T R V R L V F R  
600 GTC CAC ATC CCG CAG CCC AAC GGC CCG ACA CTG TCG CTG CAG GTG CCC TCC AAC CCC ATC  
200 V H I P Q P N G R T L S L Q V A S N P I  
660 GAG TGC TCC CAG CGC TCA GCC CAG GAG CTG CCC CTC GTG GAG AAG CAC AGC GCA GCC AGT  
220 E C S Q R S A Q E L P L V E K Q S A A S  
720 TGC CCA GTC CTC GGC GGG AAG AGS ATG GTC CTG ACT GGC CAC AAC TTC CTG CAG GAC TCC  
240 C P V L G G K R M V L T G H N F L Q D S  
780 AAG GTC GTT TTC CTG GAG AAA GCA CCA GAC GGC CAC CAC ATC TGG GAA ATC GAG GCG AAA  
260 K V V F V E K A P D G H H I W E M E A K  
840 ACC GAC GGA GAC CTG TCC AAG CCG AAT TCC TTG GTG GTC GAG ATC CCG CCT TTC CGC AAC  
280 T D G D L C K P N S L V V E I P P F R N  
900 CAG AGA ATC ACC AGC CCC CTC CAG GTC AAC TTC TAC GTG TGC AAC GGG AAG AGA AAG AGG  
300 Q R I T S P V Q V N F Y V C N G K R K R  
960 AGC CAG TAC CAG CAT TTC ACC TAC CTG CTT GCC AAC GCT CCA GTC ATA AAA ACG GAG CCC  
320 S Q Y Q H F T Y L P A N A P V I K T H P  
1020 AGT GAT GAC TAC GAG CCT GCC CTC ACC TGT GGA CCA GTG AGC CAG GGC CTG AAC CCG CTC  
340 S D D Y E P A L T C G P V S Q G L N P L  
1080 ACG AAG CCT TGC TAT GGC CCG CCA CTC GCC CTG CCA CCC CAT CCC AGC TCC TGC CTT GTG  
360 T K P C Y G P P L A L P P D P S S C L V  
1140 GCC GGC TTT CCA CCC TGT CCA CAG AGA AGC GCC GTG ATG TCT CCG CCA CCC AGC GCC AGC  
380 A G F P P C P Q R S A V M S P P P S A S  
1200 CCG AAG CTC CAC GAC CTC TCC TGT GCC CCC TAC AGC AAA GGC ATG GCC GGC CCG GGC CAC  
400 P K L H D L S C A P Y S K G M A S P G H  
1260 CTT GGA CTT CAG CCG CCA GCC GGA GGG GTC CTT GGA GGC CAG GAG GCA CCC AGA CCC CCG  
420 L G L Q R P A G G V L G G Q E A P R P G

(Continued overleaf)

1320 GGC CCA CAT CCC GGC GCT CCC CAG CCA CCA CCC CCC GGC CTG CTG CAG CCA CAG TAA  
440 G P H P G A P Q P P P P A L L Q P Q \*  
1377 ACGAAATCAAT ACGAAACCGAC CTCTCCAGCA CCAATATCCA CTATAGTTGG CAACGTGGGA CTCTCAGAAG  
1447 CTCCGCAGCA TGGAUATGAT GGCAACAAC TCTGAGTAAA CACCCGTAAC TCACACGGGC ACCTGGTACC  
1517 TCTCAGAACT GCCCACCTGCA GGCTGACGTC ATGTGTACAG ACAACCTGACT CCGCCCAGGA AGGGATGCTC  
1587 CACCTCCTGA GGGAAAGTGT CTCAACAAGC AGAAGAAAAC GGGTGGCTGG GGTGAGCGTG GGACAGCCCG  
1657 CGTGAGGCTC CCCTAAGCCA CCCCCTGCC GAGGACGCT GTCCCLAGEC CTCACCCCTC CGGCACCCCT  
1727 GAAATTTCAAG CUGGAAGTGC CTTAATTCCC CGACCACACC ATCAGGGTGT CGCAGACTCC AGCAGTGAAT  
1797 TGTCTACCG GAAAGTATT GTAGGAGCAG AAGCTAAGA CCCTTGGCCA ACAGGCTTTA TTCTTTGTAC  
1867 AAATAAGGG AATCCTGGGG GGCAGAGCAT CTGGAGGAAG CCUAGTGTCT GTTCCTCTCT GTCTTCAACC  
1937 TTCTCTGGCC GGGAGCATCA TGCTATATCC GTTCCTTAT TCAGTGCATT TCAGACTGCT CCCAGGCTC  
2007 ATCTCAGCTC TITTATAACC TTCTCTCCCC GCCCCCGTGG TACGCAAACC ATAGGGCTGT TCATTGTAGC  
2077 ATGTTAAGAT TTTGTTTTTA ATCTGGCTTC AAACACANGC ACAAGTAAGT TGAATAGCAC AAGACAGGTT  
2147 ACTGGACAAA AAAAGAAATC TCTCCGCGTC AGGCTAGATT TTGCATTTGC ATGTGTCTAC ATATATAGGC  
2217 ATATATTTAT GTATGACAA TAACAGAGGT GGTGATATAC AGCCTTCGCC CAGCAGGGGC GTCCGCATTA  
2287 GCAATAACCA GTGTCCATTT TGGAAAGACC ACCCATGCCC CCAAACCTGG CTCCCTAGCC ATTCCTGGTG  
2357 GGGAGCCCGG CCCCCTCTC CCCCGCATCT GCGTTTCGTC GCCTCCTGCA GTTAACCAAG CACTTTAGAA  
2427 GCCAGGAGAG GTGCGTTCAC AGTACCGTCT GTACCCAAT GCCAAAATTG GAAAGAAACT GTAATATGCA  
2497 TTACAGAAGG AGTGTAAATC ATTGTTCAG CTGTAAAGATG GTATTTTCAC ACAAGCCTTA TAACCTGGCT  
2567 TCAUGAACA AAACUATTAC CAAAAACAGC ATTTTCATAT GCAGCGACTG TAGTGTGTTT CCAGACTAGT  
2637 TACTGTTAAT ACGTAGGTTA GTGTAAGCTT TCTGCTGCAT TCCGTACACT TAACAGCTTA TTAGATGATT  
2707 GCCAGCAGTG ACCCGTGTGC TCACGTAACC AAAAAGCATG GTGTGACGAA CACGGTTAAC CTACTAAGTG  
2777 TGCCCTAGGA ACTCAACCCC CTCATGGAG CACGGGGGAA TCCGACCCCTC CGCCGAGGTG ACAAGCAGTT  
2847 CCCCCTACTC CAGACCTCTC CCACAGAAAG AATCACTTAA ATGTCTTCT GTCCAAATCAT GTCTTACTTT  
2917 GTAAGTCACA GAGGCGAACA TCTGAAAGCC ATATGAAACC ATTTGCACAT TACTGTGAAG AGCCGAGGAT  
2987 CCATGGCTTC GTGTGCTAG ACCTGCCCGC ATTAGCTCT GTAGGACTTG CATGAGACAT TTCTTTCGTA  
3057 GACTGTGATG GAAGGCACAC GTTCCTCTCT GTGTCAGCTA TCTCTGTAGT TAGAAGTAGG TCTTAATAAA  
3127 GCAATATTTT TTTGCCTGG

**Figure 3.13.** Nucleotide sequence and deduced amino acid sequence of porcine NFAT-2 transcription factor (1-3144 bp). Underlined sequences denote primers used for cycle sequencing.

```

Porcine ( L) ..... ..DFPPEEF PPFQHIRKGA FCDQYLSVPO H.PYPWARFR 50
NFAT2 ( 332) FVGEDLGSPP PPA--A--DY SS-----G -----A--- -.-Q--K-K
NFAT1 ( 313) SAAPSAGLPL RHLYPAV-PI G-CRCGERRN SAPESILVWP DTWPKPLV A
NFAT3 ( 327) PASCNKGLPL GAESVAPPG GSRKEVAGMD YLAVPSPLAW S.KARIG...
NFAT4 ( 336) ELCSDDQGSLL SPARETS-DD GASSQYPLKK DSCGDQPLSV PS-FT-SK-K

Porcine ..SPTFYASP SLPALDRQLP SHSGFYELRI EVQPSXSHRA EYETEGSRGA 100
NFAT2 PL--TS-M-- T-----
NFAT1 IPICSIPTVA ---P-E-P-S Q S ---P-----
NFAT3 .GHSPIFRTS A--P----- -QYEQL----- -RA-----
NFAT4 PGRHPIFRTS ---P----- A-P-CC--K- ----T-----

Porcine VKASAGGHPS VQLHGYVESE PLTLQLFIRT ADDRLLRPHA FYQVHRITGK 150
NFAT2 -----I -----L-N- --M-----G-----
NFAT1 ---PT--PV -----M-NK --G--I--G- --E--I--K---
NFAT3 ---AP---V -K-L--S.EK --T--M--G- --R--N-----
NFAT4 ---T---V -K-L--N.EK -IN--M--G- ----Y-----

Porcine TVSTTSHEAV LSNTKVLKIP LHPENNMRAI DDCACULKLR NSDIELRKGK 200
NFAT2 -----I -----S--V-----
NFAT1 --T---Y-KI WG----- -EPK---T----- -A-----
NFAT3 M-A-A-Y--- V-G-----MT----- N-----
NFAT4 --A-A-Q-II IAG----- -S-S-----

Porcine TDIGRKNTRV RLVFRVHIDQ PNCRTLSLQV ASNPTECSQR SAQELPLVEK 250
NFAT2 -----V-- -S-----
NFAT1 -----E SS--TV---T----- --H--M--R
NFAT3 -----V-- GCGKVV-V-A --V----- -QV-A
NFAT4 -----S-KV---I --I-V----- -EI--

Porcine QSAASCPVIG GKRNVLIGHN FLQDEKVVVFV EKAPDGHILW BMEAKTGDJDL 300
NFAT2 --TD-Y--V- --X--S--- -----I--- -----V--
NFAT1 -DTD--L-Y- -QQ-T---Q- TSE V T --TT--CQL- ----TV-K-K
NFAT3 Y-PSA-S-R- -EEL---S- --P---V-I -RG---KLQ- -E--TVNRLQ
NFAT4 Y-IN--S-N- -HE--V--S- --PE--II-L -GQ--RPQ- -V-G-IIRBK

Porcine CKPNSLVVEI PPRNQRTS PVQVNFYVCN GKRRRSQYQH FYVLPAMAPV 350
NFAT2 -----H-S----- -----R----- -V-I
NFAT1 SQ--M-T--- -EY--KH-RT --X-----I- -----P-- --HPV...-A
NFAT3 SNEVT-ILTV -EYS-K-VSR ---Y--S- -R---PT-S -RF--VICKE
NFAT4 CQGAHL-L-V --YH-PAV-A AV--H-L-L- ----X--S-R ---T-VLMKQ

Porcine IKTEPSDDYE PATTCGPVSQ GLEPLTKPCY GEPLALPPDP SSCIVAGPPP 400
NFAT2 -----T----- -P----- --S--PR-Y- SQQ--M-----
NFAT1 -----T-E-D -T-I-S-THG --GSQPYYPQ H-MV-ES-SC LVAIM-PCQQ
NFAT3 EPLPDSRLRG FPSASATPFC TDMDFSP-RP PY-SYPHD- ACETPYLSEG
NFAT4 EHR-EI-LSS VPLELV-KPA QTQRFSSDSG CSHDEVLSGQ RSLICSIPQT

Porcine CPQRSVAVMSP PPSASPKIHD LSCADYSKGM AGPGEL..GL QRPAGGVVGG 450
NFAT2 -----TL-PA A-GV----- --P-A-T--V -S---CHL-- PQ---EAPAV
NFAT1 FRTGLSSPDA RYQQQNPAAV -YQSK-LSP SLL-YQCPA- MAAFLSLADA
NFAT3 FGYCMPPLY QTQPE SYRP GLRMFPETRG TTGCAQFFAV STLERPFPPED
NFAT4 YASMTSSHL -QLQCRDESV SKEQHMIKSP TVHQPFQVTP TDPVGSYQP

Porcine QEAPRPGGPH FGAPQPPPEA LLQPQ..... 500
NFAT2 -DV---VAT- --S-GQ--- --PQ-----
NFAT1 HRSVLVHAGS Q-QSSALLHP SPTN-QASPV IHYSPIHQQL RCGSHQEPFH
NFAT3 FYGG-GSSFP L-L-FS--AP FRPPELPASP PLGGFFFSQS DVHELPAEGY
NFAT4 MQTNVVYNGP TCLPINASS QEFDEVLRQQ DATLSCLVNL GQQLSSIFP

```

(Continued overleaf)

Porcine	.....	.....	.....	.....	.....	550
NFAT2	.....	.....	.....	.....	.....	
NFAT1	IMYCENFAPG	CTTRPCPPPVVS	QGQRLSPPGSY	PTVIQQQMAT	SQRRAAKNGPP	
NFAT3	NKVQPGYGPC	ECAPPEQEKSR	GGYSSGFRDS	VFIQGITLEE	VSEIIGRDLS	
NFAT4	HSSNSGSTGH	LLARTPHSVH	TLPELQSMGY	HCSKTGQRSL	SSPVAUQITG	
Porcine	.....	.....	.....	.....	.....	600
NFAT2	.....	.....	.....	.....	.....	
NFAT1	VSDQKEVILPA	GVTIKQEQNL	DQTYLDDBLI	ETHLRWIQNI	L.....	
NFAT3	GFPAPPCEEP	PA.....	.....	.....	.....	
NFAT4	QPSSQLCPIT	YGPSHSGSAT	TASPAASHPL	ASSPLSGPPS	PQLQPMPIQS	
Porcine	.....	.....	.....	.....	.....	650
NFAT2	.....	.....	.....	.....	.....	
NFAT1	.....	.....	.....	.....	.....	
NFAT3	.....	.....	.....	.....	.....	
NFAT4	PSSGTASSPS	PVPRMHSQGH	STQAQSTGQS	GLSAPSSLIC	HSLCDPASFP	
Porcine	.....	.....	.....	.....	.....	700
NFAT2	.....	.....	.....	.....	.....	(82.6%)
NFAT1	.....	.....	.....	.....	.....	(47.8%)
NFAT3	.....	.....	.....	.....	.....	(46.4%)
NFAT4	PDGATVSIKP	EPEDREPHFA	TIGLQDITLD	DGKPLSDMFL	K	(37.9%)

**Figure 3.14.** Sequence comparison of the acquired NFAT sequence with other human NFAT isoforms (Accession N<sup>o</sup>s G02326, NP\_006153, Q14934 and NM\_173164 for human NFAT 1, 2, 3 & 4 respectively). A '-' denotes a homologous residue. Grey shaded sequence is 5' of the existing NFAT clone-3. The percentage homology with NFAT clone-3, as determined by ALIGN software, is shown at the end of each sequence.

```

Pig ( 1)      ..... DFPPEEPPEF QHTR.KCARG DQYLSVFOHP 50
NFATmac(337) SLALKVEPVA EDLGATPPIS -----
Human (345)  SVALKVEPVG EDLGSPPPPA --A--DYSS- ----G-- --A----
Mouse (347)  SVALKVEPVG EDLCTTPPTS      Y.T-  E      AS
Xenopus (326) RLAKKTELNT EDLACLSPIV -M-AD-TSCS QII--VQDSST --F---S-.

Pig          YFWARPR..S PTP.YASPSL PALENQLPSH SGPYELRIEV QPKSHHRAHY 100
NFATmac     -----
Human      -Q--K-KPL- --S.-M--T- -----
Mouse      -Q--K-KSL- PPS.YMSPSL -----
Xenopus    FT-NKHKS.G HTPVVRTSSL -P---A---R Y-Q---KV-- --T-----

Pig          FTRGSRGAVK ASAGGHPSVQ LHGYVESEPL TLQLFIRTAD DRLLRPHAFY 150
NFATmac     -----
Human      -----I-- ---L-N--- M----G--- -----
Mouse      -----I-- ---L-N--- T----G--- -----
Xenopus    ----- -VT-S--I-K -I--T-.K-V NV-I--G--- E-YVK-----

Pig          QVHRITGKTV STTSHEAVLS NTKVLEIPLL PNNMRAIID CAGILKLRNS 200
NFATmac     -----
Human      -----I-- -----S--V-- -----
Mouse      -----II-- -----M----- -----
Xenopus    -I----- V-A-Q-I-IT -----P-- --MT-S--

Pig          DIELRKGETD IGRINTRVRL VFRVHPQPN GRTLSLQVAS NPICSQRSA 250
NFATmac     -----
Human      -----V--S----- -----
Mouse      -----
Xenopus    ----- --A---TS -KV---T-- I-----

Pig          QELPLVEKQS AASCPVLGGK RMLTGHNEL QDSKVVFVVK APK.AEMWE 300
NFATmac     -----
Human      ----- TD-Y--V--- K---S-H--- ----I--- ----V
Mouse      ----- TD-Y--I--- K---S-H--- ----I--- ----V--
Xenopus    ----- Q---Y- VN--S-N--L EL-I--S--M P---I-L- GQ--CT--

Pig          MEAKTDGDLG KPNLSLVEIE PFRNQKITSP VQVNFYVCSG KRKRSQYQHF 350
NFATmac     -----
Human      -----R--- -----S----- --R-
Mouse      -----R--- -----S----- --R-
Xenopus    A-G-I-LKK- QM-NI----- AYHNKSVAAE ---Q----- --S-R-

```

(Continued overleaf)

```

Pig          TYLPANAPVI KTEPSDD..Y EPALTCGPVS QG..LNPLCK PCYGPPLALP 400
NFATmac     -----Y-----
Human       -----V-I- ----T--..Y ---P----- --S--PR -Y-SQQ--M-
Mouse       V I .      T . .F ---L---M- --..IS--PR -Y-SQQ-TM-
Xenopus     N-P-...VLI -Q-NLR-QNL QE-..AVS-A K RUISDL.. HIIHTNLLFQ

Pig          PDPSSC..LV AGFPP...CP QRSVMSDPE SASPKLHDLG CAYSKCMAG 450
NFATmac     -----
Human       -----TL-PAA- GVS----- P-A-T--V-S
Mouse       ---G--...- -A-...S --NTL-PT-- N----- SPA-T--LTN
Xenopus     DQRSICKT-K SLSP-GVG-- NQLTFTSSLK HEA-EISNIQ EPCT-SSPCT

Pig          PG...HLGL.. QRPAGGMVGG QEAPKPGGPH PGAPQPPPPA LLQPQ.... 500
NFATmac     -----LHPLN LSQSIVIRLT
Human       --EC----P P...EAPAV DV VAT CS-GQ... PQ.....
Mouse       --HSG----- -P--SEAPTM --V---MAIQ -NS-EQ--S- R-----
Xenopus     ADPEEMENYP DTIVSSPQKQ SMPYQGFSSD ITILPSSSLT AMPHCGQPSS

Pig          ..... 550
NFATmac     EPQP.....
Human       .....
Mouse       .....
Xenopus     ETHDCRLRDN SLSPTTIIHR QNETSGHPTD MSLGIKSELE DSDENPQTKD

Pig          ..... 600
NFATmac     ..... (95.8%)
Human       ..... (82.6%)
Mouse       ..... (82.0%)
Xenopus     IQDITLDEVA EIIERDMSH. (44.8%)

```

**Figure 3.15.** Pileup of amino-acid sequences for NFAT-2 transcription factors across a range of species. NFATmac is a porcine NFAT-2 isolated from macrophages (Accession N<sup>o</sup>s NFATmac AF069996, human NM\_006162, murine NM\_198429, *Xenopus* AB037471). A '-' denotes a homologous residue. Grey shaded sequence is 5' of the existing NFAT clone-3. The percentage homology with NFAT clone-3, as determined by ALIGN software, is shown at the end of each sequence.

Comparisons with NFAT-2 from other species, as well as the porcine NFATmac, are shown in Figure 3.15.

As discussed in Section 1.4.2, NFAT-2 is synthesised in three different splice products (Chuvpilo *et al.*, 1999). These variants differ primarily in the length of the carboxyl-terminal end, though there also differences in the N terminal domain (reviewed in Serfling *et al.*, 2000). With the possession of the 3' end, it was possible to determine that clone-3 showed closest homology to NFAT-2b. However, without the 5' end of the transcript, it was impossible to determine whether identity with this human orthologue was conserved at the N-terminal end. The porcine NFAT-2 described by Miskin *et al.* (1998) is also closest to the 2b variant in terms of sequence homology and transcript length. However, homology between clone-3 and NFATmac was quite low at the extreme 3' end of the deduced amino acid sequence (Figure 3.15). Homology was also low between the 3' untranslated regions for the two transcripts, suggesting that while they may be the same isoform, they are different alternate splicing variants.

#### **3.3.4.2 NFAT full-length PCR**

The first attempt at isolating a full-length clone utilised the ZAP Express<sup>TM</sup> library as a template for PCR. The 3' primer was based on the acquired, truncated sequence data, and the 5' on the T3 polymerase site on the pBK-CMV vector. Several attempts were made to optimise conditions, such as annealing temperature and Mg<sup>2+</sup> concentration, but no fragments of the correct size were obtained. It seemed likely that no full-length species existed to be amplified, and that an alternative method of deriving the cDNA would have to be used.

#### **3.3.4.3 Hybrid NFAT PCR-cloning**

While attempts continued to clone a full-length NFAT, a cloning strategy was employed to convert the truncated clone into a useable protein. By adding extra sequence to the PCR primers, a Kozak consensus sequence and an 'in frame' transcription start site would be added to the 5' end of the NFAT clone. The protein generated from such a modified hybrid would lack the regulatory element and nuclear export signals. Provided the DNA-binding and *trans*-activation domains remained functional, this NFAT would likely be constitutively active and give some insight into the role of NFAT in muscle. A constitutively active form of NFAT has been used

successfully in other studies in T-cells and fibroblasts (Porter and Clipstone, 2002; Neal and Clipstone, 2003), though the NFAT used in these studies represented a less severe mutation (Neal and Clipstone, 2001).

The required PCR fragments were successfully obtained, but no colonies were observed following the ligation step and transformation into *E. coli*. The reason for the failure of this final step is not immediately clear, but is possible that gene product might be toxic to the *E. coli* host. While full length porcine NFAT-2 has been cloned in *E. coli* (Miskin *et al.*, 1998), the NFAT construct described here was a radical alteration of the native protein. In any case, it was clear that this 'short-cut' was not going to be an effective substitute for a full-length clone.

#### **3.3.4.4 SMART RACE**

Amplification using the 5' RACE procedure did not produce a product, and in the end this technique also had to be abandoned. The reasons for the failure of this technique are unclear. The positive control RNA provided with the kit was reverse-transcribed and a full-length species amplified using the procedure outlined. It seems clear also that at least a fragment of the NFAT clone was also reverse-transcribed, since the internal, positive control, utilising both gene-specific primers, gave a fragment of the expected size. The failure of the full 5' RACE may be due to a need for further optimisation of the reaction conditions, or it could be due to some problem stretch of sequence making it especially difficult for the reverse transcriptase employed in first strand synthesis. This would possibly explain the difficulties in obtaining ZAP Express<sup>TM</sup> clones with the full coding sequence.

As a final thought, it may have been beneficial to pursue a simplified RACE amplification step using the original methodology described by Frohman *et al.* (1988). The Clontech SMART RACE kit enriches for full-length cDNA species using a modified reverse transcriptase that incorporates a number of deoxycytosine residues at the 5' end of first-strand syntheses that reach the end of the mRNA template. This poly-C tail serves as an adaptor, to which the SMART II oligonucleotide binds and primes for second strand synthesis in the same reaction. The original RACE method uses a gene-specific primer for first-strand synthesis, and a polyA tail added by terminal

deoxynucleotidyl-transferase. This poly-A tail serves as the template for second strand synthesis using a poly-deoxythymidine incorporating primer.

While the SMART RACE protocol is a faster method with fewer stages involved, one potential problem compared to the original method used by Frohman *et al.* (1988) is that the first strand synthesis must proceed through the entire length of the transcript, from the poly-A tail to the 5' end. In the original RACE protocol, the 5' reaction need only transcribe from the gene-specific primer to the 5' end. While this results in a fragment that must be manipulated with other pieces to reconstitute a complete cDNA clone, the shorter read-through might be more likely to succeed where the other methods had failed. Had time permitted, this may have been easier to optimise than the SMART RACE kit.

### 3.4 DISCUSSION

A full-length GATA cDNA clone has been isolated, sequenced and characterised. This clone has been identified as the porcine orthologue of GATA-2, and submitted to GenBank (Accession N<sup>o</sup> AY251012). The characterised sequence of this porcine orthologue further confirms the high degree of homology observed in GATA family members (Lowry and Atchley, 2000). At the time of writing, two other porcine GATA family members, GATA-4 and -6, have also been described (Gillio-Meina *et al.*, 2003), while a third, GATA-3, has been partially characterised (Nonneman and Rohrer, 2004). It seems highly likely that the family organisation, and therefore function, of GATA factors is conserved in the pig.

The pBK-CMV/GATA clone-7 construct is particularly well suited for conducting studies into the physiological role of porcine GATA-2. The mammalian replication origin and CMV promoter enable mammalian transfection of the construct, and over-expression of GATA clone-7. Prior to this, however, the next step is to determine whether the obtained cDNA codes for a functional protein. The first part of this goal is to test whether GATA clone-7 is translated into protein. Since the clone lacks a strong Kozak consensus sequence, according to the context ANNatgG (Kozak, 1996), there remains the slight possibility that cDNA does not represent an actual mRNA transcript.

Fortunately, the sequence data does not fall into any of the three 'warning signs' categories summarised by Kozak (2000), these being:

1. The presence of multiple, upstream ATG codons.
2. An upstream ATG codon with an open reading frame overlapping that of the putative main start codon.
3. A very weak context that lacks both of the flanking elements of a strong context. (a pyrimidine residue at position -3, or purine at position +4)

The absence of a strong Kozak consensus is unsurprising, given that GATA-2 is a transcription factor and therefore, in common with many regulatory genes, present at low copy number (reviewed by Kozak, 1996). However, evidence for the translation of a protein product of the suitable size, either by *in-vitro* transcription or from transfected mammalian cells, would provide a greater degree of confidence in later functional studies using the pBK-CMV/GATA clone-7 construct. This work is covered in Chapter Five.

Also detailed in Chapter Five is the development of isoform-specific primers and probes for real-time RT-PCR, in order to investigate the expression patterns of the mRNA transcript on which GATA clone-7 is based. The characterised sequence data contains untranslated regions that should be sufficiently isoform-specific for this task.

Only a partial NFAT sequence has been recovered, identified as NFAT-2. The transcript is most probably orthologous to the human NFAT-2b splice variant, as is another NFAT-2b orthologue previously isolated by Miskin *et al.* (1998), from porcine macrophages. It is possible that there is a different set of NFAT-2 variants in the pig from those characterised in humans. The C-terminal domain of NFAT-2a is significantly shorter than that of the two porcine NFAT-2 forms, while the NFAT-2c C-terminal region is longer with an additional transactivation domain (Scrfling *et al.*, 2000). However, with only one porcine NFAT-2 variant characterised, and NFAT clone-3 only partially so, the number and respective sizes of the NFAT-2 alternative splice forms in the pig remains unknown. That NFAT<sub>mac</sub> and NFAT clone-3 should be different transcripts is unsurprising, given the differences in source tissue. This is consistent with the observation by Sherman *et al.* (1999), that mouse NFAT-2 splice

variants are distributed in a tissue-specific manner. However, the 5' region of NFAT clone-3 would need to be isolated in order to determine whether it constitutes a novel splice form NFAT-2.

While the NFAT cloning was unsuccessful in the stated aim of acquiring a full-length clone, the sequence data obtained does allow for studies into expression patterns of the gene. The possession of the 3' UTR allows for the radiolabelling of isoform-specific fragments for Northern blot hybridisation, or for the generation similarly isoform-specific primers and probes sets for real-time PCR. This work, together with that for GATA clone-7, is described in Chapter Five.

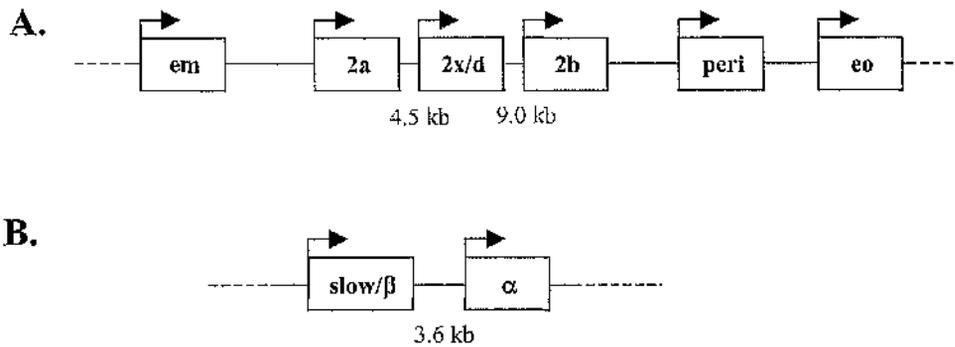
**CHAPTER FOUR - PORCINE EMBRYONIC MYHC  
PROMOTER REGION CLONING**

## 4.1 INTRODUCTION

So far, the isolation and characterisation of GATA and NFAT transcription factors has been the major focus of the work. However, the project also included a study of one of the potential targets of these factors, the porcine embryonic myosin heavy chain (emMyHC). Prior to the beginning of the study, most of the porcine myosin heavy chain (MyHC) genes had already been characterised to some degree (Chang *et al.*, 1993; Chang *et al.*, 1995; Chang and Fernandes, 1997). The embryonic form was one of the two isoforms yet uncharacterised, the other being the perinatal promoter region, since identified by Da Costa *et al.* (2000). It was decided that the cloning of the emMyHC regulatory region could prove useful in subsequent functional studies, as well as completing the information on the MyHC gene family in the pig. This work was part of a collaboration with Dr Yuh-Man Sun, at the Roslin Institute, Edinburgh, who provided much of the starting material for cloning the regulatory region of the porcine embryonic MyHC. Although the sarcomeric MyHC genes have been briefly covered in Section 1.2.1.2 it is worth covering their genomic organisation and sequence homology in greater detail, particularly those properties that were exploited in the emMyHC PCR cloning on which the subsequent genomic cloning was based. Also covered here is the background for the cloning strategies used in isolating genomic sequence, through the screening of a library of Bacterial Artificial Chromosomes (BACs) containing large (average size of 150 kilobase-pairs) inserts.

### 4.1.1 MYHC GENOMIC ORGANISATION

The eight MyHC genes important in skeletal muscle are clustered in a linear, head-to-tail arrangement in two chromosome regions (Figure 4.1). The cluster arrangement is well conserved, and is found in both humans and rodents where it was first studied (Weiss and Leinwand, 1996; Weiss *et al.*, 1999a), and also in the pig (Sun *et al.*, 2003). The first cluster contains the fast 2a, 2b, 2x and extra-ocular MyHCs, and the embryonic and perinatal developmental isoforms. This cluster is located on chromosome 17 in humans (Leinwand *et al.*, 1983), and chromosome 12 in the pig (Davoli *et al.*, 1998). The genes encoding the 2a, 2x and 2b isoforms are quite tightly clustered together, with much smaller intergenic distances than those between



**Figure 4.1.** Organisation of MyHC genes (adapted from Weiss *et al.*, 1999a). At the time of writing, this cluster organisation is conserved in all of the mammalian species studied. The intergenic distances listed are from characterised human sequence. **A.** Skeletal muscle cluster. **B.** Cardiac muscle cluster.

the embryonic to 2a or 2b to perinatal, respectively. The gap between perinatal and extra-ocular is believed to be of a similar magnitude, but is as yet uncharacterised in the pig. The second cluster contains the  $\alpha$ -cardiac MyHC and the type I/slow MyHC, also referred to as the  $\beta$ -cardiac isoform, and is located on chromosome 14 in humans (Saez *et al.*, 1987) and on pig chromosome 7 (Davoli *et al.*, 1998).

#### 4.1.1.1 Homologous Regions and Cloning Strategies

The MyHC genes share a conserved primary structure and intron/exon organisation (Weiss and Leinwand, 1996), first described by Strehler *et al.* (1986). The majority of MyHC genes are around 30 kb in length, containing 40 or 41 exons with the translational start site located in exon 3. All of these MyHC genes are highly conserved, though there is a greater degree of sequence identity between the same isoforms in different species, than in different isoforms from the same species (Weiss and Leinwand, 1996; Weiss *et al.*, 1999b). The short stretches of isoform specific sequence present a useful target for cloning strategies intended to isolate the same MyHC isoform from different species. The ATP binding site in particular is highly isoform-specific, and this has already been exploited in PCR-cloning strategies used to isolate a

number of porcine MyHC genes using sequence known from other species (Chang and Fernandes, 1996).

#### 4.1.1.2 Isolation of the emMyHC 5' UTR

The method described by Chang and Fernandes (1996) was used by Dr Yuh-Man Sun to screen a  $\lambda$  cDNA library (see section 3.1.3.2). This library was constructed using the ZAP Express cDNA synthesis kit (Stratagene) (Short *et al.*, 1988), using total mRNA from a 50 day old porcine foetus. The sequence of the longest clone isolated from the PCR cloning is shown in Figure 4.2. The 135 bp of 5' untranslated sequence was isolated, and was received from Dr Yuh-Man Sun as the basis for the further work to isolate the emMyHC regulatory region, described in this chapter.

```

1  AGTCACAGTG CCCCTTGTGC GGGTCCTCC CATCTGAGGC TCAGAGGCTC GTGTGGCCCT GCCCGCTTT
   ▼
71 GGTCTGATT TCTCACGGCT GCTGCCTCT GCTGTCCTCT GCGGTGTGA CTCTCAGCCG ACACC

135 ATG AGC AGC GAC ACT GAA ATG GAA GTG TTC GGC ATA GCC GCT CCC TTC CTC CGC AAG TCG
1   K S S D T R M E V F G I A A P F L R K S

195 GAA AAG GAG AGG ATC GAG GCG CAG AAC CAG CCC TPC GAT GGC AAA ACC TAC TGC TTC GTG
21  E K E R I E A Q N Q P F D A K T Y C F V

255 GTC GAC TCG AAG GAA GAG TAC GCC AAG GGG AAA ATT AAG AGC ACC CAG GAT GGG AAG GTC
41  V D S K E E Y A K G K I K S T Q D G K V

315 ACG GTG GAG ACC GAA GAC AAC AGG ACC CTG GTG GTG AAG CCG GAG GAT GTG TAT GCC ATG
61  T V E T E E N R T L V V K P E D V Y A M

375 AAC CCC CCC AAG TTT GAC CCG ATC GAG GAC ATG GCC AGT CTG ACG CAG CTG AAC GAG CCG
81  N P P K T E R I E D M A S L T H L N E P

435 GCC GTG CTG TAC AAC CTC AAG GAC CCG TAC ACC TCC TGG ATG AUC TAT ACC TAC TCG GGC
101 A V L Y N L K D R Y T S W M I Y T Y S G

495 CTC TTC TGT GTC ACC GTC AAC CCC TAC AAG TGG CTG CCG GTG TAC AAC CCC GAG GTG GTG
121 L F C V T V K P Y K W L P V Y N P R V V

555 GAG GGC TAC CGA GGC AAA AAG CGC CAG GAG GCC CCG CCC CAC ATC TTC TCC ATC TCC GAC
141 E G Y R G K K R Q E A P P H I F S I S D

615 AAC GCC TAT CAG TTC ATG CTG ACA GAT CGT GAA AAC CAG TCC ATT CTG ATC ACC GGA GAA
161 N A Y Q F M L T D R E N Q S I L I T G E

675 TCC GGG GCG GGA NAC ACG CTG AAC ACC AAG CGT TGT
181 S G A G X T V N T K R

```

**Figure 4.2.** Previously isolated porcine emMyHC cDNA (Acc. N<sup>o</sup>: AJ309014). The first 135 bp of the cDNA fragment (underlined) was isolated for use as an isoform-specific probe. The '▼' denotes the intron/exon boundary for intron 1.

### 4.1.2 BAC LIBRARY SCREENING

The cloning of regulatory sequences presents different problems to the isolation of gene coding sequence, such as the strategies described in Chapter Three. The cDNA libraries described there represent only transcribed, mRNA sequence. Where the sequence to be cloned falls outside of transcribed sequence, or is lost as introns during mRNA splicing, a genomic library is required. To present the total genomic DNA of a mammalian target organism in a manageable number of clones requires far larger inserts than are possible with normal plasmid vectors. A number of alternative systems have been developed for this purpose, such as  $\lambda$ -phage packaged cosmid vectors (Collins and Hohn, 1978). Though larger than normal vectors, cosmids are still constrained by the amount of DNA that can be packaged in a phage head, with around 35 to 45 kb being the upper limit. Larger inserts are possible with Yeast Artificial Chromosomes (YACs) (Burke *et al.*, 1987), up to 1 megabase-pairs (Mb) or more (Larin *et al.*, 1991). However, YAC systems suffer a high incidence of chimeric clones, in which the insert does not represent a contiguous DNA sequence (Anderson, 1993).

Bacterial artificial chromosome (BAC) systems (Shizuya *et al.*, 1992) are based on the F-factor in *E. coli*. Replication of the F' episome is tightly controlled with only one or two copies present in any one cell, greatly reducing the possibility of recombination events between inserts. In addition to its suitability for cloning large inserts, the F factor's circular, supercoiled structure makes it more amenable to manipulation than linear YAC constructs that can easily shear. The bacterial host can be grown, transformed and screened through colony lift far more conveniently than yeast cells. BAC libraries are therefore the method of choice for performing large insert screening, and were used in the embryonic MyHC promoter region described here.

### 4.1.3 AIMS

The aim of the work described in this chapter was to isolate the porcine emMyHC regulatory domain, in order to further characterise the MyHC gene family in the pig. The work was based on the 135 bp fragment already isolated, using it as a radiolabelled probe to screen a BAC library. As a long-term aim, knowledge of the upstream regulatory elements of emMyHC would be vital for determining whether emMyHC was a target for the GATA and NFAT transcription factors investigated in the course of

the study. The genomic sequence obtained was used to generate a number of emMyHC-promoter driven reporter constructs. These constructs were later used to characterise the effect of GATA-2 activity on emMyHC expression.

## **4.2. MATERIALS AND METHODS**

### **4.2.1 BAC LIBRARY SCREENING**

The Porcine Bacterial Artificial Chromosome (PigE BAC) library used was provided by the UK HGMP resource centre, constructed by Anderson *et al.* (2000). The library was filter screened and clones identified as described in the suppliers protocols, summarised briefly here. The library physically consists of around 102,000 clones in microtitre plates. These were grown up overnight in chloramphenicol selection, spotted in 4 × 4 arrays on Hybond N nylon membranes. The filters are subsequently processed as for colony lift, with SDS, denaturation and neutralisation treatments followed by UV-crosslinking, supplied ready for pre-hybridisation.

#### **4.2.1.1 BAC Clone Identification**

The 135 bp emMyHC 5' UTR fragment was labelled by the random hexamer method (see 2.2.4.1.1). As a test for isoform specificity, the labelled probe was used to probe a genomic southern blot. Genomic DNA was prepared from porcine liver tissue, and the blots prepared as described in Section 2.2.4.2.3. Once satisfied that there was a low risk of isolating different isoforms, the probe was hybridised with the BAC library filters. Hybridisation and autoradiography was as described in Section 2.2.4.2.2, using 'home-made' rather than commercial buffer due to the requirement for increased specificity and lower background. Once identified, the clones themselves were ordered from the UK HGMP resource centre, supplied on agar slope cultures containing chloramphenicol. Clones were streaked out on LB agar plates, maintaining selection pressure with chloramphenicol. Single colonies were picked, as required, to create further glycerol stocks for storage or grown up in culture to isolate BAC preparations.

#### 4.2.1.2 BAC DNA Preparations

The main complication in obtaining BAC preparations from *E. coli* culture is the large size of the insert, requiring a modification of the standard plasmid preparation methods described in Section 2.2.1.2. A modified version of the Qiagen plasmid midi kit protocol was used, summarised as follows. Single BAC colonies were picked from the overnight agar cultures, and used to inoculate a 5 ml LB starter culture, supplemented with chloramphenicol. The following morning, 0.5 ml of the starter culture was used to inoculate 100 ml LB, grown at 37 °C for 14 hours with vigorous shaking (approx. 250 rpm in an orbital shaker). The cells were divided into two 50 ml falcon tubes and pelleted at 4,500 × g for 20 min. Bacterial pellets were resuspended, lysed, and proteins precipitated by the addition of 10 ml buffers P1, P2 and P3, respectively (as described for large scale plasmid preps, section 2.2.1.2.2). Following the addition of buffer P3, the suspension was chilled on ice for 15 min, centrifuged at 20,000 × g for 30 min at 4 °C. The supernatant was removed and filtered through a cell-strainer. During the spin, a Qiagen-tip 100 was equilibrated by applying 4 ml of Buffer QBT and allowing to drain. Supernatant from the centrifugation step was pooled and decanted into the Qiagen-tip, and washed twice with 10 ml Buffer QC. The DNA was eluted with 5 ml of Buffer QF, pre-warmed to 65 °C. DNA was precipitated by the addition of 3.5 ml isopropanol, centrifuging at 15,000 × g for 30 min, 4 °C. Supernatant was removed and the pellet washed with 70 % ethanol, spun as before for 10 min, the ethanol removed and the pellet air dried. The final pellet was resuspended in 10 mM Tris (pH 8.5).

#### 4.2.2 CLONING EMMYHC REPORTER CONSTRUCTS

The BAC clone was used as the basis of a number of cloning strategies, designed to create a number of reporter constructs from the emMyHC promoter region. These constructs were to be used in testing the effect of *trans*-acting factors, such as the GATA and NFAT family members, on emMyHC expression. The basis of this strategy was the pEGFP-1 vector (Clontech), with the promoter fragments driving the expression of enhanced green fluorescent protein (EGFP). A number of approaches were used to acquire and clone fragments of regulatory sequence upstream of the emMyHC coding region.

#### 4.2.2.1 Genome Walking

The first approach used was genome walking, a method of obtaining unknown sequence from genomic DNA, based on short regions of known sequence. This work was carried out by Dr Yuh-Man Sun, using the GenomeWalker™ kit (Clontech) according to the manufacturer's protocols briefly summarised here. The BAC clone was subjected to blunt-end digestion, and the resulting fragments ligated at either end with the GenomeWalker™ adapter fragments. Long range PCR was carried out, using sense primers based on adapter-specific sequence, and gene-specific, reverse primers based on known sequence, in this case the 135 bp of untranslated sequence. PCR products were cloned using the Blunt-ended PCR Cloning Kit (Amersham Biosciences), into the pMOS-blue vector.

#### 4.2.2.2 PCR Cloning

PCR cloning was carried out to amplify part of the promoter region immediately upstream of the emMyHC gene. The sense primer was based on the 5' sequence of a 0.25 kb, *Bam*HI sub-clone of the GenomeWalker™ PCR fragment (see Section 4.3.2), containing the *Bam*HI site. The anti-sense primer was based around sequence from exon 1, with an engineered *Age*I site to allow for later cloning into the pEGFP reporter vector. The primers are shown below, with the restriction sites underlined.

Sense Primer

5' TAG GAT CCT GTC ATT TCT ATA TAT 3'  $T_m$  58.9 °C

Anti-sense Primer

5' AAA CCG GTG GAC CCG CAC AAG GGG CAC TGT 3'  $T_m$  74.8 °C

PCR conditions were as follows: denaturation for 3 min at 95 °C, followed by 35 cycles of a 45 second, 95 °C denaturation step, 45 seconds annealing at 55 °C and a 45 second extension step at 72 °C. This was followed by a final 5 min extension step, before holding at 4 °C. PCR products were purified and cloned into the TopoTA vector (2.2.1.6.2).

#### **4.2.2.3 Southern Blot Screening**

The BAC clone was subjected to restriction endonuclease digestion, the resulting fragments separated by gel electrophoresis (Section 2.2.1.4.1) and transferred to nitrocellulose filter by Southern blot (Section 2.2.4.2). The filter was screened using a PCR fragment obtained from the genome walking, the 1.6 kb fragment described in Section 4.3.2. This fragment was radiolabelled using the random hexamer method (Section 2.2.4.1.1), with probe hybridisation and autoradiography as described in Section 2.2.4.2.2. Restriction fragments positively identified by this screening process were run out again on agarose gel, excised and cloned into *E. coli*, using the methods described in Section 2.2.1.

#### **4.2.2.4 Cloning into the pEGFP-1 Vector**

PCR products and restriction fragments obtained from the promoter region cloning were ligated into appropriately digested and calf alkaline phosphatase treated pEGFP-1 vector, using the protocols described in Section 2.2.1.5. Constructs were transformed into *E. coli* (Section 2.2.1.6) and prepared by large scale plasmid preparation (Section 2.2.1.2.2).

### **4.2.3 LI-COR SEQUENCING**

Sequence data for the emMyHC promoter region was obtained using the LI-COR large-gel, automated sequencing system (LI-COR). The sequencing reaction was based on the chain-termination method (Sanger *et al.*, 1977), with the cycle-sequencing modifications described by Innis *et al.* (1988), using the Amersham Thermo Sequenase kit (Amersham Bioscience). Manufacturer's protocols were followed, summarised as follows:

#### **4.2.3.1 Cycle Sequencing**

The plasmid templates for cycle sequencing were prepared by miniprep (section 2.2.1.2.1). Custom oligonucleotides were supplied by MWG biotech, designed as described in section 2.2.3.1. For the Li-Cor work, the sequencing primers were based on T3 or T7 RNA polymerase promoter sites located on the cloning vectors.

T3

5' ATT AAC CCT CAC TAA AG 3'

T7

5' TAA TAC GAC TCA CTA TAG GG 3'

The cycle-sequencing reaction mixtures (four per DNA template) consisted of the appropriate Amersham reagent mixture (Applied Biosystems) and nucleotide (A,C,G or T), together with 200 to 500 ng of DNA template and 1 pmol sequencing primer, made up to 8  $\mu$ l with Millipore-pure water. Cycle sequencing was carried out in a thermal cycler (Perkin Elmer), using the following program: a denaturing step at 95 °C for 5 min, followed by 25 cycles of 30 s denaturation at 95 °C, 30 s annealing at 55 °C and 30 s extension at 72 °C, with a final hold at 4 °C. The reactions were terminated by the addition of 4  $\mu$ l stop solution. Reaction products were stored at 4 °C until loading, which was carried out on the same day.

#### 4.2.3.2 Large Gel Electrophoresis

The gel mixture was prepared (4% Gel Mix concentrate; National Diagnostic, 0.28 M Urea in 1.2  $\times$  TBE buffer), mixed well and chilled at 4 °C. The 66 cm glass plates were washed once with 100% ethanol, polished until dry, and once with isopropanol, again polishing until the surfaces of the plate were dry and completely free of dust, paying particular attention to the comb area and the base region read by the scanner. Bind silane (3-Methacryloxypropyltrimethoxysilane) was applied to the notched plate, using a mixture of 0.5% bind silane, 0.3% acetic acid in ethanol, spread across the comb area and gel base and allowing to dry. The glass plates were washed again with isopropanol, taking care to avoid the regions coated with bind silane. Gel plates were aligned together, with 0.2 mm spacers along each side, taking care to ensure correct alignment. The side brackets clamped on, tight enough for a good seal but while avoiding cracking the plates. The gel plates were placed in the gel pouring stand, and the gel mixture retrieved from the fridge. Ammonium persulphate (APS) was added, 400  $\mu$ l of a 10% stock, and a 4 ml aliquot taken for a base plug, the remainder returned to 4 °C. A further 40  $\mu$ l 10% APS and 4  $\mu$ l tetramethyl-1,2-diaminomethane (TEMED) was

added to the 4 ml aliquot, which was then pipetted into the base of the gel and left to polymerise for 10 minutes. The remaining gel mix was prepared, adding 40 µl TEMED and syringed between the glass plates, taking care to avoid the presence of air bubbles. The well former was placed in the top of the gel and clamped in place with 'bulldog' clips, and the gel left semi-horizontal to polymerise for 1 hour.

The gel was read in a Li-Cor model 4000L automated sequencer. The well-former was removed, and the large well flushed. The assembled gel apparatus was cleaned a final time to remove any dried-on gel, and dried thoroughly to prevent electrical leakage. The gel apparatus was mounted in the scanner, the upper and lower gel tanks fitted and filled with 1 × TBE. A 48-well 'shark-tooth' comb was carefully inserted in the top of the gel to form the wells, and 1.5 µl of reaction mixture loaded per well. The gel was run overnight at 2000 V. Sequence data was collected automatically, after manually defining the lanes. Base ambiguities were checked by visual inspection of the gel image.

#### **4.2.4 ABI SEQUENCING**

Later in the project, sequencing using the ABI PRISM became available (Applied Biosystems). ABI sequencing was used for obtaining 5' sequence data further 5' from the promoter region PCR clone. A sequencing primer was designed, based on the sequence data obtained from the PCR clone.

5' sequencing primer

5' CAC AAG TGA GGA CCA GCT CC 3'

The methods used were as described in Section 2.2.3, for the Version 1.0 chemistry (Section 2.2.3.2.1).

#### **4.2.5 SEQUENCE ANALYSIS**

Analysis of sequence data for both Li-Cor and ABI systems was carried out as described in Section 2.2.3.3. In addition, the deduced genomic sequence was analysed for the presence of possible transcription factor binding sites, using both MatInspector version 2.2 (Genomatix Software) and Match<sup>TM</sup> version 1.0 software (Biobase)

## 4.3 RESULTS

### 4.3.1 BAC LIBRARY SCREENING

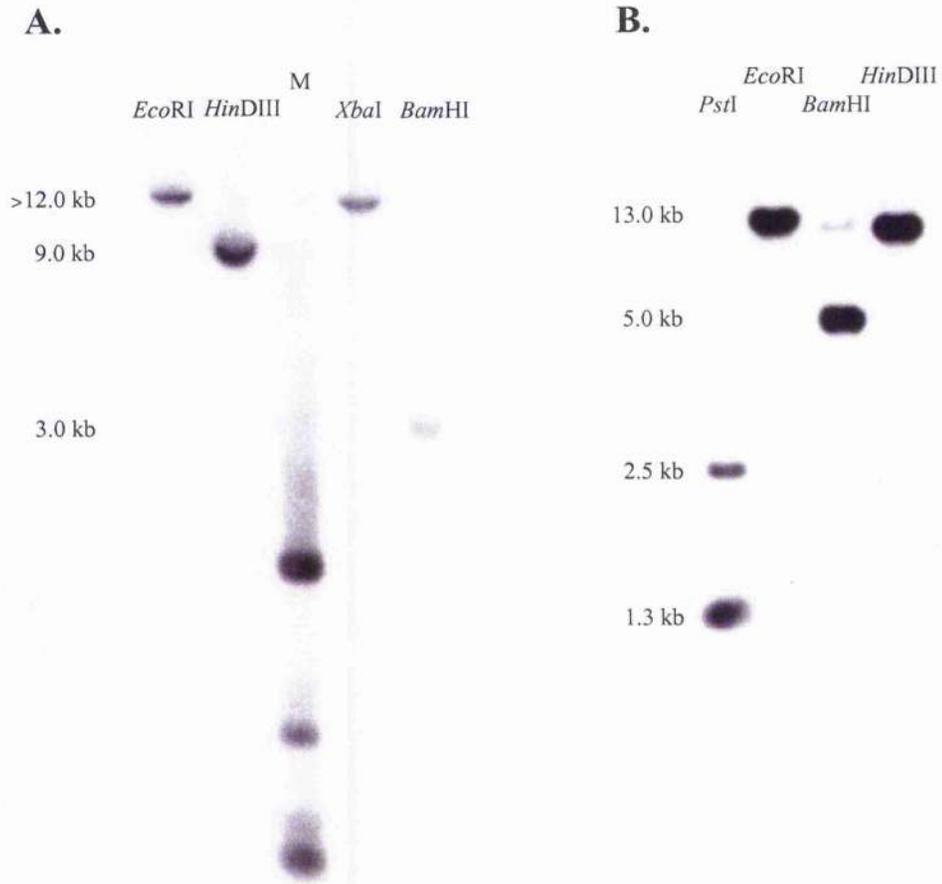
As discussed in Section 4.1.1, there is a high degree of homology between different skeletal muscle MyHC genes. There remained the possibility that a probe derived from the 5' untranslated region (UTR) of the emMyIIC gene, which might be expected to be isoform specific in other, less homologous gene families, might hybridise to different MyHC isoforms. However, screening a genomic Southern blot using the labelled 135 bp probe yielded only single bands for each of the enzymatic digests (Figure 4.3A), strongly suggesting that the probe was isoform specific. Screening the BAC filter using this 135 bp probe yielded one positive clone, subsequently obtained from the UK HMG centre.

### 4.3.2 PROMOTER REGION CLONING

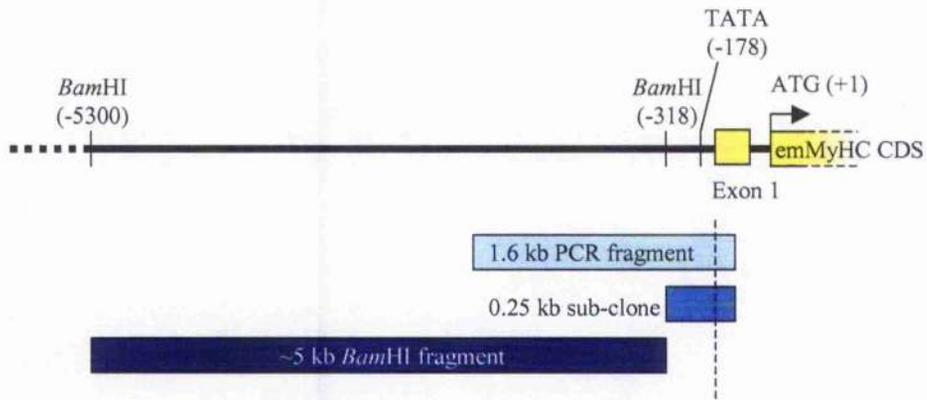
A total of three promoter constructs were obtained for the porcine emMyHC gene. A map showing their relationship to gene and the *Bam*HI sites that were used in their cloning is shown in Figure 4.4. Long-range PCR using the GenomeWalker<sup>TM</sup> kit yielded a 1.6 kb fragment, subsequently cloned into the pMOS-blue vector (Amersham-Pharmacia). The fragment was excised from the pMOS-blue vector using flanking *Hin*DIII and *Eco*RI sites present in the vector. This allowed the fragment to be religated into the corresponding sites on the pEGFP vector, in the sense orientation.

The 1.6 kb fragment was found to have an internal *Bam*HI site, used to generate a sub-clone with a smaller 0.25 kb insert. Initial sequence characterisation showed that this sub-clone contained the region immediately 5' of the emMyHC gene. In order to clone this region into the pEGFP vector, the region was amplified by PCR, with the antisense primer modified to engineer an *Age*I site into the 3' end of the resulting PCR product. The pEGFP *Bam*HI and *Age*I sites were then used to insert the 0.25 kb PCR product in the correct orientation.

Screening the digested BAC DNA with the 1.6 kb fragment identified a positive *Bam*HI fragment, of approximately 5 kb in length (Figure 4.3B). This fragment was



**Figure 4.3.** Southern Blots from the BAC library screening. **A.** Genomic Southern blot screened with the 135 bp fragment cDNA probe from the emMyHC 5' UTR, showing single isoform specificity (**M**, marker lane). **B.** Digest of BAC clone 36:L7 screened with the 1.6 kb GenomeWalker<sup>TM</sup> fragment. The 5 kb *Bam*HI fragment was isolated for generating the third EGFP reporter construct.



**Figure 4.4.** *Bam*HI restriction map of the three promoter region fragments used in the construction of reporter constructs.

immediately upstream of the 0.25 kb sub-clone containing the TATA box. To create a functional promoter construct, the 5 kb fragment was cloned into the *Bam*HI site of the pEGFP/0.25 kb clone (Figure 4.5). The orientation of the insert was checked by sequencing, using the vector T7 promoter site. A summary of the three EGFP clones is shown in Figure 4.5.

### 4.3.3 SEQUENCING

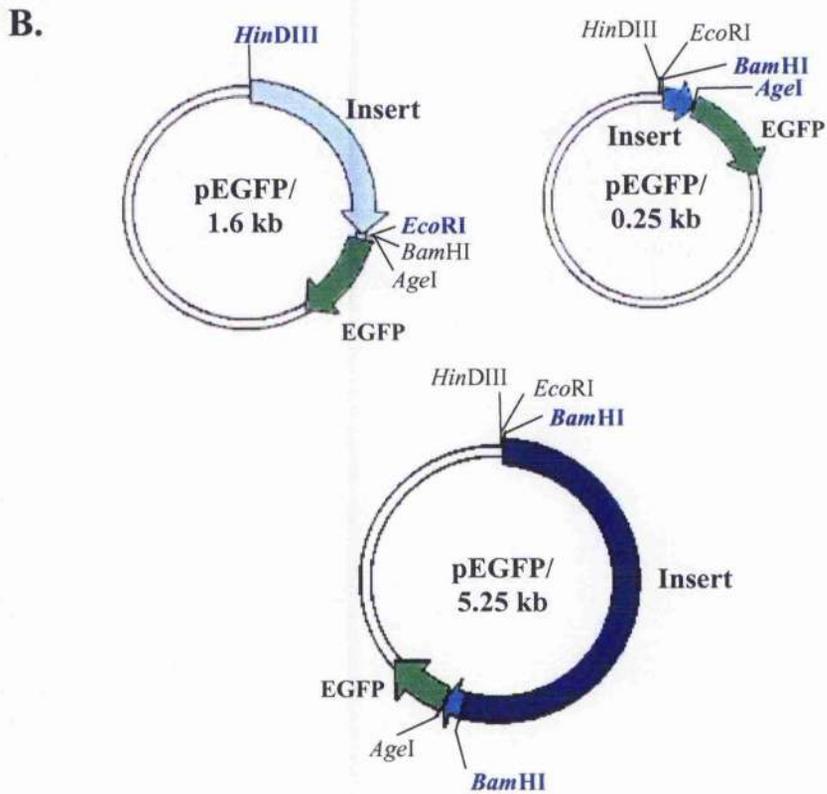
The initial sequencing upstream of exon 1 was carried out on the 0.25 kb promoter sub-clone. This sequence data was used to design a primer for subsequent sequencing further upstream using the BAC clone as a template. The furthest data 5' of the emMyHC coding regions was obtained from the pEGFP/5.25 kb clone, once it became available. This data was acquired in successive reactions, beginning with primers derived from vector sequence and continuing with oligomers based on generated sequence data. Finally, intron 1 was sequenced from a sub-clone created by PCR cloning using primers derived from the flanking exons and using the BAC clone as a template. This clone was kindly provided by Dr Yuh-Man Sun. The compiled sequence is shown in Figure 4.6. Part of this sequence was submitted to GenBank with the Accession Number AJ309014.

**A.**

```

31 CTCGAGCTCA AGCTTCGAAT TCTGCAGTCG ACGGTACCGC GGGCCCGGGA TCCACCGGTC 90
      HinDIII      EcoRI              BamHI              AgeI

```



**Figure 4.5.** Cloning strategies used to generate the pEGFP-1/emMyHC reporter constructs. **A.** Multiple cloning site of the pEGFP-1 vector (Clontech), highlighting the restriction sites used in the cloning. **B.** Restriction maps of the three reporter constructs. Restriction sites used in each cloning step are shown in blue.

Transcription Factor	Position	Strand
GATA-2	-4046	-
MEF-2	-3938	-
GATA-3	-3792	-
Myf5	-3388	+
MyoD	-3385	+
GATA-3	-3271	+
GATA-1	-3073	-
Myf5	-2840	+
Myf5	-2769	-
MEF-2	-2452	+
NFAT	-2012	-
NFAT	-1674	+
MEF-2	-1171	+
MEF-2	-756	-
Myf5	-654	+
MyoD	-651	+

**Table 4.1.** Putative transcription factor binding sites of interest found in the 5' UTR sequence of the porcine emMyHC.

The promoter sequence was compared with the emMyHC promoters from hamster and rat (Acc. N<sup>o</sup>s X56207 and X04267, respectively); this sequence comparison is shown in Figure 4.7. A high degree of homology was observed in the immediate 250 bp of the promoter regions. Through sequence comparisons, it became apparent that the translation start-site for the porcine emMyHC was located in exon 2, instead of 3, unlike all previously reported mammalian MyHC genes.

Comparisons with other species, and analysis with the Match<sup>TM</sup> and MatInspector software gave a deduced TATA box at 51 bp upstream of the transcription start site (position -51). A number of binding sites for MEF-2 and members of the MRF family were found, as summarised in Table 4.1. Of these two families, the consensus sites for MEF-2 are the more likely candidates for regulation of emMyHC activity. MEF-2 was first shown to interact with MyHC regulatory regions in studies on the  $\alpha$ -MyHC promoter (Adolph *et al.* 1993; Molkentin and Markham, 1993). For the MRFs, however, the consensus has long been that there is no direct interaction between family members and MyHC regulatory elements (Weiss and Leinwand, 1996). More recent research, such as that by Wheeler *et al.* (1999) on the role of MRFs in control of MyHC 2b, suggest that there is some direct interaction, so a role for the MRF-binding sites found in emMyHC in this study cannot be entirely ruled out.

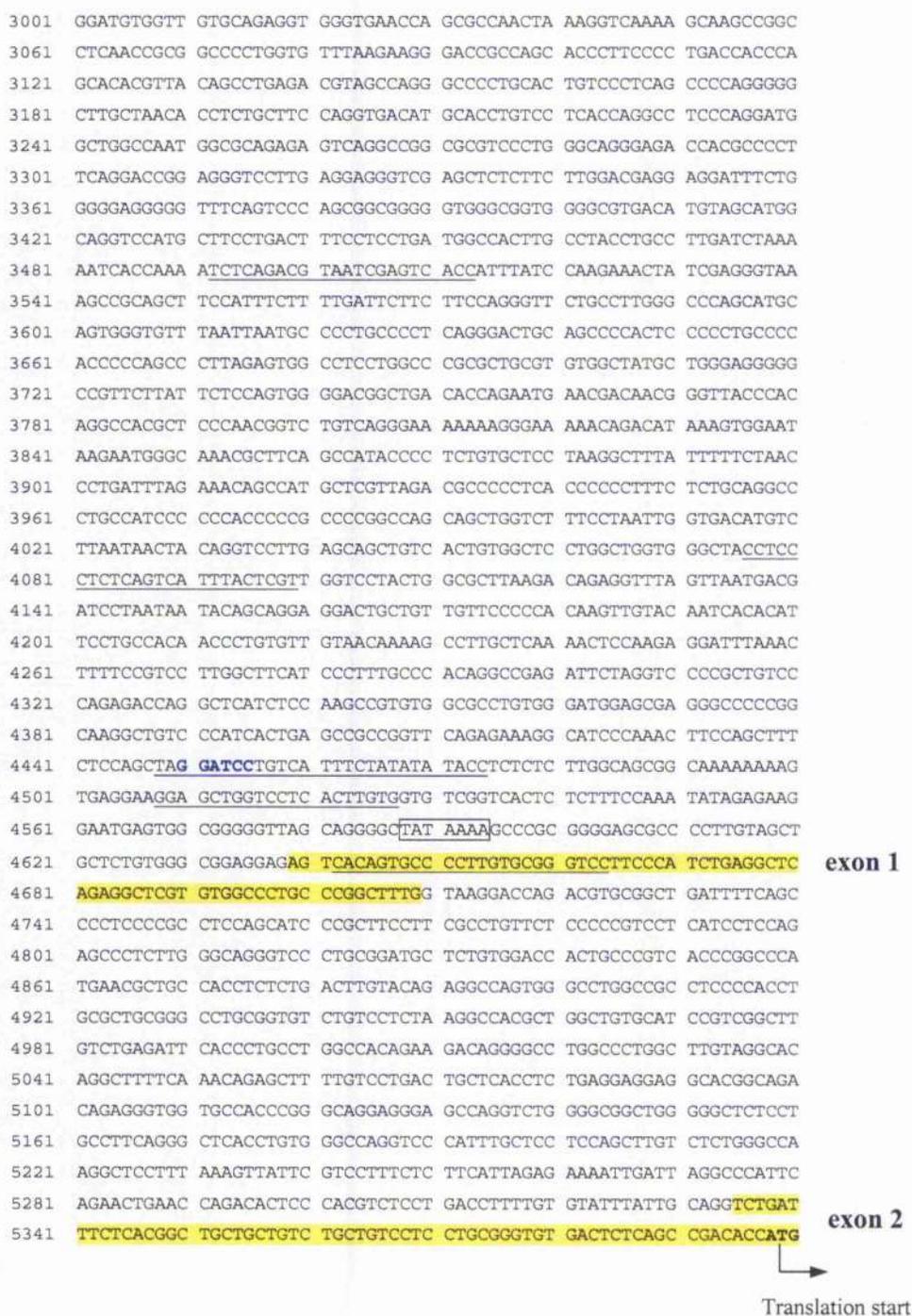
Of particular interest to this study, however, was whether any GATA or NFAT binding sites were present in the emMyHC regulatory region. One putative consensus binding site was located for GATA-2, and two NFAT sites. Three other GATA binding sites for other family members were also found: one for GATA-1 and two for GATA-3. The GATA-2 site was located in the more distal sequence, approximately 4 kb upstream of the transcription start site (position -4046). This places the site in sequence unique to the larger pEGFP/5.25 kb reporter construct. The more distal of the NFAT sites (position -2012) is also unique to pEGFP/5.25. It is more difficult to ascertain whether the second NFAT binding site (position -1674) is present on the pEGFP/1.6 kb construct without acquiring similar sequence data for this construct.

#### **4.3.2.1 Problems with LI-COR**

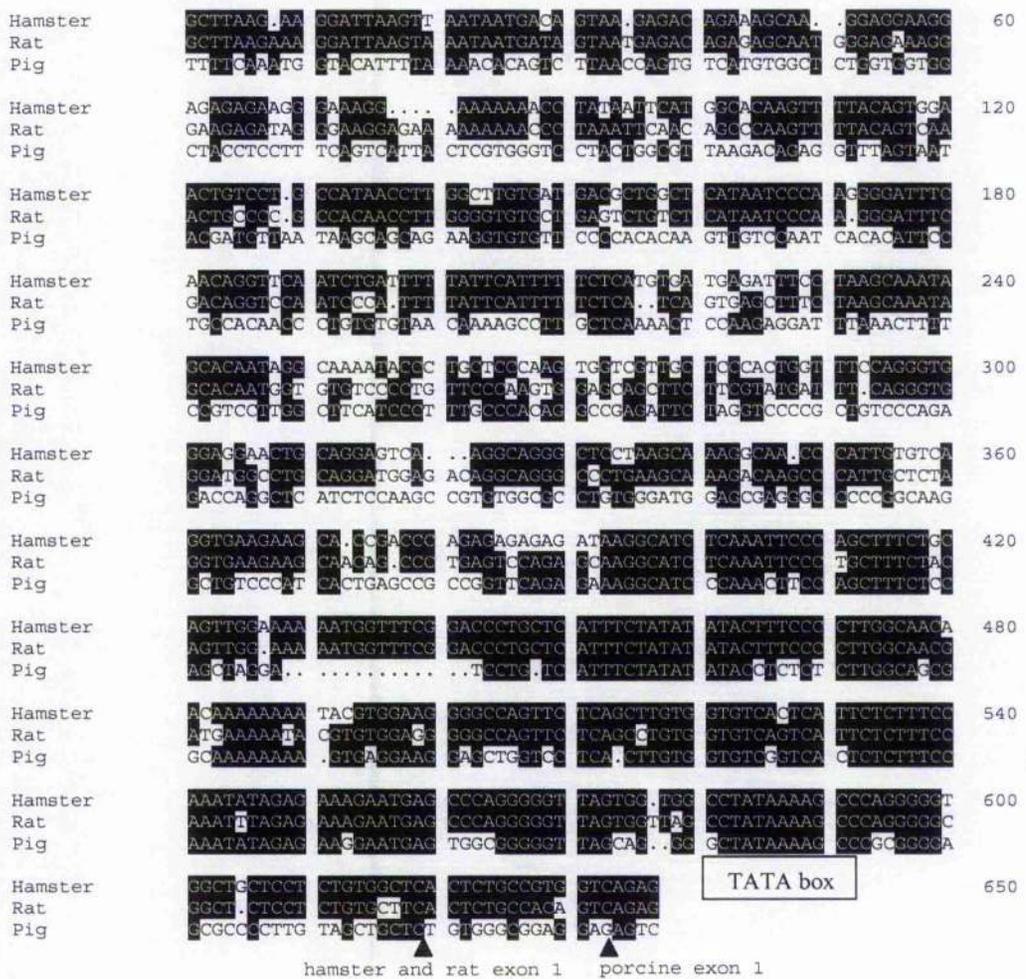
The LICOR system was only used for the earlier rounds of emMyHC regulatory region sequencing. The gel-based LI-COR sequencing method was abandoned in favour of ABI PRISM automated sequencing. There were a number of reasons for the change:

1. Four separate reactions were required per sequencing template, compared to just a single sequencing reaction required for the ABI system. Since just one failed reaction or gel-loading error out of these four could render an entire sequence worthless.
2. The pouring of the large-scale polyacrylamide gel presented numerous technical difficulties over the capillary system used by the ABI 310 and 3100 sequencers. The large, optical glass plates proved fragile, and easily cracked or broken. Minor imperfections, such as dust particles, pieces of old gel, etc could cause major errors in the laser-reading of the fluorescently labelled sequencing products.
3. The set-up of the gel and running buffer was also a source of complications, with the smallest of imperfections in the seal around the upper buffer tank sufficient to cause electrical leakage and halt the run (most often only discovered the following morning).

1 **GGATCCGAGC** CGCATCTGCA ACCTACACCA CAGCTCACGG CAACGTGCGA TCGTTAACCC  
61 ACTGAGCAAG GCCAGGGATC GAACCCACAA CCTCATGGTT CCTAGTCGGA TTCGTTAACC  
121 ACTGTGCCAC AACGAGAACT CCTGGCAGTT GACTTTTAGC AAATGCTTTT TCTGTATTTA  
181 TTGAAATGAT CATGTGGTTT TTGTCTTTTG TTGACATGTG ATTTGTGTGT GTTGAACGTG  
241 ACTTGTGATC CTAGGATGAA TCCAACCTGA TCATGGCGTA TGTGGTGTG GATTTGATTT  
301 GCTAATACTT TGTTAAGGAT TTTTGCATCT ATATTTCATCA AAGATATTGG CCCACAGTTT  
361 TCTTTTGGTG TGTCTTTGTC TGGTTTGGG ATCAGGGTGA CGGTGGCTTC ATGGAATGAC  
421 TTTGGGAGTG TTCCCTCTTC TTCAGTCTTT TGGAGAGATT TGAGAAGGAC CAGTATAATT  
481 CTTTCTTCCT CAGGGAAGCT GTCCTGGACT TTTGTTTGCA GGGAGTTTTT TGTGTGTGTA  
541 TGTTTTATTA CAGACTCTAT TTCACTTCTG ATGATTGGTT GGTCTGTTCA **TGTTATCTAT**  
601 TTCTTCTTAA TTGAGTTTGT GCAGGATGTA TATTTCTAGA AAGTTGTCCA TTTCTTCTAA  
661 TCATCCGTCC TGTTGGCATG TAATTGTTCA CAGTATCTC TTATGATTTT TTTATATTTT  
721 TGCAGTGTG GCTGTTATTT CTCCTCTTTC ATTCCTTATT TTGTTTATTT GGGTCCCCTT  
781 TCTTGAAAAG CACAACCTTA AATGAAAATC CAGTGTIACC CTTCAATTTT AAGTTAAGG  
841 CTTAAATCTT CATCCACCAC CATGGCTCCA GTTGGCCACA GAGCTGCTG TTCTCCATCC  
901 CCTCCCTGTG GTGACCCCTT GGTGACCTTT GGCCTTACC TTCCACAGCC TCATGCCCAT  
961 GGCAGACTGA TCTGCTTGGC TCCTTCTCGA TGGCATTGTT CAGAGGGGTG CATTACTCTT  
1021 CATTCTGTG CCCAAATCCC CTGGCAGGGC CCTGGGCCAT GCCTGCCCTT TAGAGCCTTC  
1081 TTTCCCTTGG AATGCCGTC TTGTCCCTC CTCTCCGAG CATTCTTCCC CGCTTCAGTC  
1141 ATGTGCTCC ATGCTCCCAA CCACAAGGC CCATCAATCT TCTGAATCAC AGGCCGACGA  
1201 GGGACAGCAG CCACAGCCGC CCCACCCAG GTAAGGGACT GACTCCGCTT TGCCAGCAGC  
1261 TGTGTCTCCA GCCTTCTCTC GCTCGCATCT CTAGTCTGT CCGCTGGCCT TCAGTGTGTG  
1321 TGGAATCCTC TCTTACCTG CTCACGTTCT GGCACCAGCT CGTACAGAT TTCACGTACA  
1381 GATTGAGGCT GGACGTTTTT AAGAAGTTG CTCTGACATC ACGAACTTGT GGCTCCCCTC  
1441 GTAATTCGG AACCACCCAC TCCCAGACT GCTCGCGTGT AGACAGCGTG GTGTTTCGCA  
1501 GCGTGGCTCT GACATCAAAT TCAAGTGCTT CATAATCCTG GATGGTTAGT ACCACACCAT  
1561 GTGCTTATCT AAGTGTATCT CCACAGCGTC CGTAGTAGAA ATTGGGGAAA GTGGATGTTT  
1621 GTTACAGGAG AGTTGTGAGC ACGCTCTAAT TTCTACCTGG GGCTCTGAGC ACTGGTTCAG  
1681 GACACCCAC CCTTGGCCTC AGGCTGGGAA TGGTCTTGGC TCAGAATAAC CAGGCTCCCC  
1741 ACTTGCAGTG ACACCTAATC CCCCTTGGC GGGACGTGGA GAAGGCCCTT GATCCAGAG  
1801 GCATCTGCTG CGAGGCTGAC TTCATGCCCT GGCAGAGGGC AGGGCTGCAG AACTCAGGGT  
1861 CCACATGCGG AATGGAGGCA GATGGCACAG GAGGAAGCTC AGGCTCCAGG AGGGGCCAG  
1921 CTGTGCTCTG GTCAGCGCCT CCCGAGCTGC TCGCATCTT GAGACAAATC GTTTTCTTCC  
1981 AGCATATCCG GGAGTAAAGG TGGCTCTCTG TCCAGGCCTG GGGGTCAGCT CTAGATGGGT  
2041 GATGGCCGAG AGAACAGGGC TCCTCCTCTT CCTGGAAGGC TGGCTACTAA CACCCTGGGA  
2101 AGTCGTTCTG CTCATCAGAG GAGTCCCACA CCAGCTGGCC CAAAAGCTGC CTGTTAAGC  
2161 ACCAAGAGGT TCTCTCTTT GGATGTAAAT CTTTCTAAAA AATACATATA TATTTCTTTC  
2221 TGCCTCCTCA GGTCAATAAA ACCAGTGTCC AATGTGATGT ACTCAATGCC ATCAAGAGGA  
2281 CCAGTCGTAG TGGGCAGCAA CACACAGAAG CAAGCCTTCA GTCTCCAGCG AGTACATGC  
2341 AGCACCAGCG ACAGCGTGCC TGACATAATA TCCAGCCGAA TGCACGTAGC TGGTCTGCCA  
2401 GGCAGCTCTA TCACCCCAT TTTACAGATG AGAAAACAGA GGCTCAGAGA AGTTAGGTGC  
2461 ACTGCCAGCC ATCACACGGT AGCGAGAGTG AGCGCCAGGT CCACAGCCGG GCCCGTCTGG  
2521 CCCGACGCAC CACAGCCTTC AGGCTGCAGT TCCCACCGCC CCTCCCTCCT TATGGGTGTT  
2581 TTCCGGGGCA CCTTGCTTCC GGATTCTTGA CCTCCGTTTT TACGGCTGTT **TCCTATGTGG**  
2641 TGGTTATTGC CGTGCTTCC ACCCTCAGGG TTCTCTGGC GAGCGGAGCT **CCCTGCCTAA**  
2701 CTCGGACCCT CGTTCCATGG CCCGGCAGG CTCTTCCCC TCGTGCCTTC CAGCCCCTA  
2761 TGTGAGAAG CAGGCCGTCA GGCAGATGAC CCGAGACCTC CCAGCCGAGC CTCTGTGCTC  
2821 GTGGAGGACG TGTGACGGAG AGAAGGAAGA CAGGCCGGCT GACGGGGGAA AAGCTGGGGT  
2881 GGGAGGCCGT CAGCCTTGG CTCGCCTTGG GCTCAGGGAT GCGACGTGGC TTCACGCTTG  
2941 ACAAGAAGCT CAAAGAAATC CTGTGTGGAA **AACGTCTCT** CCGTGTCCC GAGGTGGGAT



**Figure 4.6.** Genomic sequence 5' of the porcine emMyHC coding region, including exons 1 and 2 (Acc. N<sup>o</sup> AJ309014). Sequences used for primer design, for amplification of the promoter region and for upstream sequencing are underlined. Exonic sequences are highlighted yellow, and the TATA box outlined. The GATA-2 and NFAT consensus binding sites are shown in green, *Bam*HI sites in blue.



**Figure 4.7.** Multiple alignments of the immediate emMyHC promoter regions of hamster, rat and pig, showing the high degree of homology (black shaded sequence) in the proximal 250 bp (Hamster and rat Acc. N<sup>o</sup>s X56207 and X04267, respectively).

## 4.4. DISCUSSION

The porcine emMyHC gene is the last of the major skeletal muscle isoforms to be characterised in the pig. As such, the isolation and sequencing of the 5' regulatory region of this gene is among the final pieces in describing the porcine sarcomeric MyHC family. The findings described here were predictable, in that a high degree of sequence identity was observed between the porcine emMyHC regulatory domain, and previously isolated orthologues. However, the presence of the transcriptional start site on exon 2 is a novel finding not previously characterised in any other MyHC isoforms, which have start sites in exon 3 (Weiss and Leinwand, 1996). This feature has since been observed in the human orthologue, following the complete characterisation of human genomic data, and similarly in the chimpanzee (Ensemble genes: ENSG00000109063 and ENSPTRG000000008773 respectively). At the time of writing, human, chimpanzee and porcine emMyHC genes are the only MyHC genes known to have a start of translation on exon 2, though the functional significance of the difference is unknown. It is likely that other emMyHC orthologues may be found to have a similar gene structure.

The emMyHC isoform is characteristic of a specific stage in development, both in embryonic myogenesis and in the early stages of muscle regeneration. Since GATA-2 is known to be involved in muscle hypertrophy, it is possible that emMyHC might be a target for this transcription factor. This hypothesis is supported by the presence of a consensus GATA-2 binding site within the 5' regulatory region. One way to test this further would be to make use of the three emMyHC reporter constructs in conjunction with the GATA-2 clone described in Chapter Three. If GATA-2 does affect emMyHC expression via the consensus binding site uncovered in this study, it might be expected that overexpression of GATA clone-7 should have some effect on the activity of the pEGFP/5.25 kb construct. A preliminary investigation into the effect of the porcine GATA-2 on the emMyHC reporter constructs is described in Chapter Five.

GATA-2 is just one of a number of potentially interesting regulatory sites listed in the 5' UTR of the porcine emMyHC (Table 4.1). The possibility of MEF-2 interaction with the emMyHC regulatory regions is especially relevant given the co-operative role of GATA-4 and MEF-2 in cardiogenesis (Morin et al., 2000). Though an investigation

into the effect of MEF-2 on emMyHC is beyond the scope of this study, it might be speculated that GATA-2 and MEF-2 might similarly co-operate in regulating emMyHC. While a study into the role of NFAT-2 on emMyHC function could not be carried out without the full-length NFAT clone, the discovery of consensus binding sites leaves open the possibility that emMyHC may be regulated by calcineurin/NFAT signalling.

**CHAPTER FIVE - GATA-2 AND NFAT-2  
FUNCTIONAL AND EXPRESSION STUDIES**

## 5.1 INTRODUCTION

Chapters Three and Four have focussed on the cloning, respectively, of *trans*- and *cis*-elements involved in fibre-specific gene expression. This Chapter describes the studies that used these materials to investigate the potential roles for GATA-2 and NFAT-2 in porcine muscle. One such step, and the first study listed here, is to investigate its tissue-specific and developmental patterns of expression. As a means to accomplish this aim, the putative untranslated regions of GATA clone-7 offer isoform-specific targets for the design of primer and probe sets for quantitative real-time RT-PCR. Though lacking the complete coding region, the NFAT clone-3 still possesses 3' untranslated sequence that can be used as the basis for a similar study into NFAT-2 expression patterns. The possession of an apparent full-coding sequence for porcine GATA-2, cloned into a CMV promoter-driven expression-vector, allows for further studies into GATA-2 function. Before the pBK-CMV/GATA clone-7 construct can be used, however, it is necessary to confirm that the cDNA insert represents an mRNA transcript, and will be transcribed and translated in mammalian cells.

To a certain extent, the expression patterns of GATA-2 and NFAT-2 have previously been characterised in studies in other species. While both factors are the only isoforms of their respective families known to be expressed in mature skeletal muscle fibres (Abbott *et al.*, 1998; Musaro *et al.*, 1999), they are also expressed in a wide range of other tissues (Hocy *et al.*, 1995; Masuda *et al.*, 1995; Kumano *et al.*, 2001; Patient and McGhee, 2002). While briefly mentioned in Section 1.4, the expression patterns and tissue-specific functions of GATA-2 and NFAT-2 are considered here in greater depth. In addition, previously characterised functions of GATA-2 in muscle are also considered, to provide a context for the results that were obtained.

### 5.1.1 DISTRIBUTION OF GATA-2

The transcription factor GATA-2 is expressed in a wide range of tissues, first characterised by Yamamoto *et al.* (1990) in studies in cells of the erythroid lineage in the chicken. It was found that GATA-2 was expressed at lower levels than GATA-1, but with a far more extensive tissue distribution. While GATA-1 was largely confined to cells of the erythroid lineage, GATA-2 was expressed in developing brain, liver and cardiac muscle, in adult kidney and fibroblasts, and in all stages of the erythroid

	Tissue	References
All Stages	Erythroid lineage	(Yamamoto <i>et al.</i> , 1990)
Development	Placental trophoblasts	(Ng <i>et al.</i> , 1994; Ma <i>et al.</i> , 1997)
	Brain, Liver, Heart	(Yamamoto <i>et al.</i> , 1990)
	Urogenital system	(Zhou <i>et al.</i> , 1998b)
	Pituitary gland	(Dasen <i>et al.</i> , 1999)
	Spinal cord	(Zhou <i>et al.</i> , 2000)
Adult	Kidney, fibroblasts	(Yamamoto <i>et al.</i> , 1990)
	Epithelial cells	(Lee <i>et al.</i> , 1991; Dorfman <i>et al.</i> , 1992)
	Adipose tissue	(Tong <i>et al.</i> , 2000)
	Skeletal muscle	(Musaro <i>et al.</i> , 1999)

**Table 5.1** Tissue distribution of GATA-2 during development and in the adult.

lineage. The importance of GATA-2 in haematopoiesis was confirmed by Tsai *et al.* (1994), who found that *Gata-2* knockout mice die mid-gestation due to haematopoietic failure. While the majority of further studies focussed on the role of GATA-2 in erythropoiesis (reviewed in Orkin, 1995; Patient and McGhee, 2002), a wider picture of GATA-2 expression has been provided by a number of studies. The role of GATA-2 in the development of other tissues has been obscured by the early lethality of the *Gata-2* *-/-* phenotype, but studies have revealed a role for GATA-2 in urogenital development (Zhou *et al.*, 1998b), pituitary cell fate (Dasen *et al.*, 1999), placental trophoblasts (Ng *et al.*, 1994; Ma *et al.*, 1997) and neurogenesis (Zhou *et al.*, 2000). In adult tissues, GATA-2 expression was also found in epithelial cells (Lee *et al.*, 1991; Dorfman *et al.*, 1992), adipose tissue (Tong *et al.*, 2000), and in skeletal muscle (Musaro *et al.*, 1999). The tissue distribution of GATA-2 is summarised in Table 5.1.

### 5.1.2 DISTRIBUTION OF NFAT-2

NFAT-2, also commonly referred to known as NFATc or NFATc1, is the NFAT family member most closely associated with adult muscle fibres (Abbott *et al.*, 1998). As with other members of the NFAT family, NFAT-2 was first characterised in the immune system (reviewed in Rao *et al.*, 1997), specifically in T cells, thymus, spleen and peripheral blood lymphocytes (PBL) (Hoey *et al.*, 1995; Masuda *et al.*, 1995). NFAT-2 expression has also been characterised in the endothelium of the developing heart, and is essential for heart valve formation (de la Pompa *et al.*, 1998; Ranger *et al.*, 1998). *Nfat-2* *-/-* mice die *in utero* from congestive heart failure caused by defective valve

	Tissue	References
All Stages	T-Cells, thymus, spleen, PBL	(Hoey <i>et al.</i> , 1995; Masuda <i>et al.</i> , 1995)
Development	Cardiac endothelium and developing heart valves	(de la Pompa <i>et al.</i> , 1998; Ranger <i>et al.</i> , 1998; Johnson <i>et al.</i> , 2003)
Adult	Skeletal muscle	(Abbott <i>et al.</i> , 1998)
<i>In vitro</i> studies	Differentiating osteoclasts Vascular smooth muscle Glomerular mesangial cells	(Ishida <i>et al.</i> , 2002; Takayanagi <i>et al.</i> , 2002) (Wada <i>et al.</i> , 2002) (Sugimoto <i>et al.</i> , 2001)

**Table 5.2** Tissue distribution of NFAT-2 during development and in the adult.

development. In addition to this developmental role, NFAT-2 expression has also been demonstrated in a number of cell types, in *in vitro* studies. These include differentiating osteoclasts (Ishida *et al.*, 2002; Takayanagi *et al.*, 2002), vascular smooth muscle cells (Wada *et al.*, 2002), and kidney glomerular mesangial cells (Sugimoto *et al.*, 2001). These *in vitro* studies do not specify a developmental stage, but the processes mediated by NFAT-2 leave open the possibility of a role either in development or the adult maintenance of the respective tissue. The distribution of NFAT-2 is summarised in Table 5.2.

As discussed in Section 1.4.2, the human NFAT-2 has three splice variants (Park *et al.*, 1996; Chuvpilo *et al.*, 1999). Two of the splice products are known to be expressed in skeletal muscle (Hoey *et al.*, 1995, Masuda *et al.*, 1995), with the third characterised in T cells only (Chuvpilo *et al.*, 1999). Many studies in both muscle and non-muscle tissues do not specify which splice variant was observed, and so the exact distribution of these splice variants, together with their functional significance, remains unclear at present. The NFAT clone isolated during the work described in Chapter Three showed closest homology to the human NFAT-2b (Section 3.3.4).

### 5.1.3 GATA-2 FUNCTION IN MUSCLE

GATA-2 was first characterised in skeletal muscle in the study on IGF-1 induced muscle hypertrophy by Musaro *et al.* (1999), in which GATA-2 was upregulated in response to IGF-1 expression in a transfected rat myoblast cell line. GATA-2 upregulation was also induced by calcineurin expression, and in both cases was

abolished by CsA administration. Even though a role for calcineurin/NFAT in muscle hypertrophy was later disputed (Glass, 2003a), the initial finding that GATA-2 is upregulated in hypertrophying muscle was further supported in whole-muscle studies in IGF-1 transgenic mice (Musaro *et al.*, 2001). In addition, GATA-2 was also identified in muscle regeneration in rats, following damage through bupivacaine injection (Sakuma *et al.*, 2003). This study showed co-precipitation of GATA-2 and NFAT-2, and that regeneration was affected by CsA, linking the activity of GATA-2 in the regenerative pathway with calcineurin signalling. GATA-2 has not been demonstrated to have a role in the expression of slow-fibre specific genes. However, GATA-4 has been shown to bind to the slow/ $\beta$  MyHC promoter and upregulate this gene (Wang *et al.*, 1998), and it remains possible that GATA-2 may have a similar role in skeletal muscle.

#### **5.1.4 AIMS**

There were three parts to the work described in this Chapter. The first was to study the tissue distribution of both GATA-2 and NFAT-2 by quantitative real-time RT-PCR, in order to analyse the expression patterns for the two transcription factors in the context of published data. The temporal distribution in developing muscle was also investigated, something not previously reported for GATA-2 and which could potentially indicate a novel role in myogenesis for this factor. The second goal was to produce and characterise the translation products of the novel GATA-2 clone, as a prerequisite to use in further functional studies.

Finally, two studies were undertaken to test the possible relationship between GATA-2 activity and MyHC expression. One approach, the more comprehensive of the two, was to characterise the expression of the different MyHC genes in porcine muscle fibres transfected with the pBK-CMV/GATA clone-7 construct, and to compare these patterns with untransfected myotubes. This required the establishment of porcine primary myoblast cultures and the development of differentiation protocols. The second approach, focussing more directly on the relationship between GATA-2 and emMyHC, was a preliminary investigation into the effect of the CMV promoter-driven GATA-2 overexpression on the activity of the emMyHC/luciferase reporter constructs generated in the work described in Chapter Four.

## 5.2 MATERIALS AND METHODS

### 5.2.1 QUANTITATIVE REAL TIME RT-PCR

For the most part, quantitative real-time RT-PCR was carried out exactly as described in the General Methods chapter, section 2.2.6. This section lists the specific primer and probe sets used, and the origins of the tissue samples assayed.

#### 5.2.1.1 Design of Porcine GATA-2 and NFAT-2 probes

The TaqMan real-time PCR primer and probe sets were designed around the 3' untranslated region of both GATA-2 and NFAT-2 clones using Primer Express<sup>®</sup> v.2.0 software, as described in the manufacturer's protocols (Applied Biosystems). RT-PCR primer and probe sets were as listed in Table 5.3. Also included in this table is the porcine  $\beta$ -actin primer and probe set, designed previously by the same method, which was used to normalise GATA-2 and NFAT-2 results by the relative standard curve method (Section 2.2.6.5).

Gene		Sequence (5' to 3')
GATA-2	Sense	CTG GGA GGC TTC AG
	Anti-sense	CTT GCC CCC TCC TTT CAT CT
	Sense probe	TTT CCT GCA TGG ACA CCT GTT TGG AGA
NFAT-2	Sense	TTT GTA AGT CAC AGA GGC GAA CA
	Anti-sense	CCA TGG ATC CTC GGC TCT T
	Sense probe	CTG AAA GCC ATA TTG AAC CAT TTG CAC ATT ACT
$\beta$ -actin	Sense	CCA GCA CCA TGA AGA TCA AGA TC
	Anti-sense	ACA TCT GCT GGA AGG TGG ACA
	Sense probe	CCC CTC CCG AGC GCA AGT ACT CC

**Table 5.3.** GATA-2, NFAT-2 and  $\beta$ -actin primer and probe sets designed for TaqMan real-time PCR.

#### 5.2.1.2 Preparation of Samples

Pigs were slaughtered by captive bolt, followed by severance of major neck blood vessels. Tissue samples for mRNA quantification were taken within 30 min of death and snap-frozen in liquid nitrogen. Direct extraction of mRNA from the tissue samples, quantitative real-time RT-PCR and data analysis was carried out as described in Section 2.2.6.

To investigate GATA-2 and NFAT-2 expression across a range of tissue types, a range of tissue samples were taken from a six-week-old, Large White cross-bred animal. To analyse differences between *longissimus dorsi* (LD) and *psoas* muscles (fast- and slow-twitch muscles respectively), pooled cDNA from a panel of six, adult (22 week-old) Duroc pigs was used. Finally, for the foetal stages of development, pooled cDNA was used from the LD muscles of three Large White cross-bred animals each at 35, 49, 63, 77 and 91 days gestation.

## **5.2.2 PROTEIN ANALYSIS**

Porcine GATA-2 transcription-translation was performed by Western blot analysis of GATA-2 transfected C2C12 cells and by an *in vitro* transcription/translation system. In both approaches, the primary objective was to test whether the pBK-CMV/GATA-2 construct was transcriptionally functional and able to generate a viable protein in culture.

### **5.2.2.1 Preparation of Cell Extracts**

#### 5.2.2.1.1 Cell Culture and Transfections

C2C12 myoblasts were cultured as described in section 2.2.5.3. Cells were transfected with pBK-CMV/GATA clone-7 (Section 3.3.3.1) and a pCMV-IGF-1 expression construct. Stable transfections were carried out as described in Section 2.2.5.2, with the cells maintained in 1 mg/ml G418.

#### 5.2.2.1.2 Cell Harvesting

Culture medium was removed and the cells washed twice in ice cold PBS. Further PBS was added, and the adherent cells scraped free. The resulting suspension was removed to a pre-cooled 50 ml Falcon tube, spun down gently to collect the cells, supernatant removed and the cell pellet resuspended in 1 ml of ice-cold PBS. This was transferred to a 1.5 ml microcentrifuge tube, spun at  $10,000 \times g$  for 5 min in a rotor chilled to 4 °C. The supernatant was removed and the cells snap-frozen in dry ice. Cell pellets were stored at -70 °C until use.

#### 5.2.2.1.3 Preparation of Nuclear Extract

Cells were resuspended in lysis buffer (10 mM Tris, pH 7.9, 10 mM NaCl and 2 mM MgCl<sub>2</sub>, supplemented with 0.5 mM PMSF and 1 mM DTT immediately prior to use), at five times the volume of the cell pellet, and incubated on ice for 5 min. Cells were lysed with the addition of detergent (50 µl/ml 10% NP40), and the lysate kept on ice a further 10 min. The lysate was spun down at 3,000 rpm for 5 min, and the pellet resuspended in cold buffer (20 mM HEPES, 0.42 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 25% glycerol, supplemented immediately prior to use with 18.5 µl protease inhibitor mix (PIM), 0.5 mM PMSF, 1 mM DTT). The resuspended pellets were incubated with slow rotation at 4 °C for 30 min, then centrifuged again at 10,000 × g for 10 min at 4 °C. The supernatant of nuclear extract was transferred to a fresh tube, snap frozen and stored at -70 °C until use.

#### 5.2.2.1.4 TCA Precipitation of Proteins

Precipitation of protein by the addition of trichloroacetic acid (TCA) was used in instances where the protein content of the samples was too dilute for loading onto SDS-PAGE gels. Samples were precipitated with the addition of 0.25 volumes of TCA solution (100% w/v trichloroacetic acid, 0.4% sodium deoxycholate), vortex mixed and placed on ice for 15 min. Following this incubation, the samples were spun at 10,000 × g for 15 min, and the supernatant carefully removed. To remove residual traces of TCA, 0.5 ml of acetone was added, vortex mixed and spun again at 10,000 × g for 5 min. The supernatant was removed and samples vacuum-desiccated. Samples were resuspended in SDS-protein sample buffer, boiled for 5 min and loaded as described in the SDS-PAGE protocol outlined below.

#### **5.2.2.2 SDS-PAGE**

The separation of proteins according to their molecular weight, and subsequent visualisation was carried out using SDS-polyacrylamide gel electrophoresis as described by Laemmli (1970).

Gel plate, comb and spacers were cleaned and carefully assembled. The separating (or resolving) gel was prepared with 1.5 ml of 4 × Lower buffer (1.5 M Tris at pH 8.8, 0.4% SDS) and between 5 to 15% acrylamide, made up to 5 ml with water.

Polymerisation of the gel was started with the addition of 11  $\mu$ l tetramethyl-1-, 2-diaminomethane (TEMED) and 30  $\mu$ l ammonium persulphate (APS), mixed by vortex, and the gel applied between the glass plates with a Pasteur pipette. Isobutanol (2-methyl-1-propanol) was layered on top, and the gel left to polymerise for 1 hour at room temperature. The isobutanol was washed out with distilled water, and the loading comb placed between the glass plates. The stacking gel, consisting of 1.25 ml Upper buffer (0.5 M Tris, pH8.8, 0.4% SDS), 0.7 ml 30% acrylamide and 3 ml water, was induced to polymerise as above, and applied to the gel. This was left for 30 min at room temperature, for the polymerisation to complete.

The polymerised gel was cleaned and placed in the running tank, with the lower reservoir filled with protein running buffer (10 mM Tris, 76.8 mM glycine, 0.1% SDS, pH 8.3). This was then sealed with 2% agarose. The gel comb was carefully removed, and the upper reservoir filled. Samples were prepared, if necessary precipitating with TCA as described above. Otherwise, an equal volume of sample buffer (250 mM Tris-HCl, pH 6.8, 2% SDS, 20%  $\beta$ -mercaptoethanol, 40% glycerol, 0.5% bromophenol blue) was added, the mixture boiled for 5 min and the samples loaded immediately. Gels were run at 100 volts for approximately 40 min until the dye front reached the interface, then at 160 volts until sufficient separation was obtained.

### **5.2.2.3 Western Blotting**

Western transfer of proteins from SDS-polyacrylamide gels onto polyvinylidene fluoride membranes (Schleicher and Schuell UK) was carried out using a modification of the procedure of Towbin *et al.* (1979). The resulting Western blots were probed for the presence of GATA-2 using a mouse monoclonal IgG, anti-GATA-2 antibody, CG2-96 (Santa Cruz Biotechnology). Detection was by the enhanced chemiluminescence (ECL) method (Amersham). This method utilises a secondary antibody conjugated with horseradish peroxidase (HRP), which catalyses the oxidation of luminol by hydrogen peroxide in a light-emitting manner. Chemiluminescence is increased by chemical enhancers, to the point where it is detectable by autoradiography through blue-light sensitive film.

#### 5.2.2.3.1 Transfer onto PVDF membrane

The PVDF membrane was soaked in 100% methanol for 5 seconds, followed by water for 5 min, then in transfer buffer (25 mM Tris, 20% v/v methanol, 1.1% w/v glycine, pH 8.3) for 15 to 20 min. The SDS gel was soaked in transfer buffer for 15 to 20 min, and placed atop the membrane. Membrane and gel were sandwiched between two sets of three sheets of blotting paper, pre-soaked in transfer buffer.

#### 5.2.2.3.2 Antibody Probing

Once blotted, the filter was washed for 10 min in TBS (25 mM Tris at pH 7.6, 150 mM NaCl), and blocked for 30 min in 500 ml milk buffer (MB) (1 × TBS, 0.5% Tween-20, 4% powdered milk). The filter was probed with the primary antibody, mouse anti-GATA-2, for 2 hours at room temperature or overnight at 4 °C. The filter was then washed, three times for 2 min in MB, and probed with the secondary antibody, conjugated to horseradish peroxidase, for 1 hour at room temperature. The filter was washed a further five times in MB, three times for two minutes and twice for 15 min, before a final 5 min wash in TBS.

#### 5.2.2.3.3 Development using ECL

The development of the Western blot was carried out as described in the Amersham ECL protocols. Equal volumes of the two detection reagents were mixed, sufficient for 0.125 ml/cm<sup>2</sup> of filter, and pipetted onto the PVDF membrane. After 1 min, excess reagent was drained off and the filter Saran wrapped. The filters were exposed to ECL Hyperfilm<sup>TM</sup> (Amersham) in a darkroom, beginning with an exposure time of 1 min. Ideal exposure time, depending on the sample and background levels, varied between 5 s to 15 min.

#### **5.2.2.4 *In-Vitro* Transcription Translation**

The TNT<sup>®</sup> coupled reticulocyte lysate system (Promega) was used in the *in vitro* transcription translation of the novel GATA-2 clone, using T7 RNA polymerase. The procedure was carried out as described in the manufacturer's protocol, briefly summarised as follows.

The rabbit reticulocyte lysate was rapidly thawed and placed on ice. To prevent degradation of the lysate components, this reagent was not subjected to freeze/thaw more than twice. The reaction components, consisting of the TNT<sup>®</sup> lysate and reaction buffer, T7 RNA polymerase, amino acid mixture, RNasin ribonuclease inhibitor and DNA template, were assembled in a microcentrifuge. For the DNA template, 1 µg of GATA-2 plasmid DNA was used, purified by large scale plasmid preparation (section 2.2.1.2.2). The volume was made up to 50 µl with nuclease-free water, and mixed gently by pipetting. The reaction mixture was incubated for 90 min at 30 °C. Transcription translation products were visualised by SDS-PAGE largely as described in section 5.2.2.3.

### **5.2.3 PRIMARY CELL CULTURES**

The harvesting of muscle tissue for satellite cells, and the subsequent culture and maintenance of the primary cell culture was as described in section 2.2.5. Primary cell cultures were prepared from LD and *psoas* tissue samples, from a six-week-old, Large White cross-bred animal.

#### **5.2.3.1 Testing for Differentiation**

A potential problem in the use of primary muscle cell preparations as a source of myoblasts is the presence of contaminating non-myogenic cells, such as fibroblasts (Baroffio *et al.*, 1995). Before using the primary satellite cells as a model for the effects of GATA-2 on fibre type, a sample culture was tested for the capacity to differentiate normally. One 75 cm<sup>2</sup> flask of primary cells, from both the LD and *psoas* was grown to confluency, switched to differentiation medium and cultured for a further seven days (as described section 2.2.5.1.2). The cells were harvested for mRNA and cDNA prepared for quantitative real-time RT-PCR as described in Section 2.2.6. The cDNA was assayed for the presence of  $\alpha$ -actin and for pre- and post-natal MyHC expression, using the primer and probe sets listed in Table 5.4.

Gene		Sequence (5' to 3')
Skeletal $\alpha$ -actin	Sense	CCA GCA CCA TGA AGA TCA AGA TC
	Anti-sense	ACA TCT GCT GGA AGG TGG ACA
	Sense probe	CCC CGC CCG AGC GCA AGT
$\beta$ -actin	Sense	CCA GCA CCA TGA AGA TCA AGA TC
	Anti-sense	ACA TCT GCT GGA AGG TGG ACA
	Sense probe	CCC CTC CCG ACC GCA AGT ACT CC
Slow MyHC	Sense	GGC CCC TTC CAG CTT GA
	Anti-sense	TGG CTG CGC CTT GGT TT
	Sense probe	CCF CTT TCT TCT CCC AGG GAC ATT CGA
emMyHC	Sense	CCC GGC TTT GGT CTG ATT T
	Anti-sense	GGT CTC GGC TGA GAG TCA
	Sense probe	TGC TGC TGT CTG CTG TCC TCT GCC
periMyHC	Sense	CGA GCC CTC CTG CTT TAT CTC
	Anti-sense	TGC CAG ATG AAA ATG CAG GTT
	Sense probe	CCA AGA GCC CAG AGT GTT AGG CAC TTC C
2aMyHC	Sense	TTA AAA AGC TCC AAG AAC TGT TTC A
	Anti-sense	CCA TTT CCT GGT CGG AAC TC
	Sense probe	TTC CAG GCT CCA TCT TCT CAC TTG CTA AG
2xMyHC	Sense	AGC TTC AAG TTC TGC CCC ACT
	Anti-sense	GGC TGC GGG TTA TTG ATG C
	Sense probe	AGC CCA GTC AAA GAC CCT TTG AGA TGC A
2bMyHC	Sense	CAC TTT AAG TAG TTG TCT GCC TTG AG
	Anti-sense	GGC AGC AGG GCA CTA GAT GT
	Sense probe	TGC CAC CGT CTT CAT CTG GTA ACA TAA GAC G

**Table 5.4.** Porcine primer and probe sets designed for TaqMan real-time PCR.

### 5.2.3.2 Primary Cell Transfections

Transient GATA-2 transfections were set up in six-well plates, using a liposome transfection method with the lipofectamine reagent (Invitrogen) as described 2.2.5.2. One pair of wells was transfected with the pBK-CMV/GATA clone-7 construct, another with the insertless pBK-CMV vector. As an additional control, a further pair of untransfected wells was harvested prior to differentiation. The remaining wells were switched to differentiation medium, as described in 2.2.5.1.2, to induce the cells to differentiate. The cells were maintained for a further five days before harvesting for mRNA. The mRNA extraction, reverse transcription and qualitative RT-PCR were all carried out as described in Section 2.2.6. The same primer and probe sets were used as for the preliminary test (Table 5.4).

### 5.2.3.3 Magnetic Antibody Cell Separation

One method investigated as a means of improving the yield of myoblasts in the primary cell extracts was the use of antibody-conjugated magnetic Dynabeads (DynaL Biotech). A positive selection method was used, following the manufacturer's protocol derived from those described by Drake and Loke, (1991). In this method, the desired cells are labelled with the appropriate antibody. The cells are then rosetted with the magnetic Dynabeads, which are coated with a secondary antibody reactive against this primary antibody. On the application of a magnetic particle collector, the desired cells are separated from the mixture, and unwanted cells removed by a series of wash steps.

The 5.1 H11 antibody (mouse IgG) was used as the primary antibody for selecting myoblasts. This is a myoblast-specific antibody raised by Walsh and Ritter (1981), against neural cell adhesion molecule (NCAM). NCAM is a cell adhesion molecule first characterised in nervous tissue, but since found in a number of cell types including developing or regenerating skeletal muscle (reviewed in Baldwin *et al.*, 1996). While 5.1 H11 was originally characterised as human myoblast specific, cross-species reactivity for porcine myoblasts was demonstrated by Blanton *et al.* (1999). The antibody was prepared from hybridoma cultures obtained from the Developmental Studies Hybridoma Bank at the University of Iowa, according to supplier's protocols. The beads used were M-450 Dynabeads, coated with sheep anti-mouse IgG.

The cell culture to be sorted was trypsinised, resuspended in 1 ml growth medium and transferred into a 15 ml Falcon tube, and a sample counted in a haemocytometer in order to calculate the correct volume of beads to add for a ratio of five beads to each cell. The beads were thoroughly resuspended in their storage buffer, and the required volume transferred to a microcentrifuge tube for reconditioning in growth medium. The tube was placed in a Dynal MPC for 1 min until the beads had settled out of suspension. Storage buffer was then removed, the tube taken out of the MPC and the beads resuspended in 1 ml growth medium. The reconditioning step was repeated once more, and the beads stored on ice until used.

The cells were incubated with a 1:10 dilution of the 5.1 H11 primary antibody for 30 min at 4 °C, with continuous rotating and tilting throughout the incubation. The cells

were washed twice with growth medium, spinning down at  $400 \times g$  for 5 min and resuspending in fresh growth medium each time. The washed, primary antibody-coated cells were then rosetted with the Dynabeads,  $4^{\circ}\text{C}$  for 30 min with continuous rotating and tilting. To collect the cells rosetted to the Dynabeads, the tube was placed in a DYNAL MPC for 2 min and the supernatant pipetted off. Cells were washed four times in growth medium, and transferred to culture flasks. The selected cells were grown in PromoCell Skeletal Muscle Growth Medium at  $37^{\circ}\text{C}$  to confluency, and then passaged into chambered slides for desmin staining.

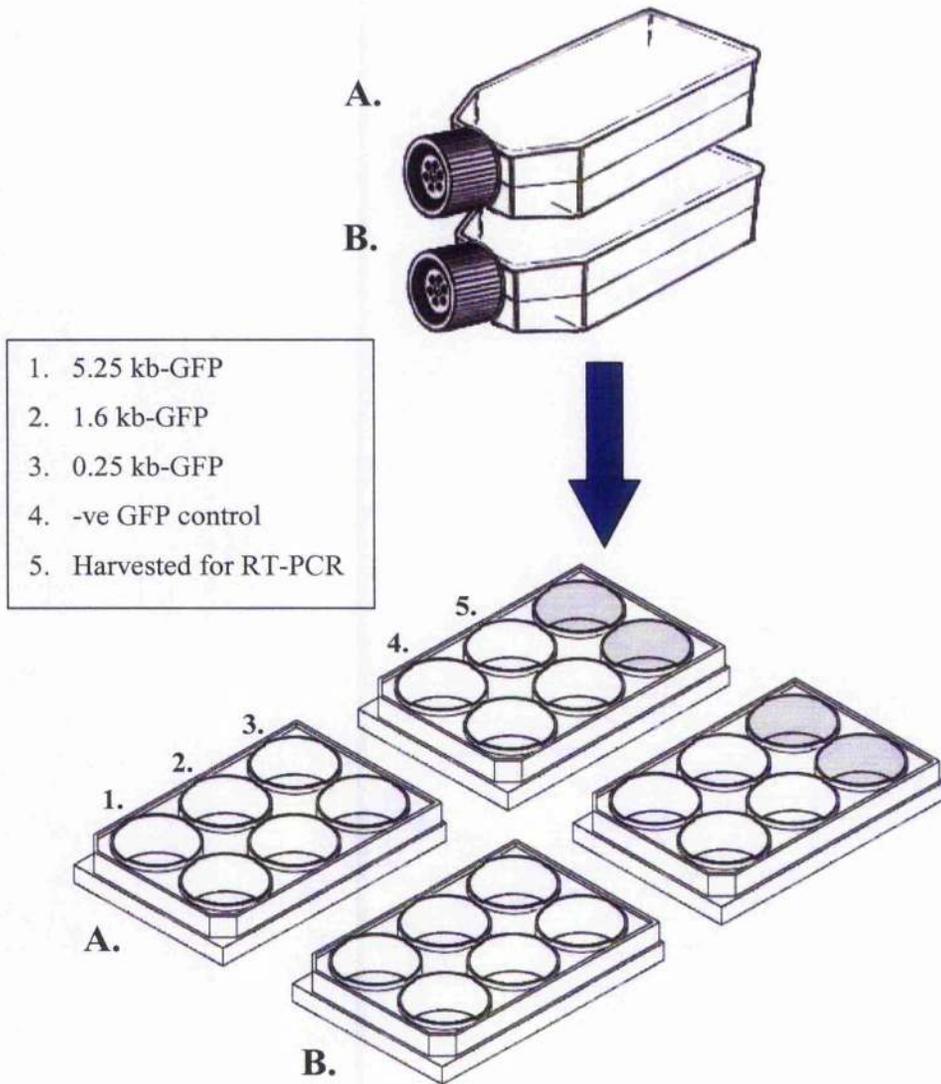
#### **5.2.4 C2C12 CULTURES**

C2C12 cells were cultured and maintained as described in section 2.2.5.3. Two stably transfected C2C12 lines were generated by lipofectamine transfection and maintained in appropriate selection media, as detailed in section 2.2.5.2. The first cell-line consisted of C2C12 cells transfected with the pBK-CMV/GATA-2 construct, referred to as C2C12/GATA-2. The second cell-line, generated for use as a control, was transfected with the pBK-CMV vector without the insert, referred to as C2C12/CMV.

##### **5.2.4.1 Promoter Construct Transfections**

A summary of the experimental set-up for the emMyHC/GFP promoter constructs is shown in Figure 5.1. Transient transfections were carried out using lipofectamine, as detailed in section 2.2.5.2, in six-well plates. The cells were induced to differentiate by switching to DMEM supplemented with 2% horse serum (section 2.2.5.3), and maintained for five days. After this period, the cells were examined under fluorescence microscopy for differences in fluorescence intensity between C2C12/GATA-2 and C2C12/CMV cells.

## C2C12 Cultures



**Figure 5.1.** Experimental set-up for transient emMyHC/GFP transfections in C2C12 cells (shaded wells unused). **A.** C2C12/GATA-2 cells. **B.** C2C12/CMV cells. **1-3.** Wells transfected with the pEGFP-1/emMyHC-promoter reporter constructs. **4.** Wells transfected with insertless pEGFP-1 vector. **5.** Undifferentiated wells harvested for quantitative real-time RT-PCR to assay for levels of GATA-2 expression.

## 5.3 RESULTS

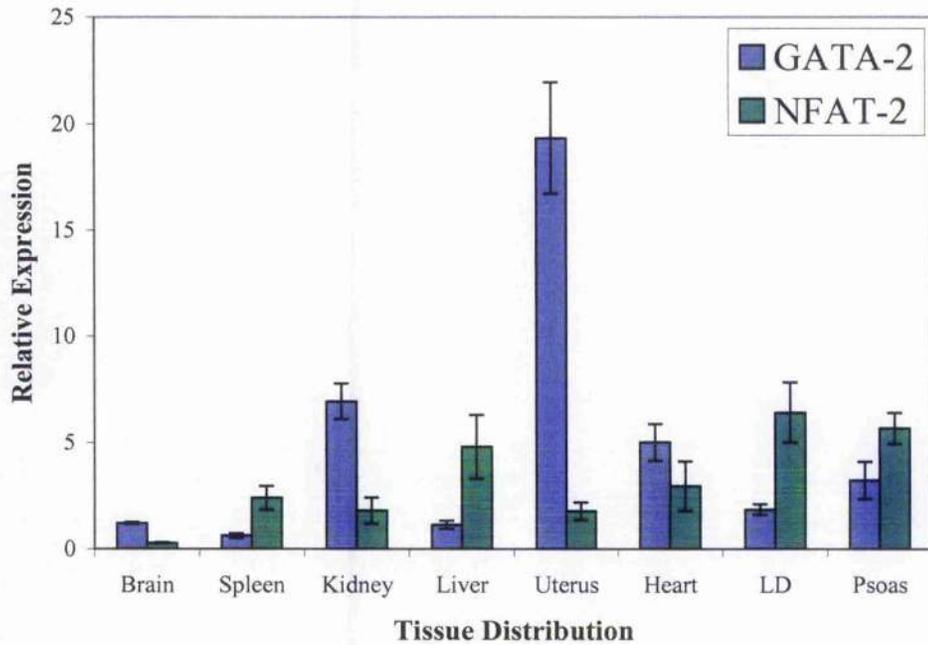
### 5.3.1 MRNA EXPRESSION

#### 5.3.1.1 GATA-2

The mRNA expression data obtained by real-time RT-PCR is summarised in Figures 5.2 to 5.4. Tissue distribution of GATA-2 mRNA was found to be widespread (Figure 5.2), with low levels of expression in most of the tissues assayed. This pattern is similar to the earliest observations of GATA-2 distribution made by Yamamoto *et al.* (1990). However, the high abundance of GATA-2 in uterine tissue has not been previously reported.

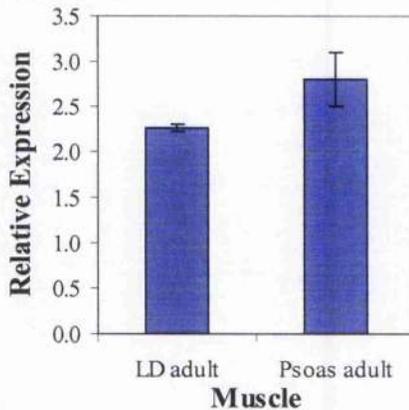
GATA-2 mRNA was found in both LD and *psaos* skeletal muscles of the 22-week post-natal animal (Figure 5.3A), though at low levels compared to NFAT-2. Previous studies have reported that GATA-2 is normally absent from skeletal muscle, except as a marker for IGF-1 mediated hypertrophy (Paul and Rosenthal, 2002). This study did not address the presence of GATA-2 protein in the tissues assayed, and it is possible that translation may be repressed. The differences in GATA-2 expression between muscles of predominantly different fibre types were slight, and do not appear to provide evidence for a role of GATA-2 in promoting either the fast or slow fibre phenotype.

The levels of GATA-2 expression in LD muscle increased with gestational age, as shown in Figure 5.4A. From day 91 of gestation to 22-weeks of post-natal growth, GATA-2 expression increased almost twenty-fold, with relative expression levels of 0.12 at 91 days versus 2.26 at 22 weeks post-natal (arbitrary units). This previously unreported, stage-specific increase of GATA-2 points to a role in development. The increase in levels of GATA-2 continued in post-natal growth, with expression levels appreciably higher in 22-week-old LD than in six-week-old.

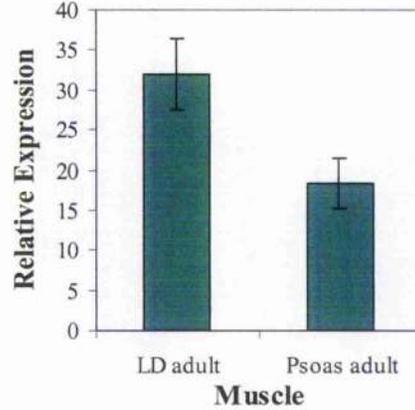


**Figure 5.2.** Tissue distribution of GATA-2 and NFAT-2 expression in a six-week-old pig. GATA-2 tissue distribution was noticeably high in uterus, kidney, heart and skeletal muscle. NFAT-2 expression was more evenly distributed, with the highest levels in skeletal muscles and in liver.

**A. GATA-2**

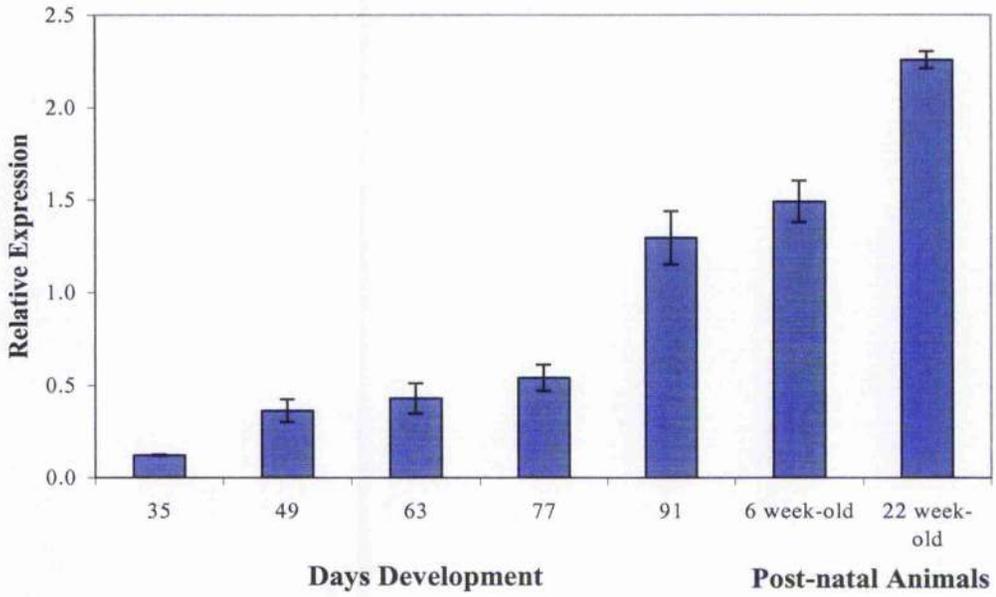


**B. NFAT-2**

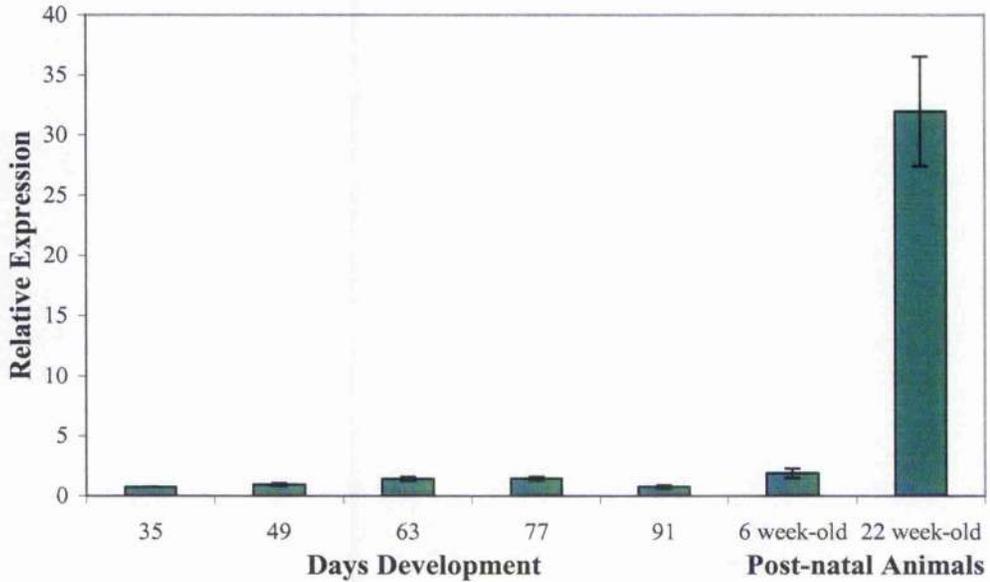


**Figure 5.3.** Relative expression of GATA-2 and NFAT-2 expression in fast (LD) and slow (*psoas*) muscles. **A.** GATA-2 **B.** NFAT-2.

### A. GATA-2



### B. NFAT-2



**Figure 5.4.** Expression of GATA-2 and NFAT-2 in different stages of pre- and post-natal porcine development, in LD muscle. **A.** GATA-2 expression increased steadily throughout pre- and post-natal development. **B.** NFAT-2 remained at much the same level throughout development and in the young animal, but was greatly upregulated in the 22-week-old adult.

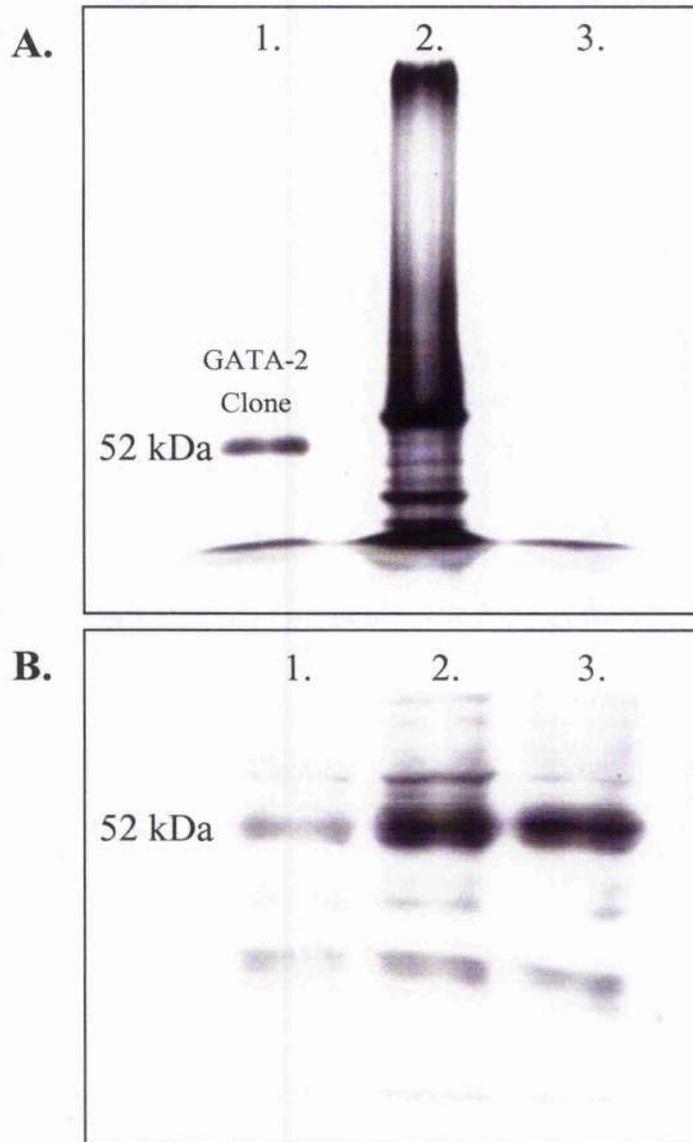
### 5.3.1.2 NFAT-2

The tissue-distribution of the porcine NFAT-2 mRNA, as determined by the real-time RT-PCR in a six-week-old pig, is shown in Figure 5.2. Of the tissues assayed, NFAT-2 expression was highest in skeletal muscles and liver. Expression at lower levels was also observed in spleen, kidney and heart, with all of these tissues previously reported to express NFAT-2 (see Section 5.1.2). Similar levels of NFAT-2 expression were observed in uterine smooth muscle, though this is also predictable to some degree given the previously reported expression of NFAT-2 in vascular smooth muscle (Wada *et al.*, 2002). The observation of elevated NFAT-2 expression in liver was not expected. If this corresponds with elevated NFAT-2 protein in this tissue, then this may signify a previously unreported role for the NFAT-2 isoform in liver.

The comparison between NFAT-2 levels in 22-week-old LD and *psoas* is shown in Figure 5.3B. It was found that NFAT-2 expression was two-fold higher in the predominantly fast muscle, the LD, than in the predominantly slow-fibre *psoas* muscle. This result was unexpected given the widely reported role of NFAT-2 in mediating the slow-fibre phenotype (Chin *et al.*, 1998; Schulz and Yutzey, 2004). In both LD and *psoas*, NFAT-2 expression was higher than GATA-2. Figure 5.4B shows changes in NFAT-2 expression in LD during gestation and post-natal growth. The expression level in 22-week-old LD muscle was up to 40-fold that of gestational LD, with relative expression levels of 0.74 at 91 days, versus 31.95 at 22 weeks post-natal (arbitrary units). In contrast to the pattern observed in GATA-2 expression in LD muscle, pre-natal expression of NFAT-2 showed no discernible pattern, up to and including the six-week-old. It appears that the porcine NFAT-2 is restricted to a role in fully developed muscle, as previously reported (Abbott *et al.*, 1998; Schulz and Yutzey, 2004).

### **5.3.2 PROTEIN ANALYSIS**

The results of the protein analysis are shown in Figure 5.5. The *in vitro* transcription/translation showed a band of the correct size of 52 kDa, as predicted from the primary nucleotide sequence for GATA clone-7 (Figure 5.5A). As further confirmation of a translated protein product for the construct, Western blotting showed a 52 kDa band, upregulated over untransfected controls, was observed in the pBK-CMV/GATA clone-7 transfected C2C12 cells (Figure 5.5B). This same band was also



**Figure 5.5.** SDS-PAGE analysis of the porcine GATA-2 clone. **A.** *In vitro* transcription-translation run with luciferase as a marker. Lanes: 1) GATA-2 *in vitro* transcription translation. 2) Luciferase marker lane. 3) Control lane. **B.** Western blot of nuclear extract from C2C12 cells, probed with anti-mouse GATA-2. Lanes: 1) Non-transfected. 2) Transfected with IGF-1 expression construct. 3) Transfected with GATA-2 expression construct. Both the IGF-1 and GATA-2 transfected cells show upregulation of a 52 kDa protein, corresponding to the predicted size of the novel GATA-2 clone.

upregulated in the IGF-1 transfected cells, and most likely correlated to the increased expression of endogenous, murine GATA-2, induced by the over-expression of IGF-1. This is in accord with previous observations in both muscle cell culture and transgenic mice, in which GATA-2 was induced by IGF-1 (Musaro *et al.*, 1999; Musaro *et al.*, 2001).

### 5.3.3 PRIMARY CELL CULTURE

#### 5.3.3.1 Initial Characterisation

The threshold cycle ( $C_T$ ) values obtained by quantitative RT-PCR for the initial differentiated primary cell cultures are shown in Table 5.5. Expression of  $\alpha$ -actin and the em-, peri- and slow-isoforms of MyHC was detected, consistent with the early onset of differentiation as described by Sun *et al.* (2003). From this it was assumed that a significant proportion of the harvested cells were myoblasts and that the culture conditions were favourable for their growth and subsequent differentiation.

	Average $C_T$	
	LD	Psoas
$\alpha$ -actin	22.30	24.27
emMyHC	23.65	24.71
slow MyHC	32.51	33.11
periMyHC	35.05	37.81
2a MyHC	37.42	38.40

**Table 5.5.** Average  $C_T$  readings for the initial characterisation of myoblast cultures derived from primary tissue extractions.

#### 5.3.3.2 Problems with Differentiation

Despite these earlier, promising results, subsequent GATA-2 transient transfection experiments using primary cell culture isolates failed to show expression patterns consistent with differentiation. Expression of  $\alpha$ -actin, a marker of differentiating myocytes, was absent in all the cDNA preparations assayed. The absence of differentiation was found in both GATA-2 transfected cells, controls and in untransfected cells brought out of storage to test whether the transfection procedure was interfering with differentiation. From this, it was concluded that the method of

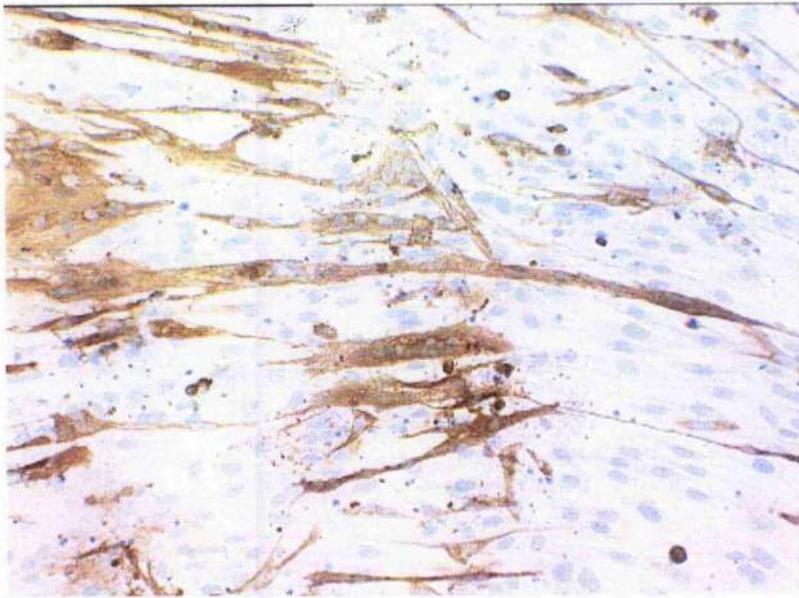
obtaining differentiated cell culture was of low efficiency, and that the earlier success was not representative of the technique used. No mRNA was detectable for any of the MyHC isoforms, the only exception being emMyHC. The embryonic isoform was observed at very low levels, at around the lower limit for detection ( $C_T$  readings between 30 to 35). No consistent differences were observed in emMyHC levels between GATA-transfected and untransfected cells, though this may simply have been due to the low levels involved.

The underlying problem with the primary cell cultures was the presence of non-myoblast cell populations, as determined by desmin antibody staining (Figure 5.6). It was considered that the most probable explanation for the failure of the primary cultures to differentiate and form myoblasts in low serum conditions was the low percentage of myoblasts. The percentage of desmin-positive cells varied quite widely between different primary cell cultures. Initially, it appeared that the length of time in culture might be a contributing factor, given the success of the initial characterisation, described in Section 5.3.3.1. This raised the possibility that the myoblasts were simply outgrown by desmin-negative cell types, through the course of multiple cell passages. Further extractions from porcine tissue showed low populations of desmin-positive cells and lack of differentiation, even when induced to differentiate after a low number of passages. It was apparent that percentage of myoblasts in culture varied according to the particular isolate, as well as time spent in culture, and that the method used to isolate primary myoblasts was inadequate.

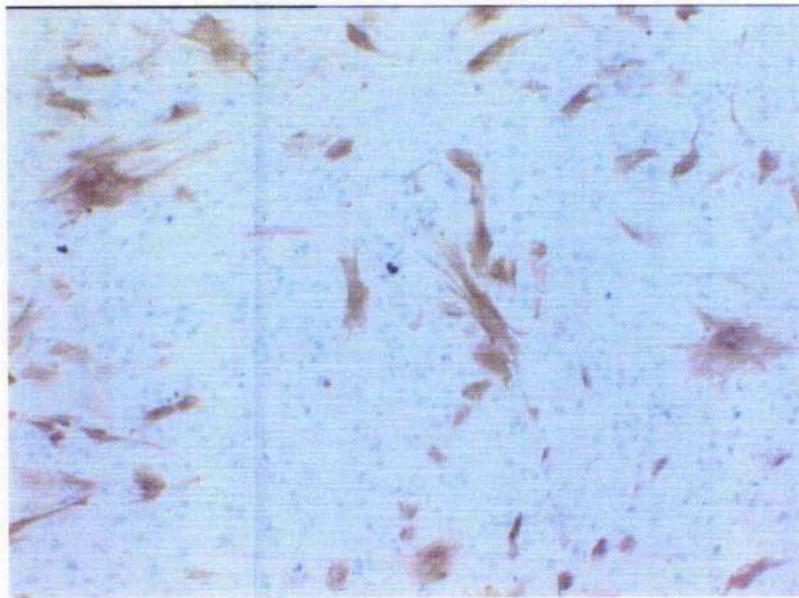
### **5.3.3.3 Cell Separation**

Separation of the cells using the 5.1 H11 antibody conjugated to Dynabeads was tested as a means of enriching the primary cell cultures for myoblasts. However, comparisons between antibody-selected cells and non-sorted cells from the same primary cell line showed no increase in the proportion of desmin-positive cells. The affinity of the 5.1 H11 antibody for porcine myoblasts has been demonstrated previously (Blanton *et al.*, 1999), and so the failure of the selection process may have been due to the antibody fraction used. Given sufficient time, further optimisation of the technique may yield better results.

**A.**



**B.**



**Figure 5.6.** Examples of desmin staining of primary cultures from porcine muscle, showing the variability of myoblast differentiation. Brown-stained cells are desmin positive. **A.** Differentiated myoblasts (cultured in 2% HS for 10 days) in the process of forming myotubes. **B.** Differentiated myoblasts (treated as above), with insufficient population of myoblasts.

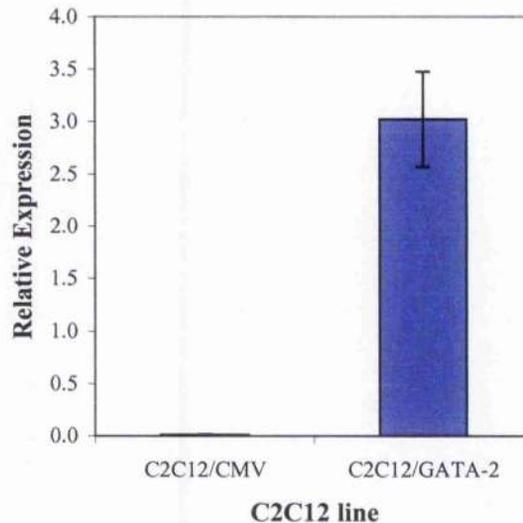
### 5.3.4 C2C12 MYOBLAST CULTURE

The use of the C2C12 mouse myoblast cell line offered several advantages over the primary cell culture, chiefly the homogenous cell population and the relative ease of maintenance of the cell line. The chief disadvantage was species difference, and the possibility that the acquired porcine GATA-2 might not be functional in the mouse myoblasts needed to be considered. A pileup of murine and porcine GATA-2 is shown in Figure 5.7. As can be seen, the two proteins show considerable conservation in the functional zinc finger domains, sufficient that the porcine GATA would most likely be functional in the C2C12 myoblasts.

Pig	MEVADEQPRW	MAHFAVLNAQ	HPDSHFHFLA	HNYMEPAQLL	PPDEVDVFFN	50
Mouse	-----	-----	-----	-----	-----	50
Pig	HLDSQGNPY	ANPAHARARV	SYSPAARLT	CGQMRPHLL	HSPGLEWLDG	100
Mouse	-----	-----	-----	-----	-----	100
Pig	GKAALSAAAA	HHHNPWTVNP	FSKTPHPSA	AGFPQPLEV	YFGAGGGGGG	150
Mouse	-----	--S--S-	-----	--S-----	----A--I--	150
Pig	GSGSSVASLT	PTAAHSGSHL	FGFPPTPPKE	VSPDPSTGA	ASPASSSAGG	200
mouse	-----	-----	-----	-----	-----	200
Pig	SAARGEDKDG	VKYQVSLTES	MKMEGSPLEK	PGLAANGTQP	ATHPIPIIYP	250
mouse	-V-----	-----S-	---G-----	---T-----	-----	250
Pig	SYVFAAAIDY	SSGLEPIPGGF	LGGRASSFTF	KORSKARCS	<u>EGRECVNCGA</u>	300
Mouse	-----	G-S-----	-----	-----	-----	300
Pig	<u>TATPLWRRDG</u>	<u>TGHYLCNAG</u>	<u>LYEKMNQNR</u>	<u>PLIKPKRRLS</u>	<u>AARRAGTCCA</u>	350
Mouse	-----	-----	-----	-----	-----	350
Pig	<u>NCQETTTTLW</u>	<u>RRNANGDPVC</u>	<u>NACGLYYKLH</u>	<u>NVNRPLTMKK</u>	<u>EGIQTRNRKM</u>	400
Mouse	-----	-----	-----	-----	-----	400
Pig	SNKPKRNRKG	ABCPEELSKC	MQEKASFFSA	AALAGHMAPV	GHLPPFSSHG	450
Mouse	-S--S--	-----	---S---	-----	-----	450
Pig	HILPPTPIH	PSSSLSPGHP	HSSMVTAMG			480
Mouse	-----	-----	-----			480

**Figure 5.7.** Pileup comparison of the porcine GATA clone and murine GATA-2 (Accession N<sup>o</sup>: AK004675) highlighting mismatches. A '-' denotes a homologous residue. Underlined residues denote GATA zinc finger motifs, i.e. the DNA binding and sequence recognition domains.

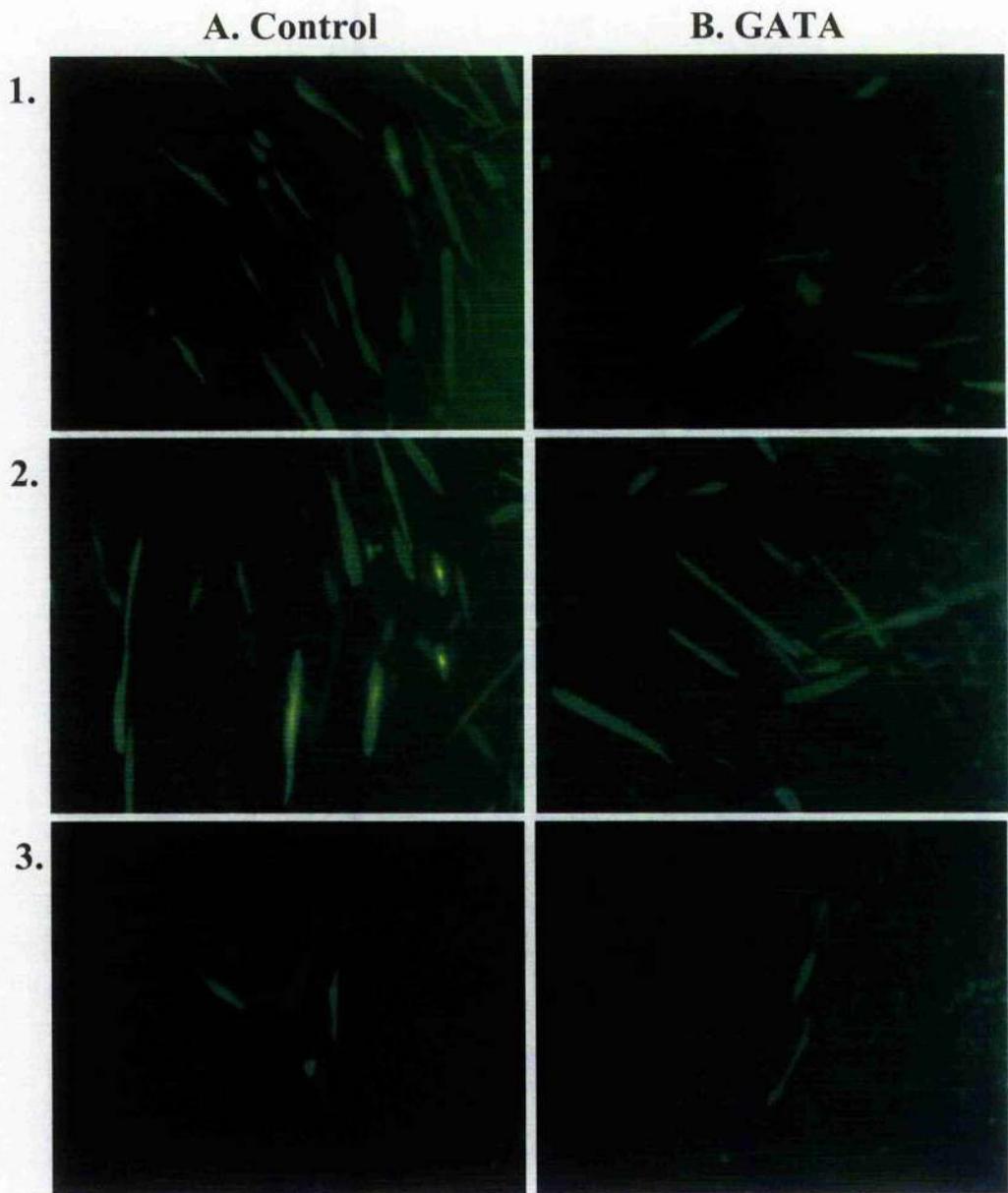
The expression of the pBK-CMV/GATA clone-7 construct in the stably transfected cell -lines was verified by RT-PCR. The expression of porcine GATA-2 mRNA was not detected in the control C2C12/CMV cell-line (Figure 6.4).



**Figure 5.8.** Verification of GATA-2 expression in the C2C12/GATA-2 cell line.

The results of the emMyHC/GFP transient transfections are shown in Figure 5.9. Unfortunately, time constraints prevented a more thorough analysis of differences in fluorescence between the C2C12/GATA-2 and wild-type C2C12 populations. A more thorough study would require a numerical measurement of fluorescence intensity in each cell, and plotting the frequency distribution of this intensity for over one hundred cells in each treatment well. This way, a meaningful measurement might be obtained of the different distribution patterns between the the C2C12 and C2C12/GATA populations than can be obtained simply by eye as in the preliminary study here. With this in mind, any conclusions drawn from the reporter study must be considered with caution, as preliminary data in need of further confirmation by a more detailed experiment.

As expected from previous work using these constructs (Dr Yuh-Man Sun, personal communication), the 0.25 kb-emMyHC/GFP reporter construct resulted in low levels of GFP expression compared to the constructs containing 1.6 kb and 5.25 kb of the



**Figure 5.9.** Effect of GATA-2 on emMyHC reporter constructs. **A.** Control C2C12 myoblast cell line, transfected with empty pBK-CMV vector. **B.** C2C12/GATA cell line, transfected with the pBK-CMV/GATA construct. **1, 2 & 3.** Transient co-transfections with the pEGFP-1/5.25 kb-, 1.6 kb- and 0.25 kb-emMyHC reporter constructs respectively.

promoter region, respectively. No difference in reporter activity was observed between GATA-transfected and untransfected cells either in the 0.25 or 1.6 kb reporter constructs. For the 5.25 kb construct, fluorescence intensity was lower in the C2C12/GATA-2 cells than in the control cell-line. This would indicate that GATA-2 does repress emMyHC, with the GATA-binding site somewhere between 1.6 to 5.25 kb upstream of the TATA box.

## 5.4 DISCUSSION

### 5.4.1 EXPRESSION PATTERNS

The tissue distribution patterns of the GATA-2 and NFAT-2 transcripts provide some insight into the function of these factors, although there are limits to the conclusions that can be drawn. Different levels of mRNA transcript do not necessarily equate to differences in the levels of protein. For instance, while NFAT-1 mRNA is quite abundant in brain and heart, NFAT-1 protein is absent from extracts of these tissues (Ruff and Leach, 1995; Wang *et al.*, 1995). The advantage of the quantitative real-time RT-PCR approach used in the expression studies is its sensitivity, and the capacity for a high throughput of different samples. However it is limited in that only mRNA levels have been quantified. Whether these reflect the levels of protein is yet to be verified.

This said, the expression data presented here provides a comprehensive study of the tissue distribution of GATA-2 and NFAT-2 in the pig, with a couple of unexpected findings that may point to previously uncharacterised functions for these transcription factors. For the most part, levels of the two transcription factors in the tissues of the six-week-old animal seemed to fit in with those previously described (see Sections 5.1.1 and 5.1.2). There was evidence of a persistence of developmental patterns of expression, however, such as the presence of GATA-2 and NFAT-2 in the heart and the low levels of NFAT-2 expression in muscle, compared with the 22-week-old. This may be related to the young age of the animal from which the samples were derived. A similarly comprehensive study in the 22-week-old would be a useful comparison, though samples were not available in this study.

The functional significance of the relatively high levels of GATA-2 in uterine smooth muscle and NFAT-2 in liver is unclear at the time of writing. That NFAT-2 is expressed in liver is the least surprising of the two observations, given the widespread expression of this factor and the ubiquitous nature of NFAT signalling (Hogan *et al.*, 2003). The expression of GATA-2 in the uterine smooth muscle, and at such relatively high levels, is not as easy to explain in the light of existing data on the role of GATA-2. From its first characterisation, GATA-2 expression has been reported as being quite widespread, but at low levels (Yamamoto *et al.*, 1990). In this study, GATA-2 expression in uterine smooth muscle was quite high in comparison with other GATA-2 expressing tissues. Also, GATA expression in smooth muscle tissue, such as the uterus, is generally thought to be restricted to the second 'sub-family' of GATA factors, GATA-4, -5 and -6 (reviewed in Patil and McGhee, 2002). The discovery of such relatively high levels of GATA-2 in the uterus may indicate that the distinction drawn between the two sub-families is not quite as clear-cut.

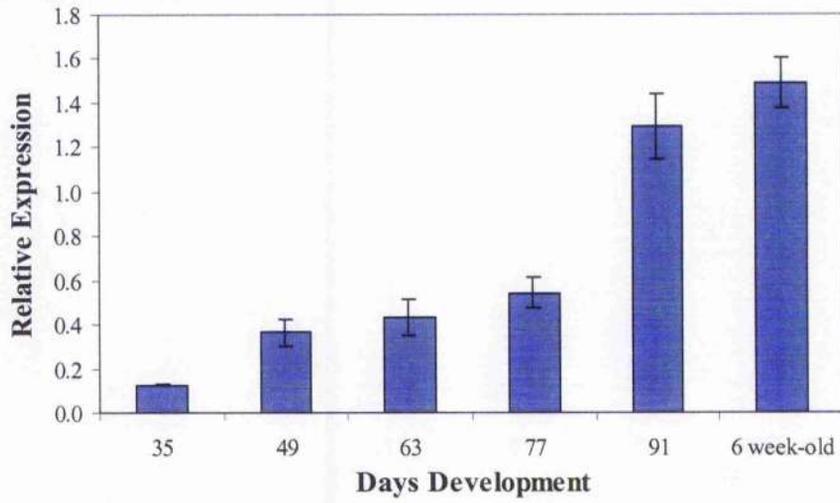
The upregulation of GATA-2 expression during development corresponds with a previously observed decrease in emMyHC expression (Sun *et al.*, 2003). A comparison is shown in Figure 5.5. While this in itself is only suggestive of a role for GATA-2 in the repression, it did provide further basis for the studies into the functional role of GATA-2, namely the effect of GATA clone-7 expression on the emMyHC-GFP reporter constructs.

#### **5.4.2 GATA-2 FUNCTION**

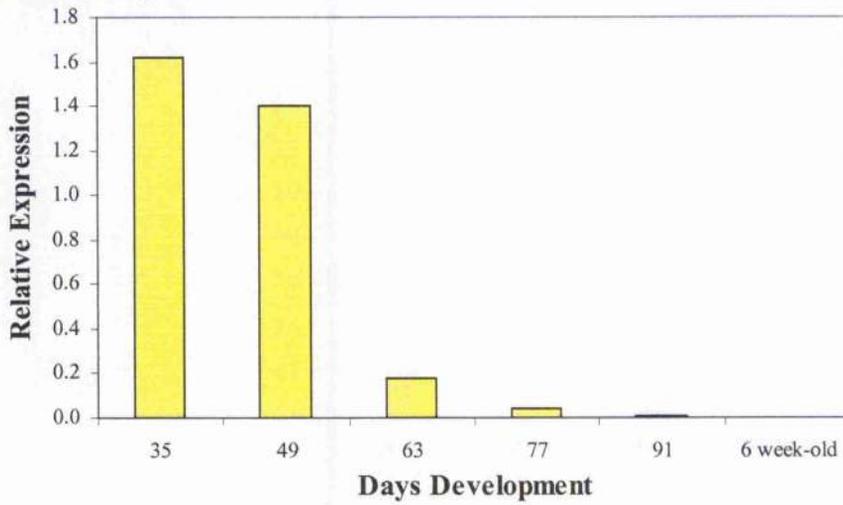
Despite the reservations expressed in Chapter Three regarding the absence of a strong Kozak consensus sequence, the GATA-2 clone was shown to be functional. The translation of a protein corresponding to the predicted size is the most important piece of evidence that the assessment of the main reading frame for GATA clone-7 given in Chapter Three is correct. The confirmation that the GATA clone-7 cDNA corresponds to an mRNA species lends support to the further studies to determine its biological function.

Though somewhat limited, the preliminary C2C12 study using the emMyHC reporter constructs provides some evidence in support of the conclusions drawn from the

### A. GATA-2



### B. emMyHC



**Figure 5.10.** Comparison of developmental patterns of expression between the GATA-2 clone (see also Figure 5.4) and emMyHC (Sun *et al.*, 2003) during gestation and early growth.

expression data discussed above, namely that GATA-2 represses emMyHC expression. However, a more in-depth study as laid out in Section 5.3.4 is required to confirm this finding. Since the repression of reporter activity seems to be restricted to the pEGFP/5.25 kb construct, this supports the conclusion made in Chapter 4 that the putative GATA-2 consensus site at position -4 kb upstream binds GATA-2 and plays a role in emMyHC transcription. Again, this is a preliminary observation in need of further confirmation.

The initial studies using the primary cell culture remain something of a disappointment. The problems with cell culture appear to stem from a lack of a suitable source of porcine satellite cells. There are a number of ways that this could be addressed in future work. Firstly, it is apparent that the age of the animal is crucial. Satellite cell cultures have been shown to be highly sensitive to the age of the animal with one week after birth being ideal (Cardasis and Cooper, 1975). Owing to restrictions during the time period of the project on the movement of animals due to the foot and mouth crisis, pigs younger than the six-week-old animal used were not available. It soon became apparent that the stop-gap measure of culturing myoblasts from older animals was not a viable alternative. It is possible that the antibody separation and attempts to purify myoblast cultures may have failed because the population of myoblasts in the cell isolates was insufficient. That having been said, repetition of the separation technique with a different antibody extraction may have yielded better results, owing to the inherent variability in such preparations.

## CHAPTER SIX - GENERAL DISCUSSION

## 6.1 INTRODUCTION

Changes in muscle phenotype, whether in development, regeneration or in response to changes in physiological demand, are brought about through the co-ordinated expression of fibre-specific genes. The signal transduction pathways believed to be responsible for mediating these patterns of gene expression have been the subject of many recent studies, as covered in Chapter One. Musaro *et al.* (1999) identified the transcription factors GATA-2 and NFAT-2 as having a role in the control of muscle phenotype, specifically in IGF-1 mediated muscle hypertrophy. Though these findings have been the subject of recent debate (reviewed in Schiaffino and Serrano, 2002; Glass, 2003), roles for both factors in various aspects of muscle biology have been further supported in later studies. GATA-2 and NFAT-2 have been identified as having a role in muscle regeneration (Sakuma *et al.*, 2003), while several studies have supported a role for NFAT-2 in the fast-slow change in phenotype (Liu *et al.*, 2001; Kubis *et al.*, 2002).

As major determinants of muscle fibre properties, such as shortening velocity and ATP utilisation, the MyHC isoforms are likely targets for transcription factors controlling fibre type. The aim of the study described in this Thesis was to investigate possible roles for GATA-2 and NFAT-2 in the control of MyHC isoform expression. As discussed in Chapter Three, a porcine GATA-2 orthologue was cloned and isoform-specific sequence data obtained for a porcine NFAT-2 orthologue. The latter was truncated and incomplete, but most likely corresponding to the NFAT-2b isoform. The investigation of one of the embryonic MyHC (emMyHC), one of the last remaining uncharacterised isoforms in the pig, revealed a novel transcript structure, with the translation start site located on exon 2.

Quantitative real-time RT-PCR studies confirmed earlier observations of widespread tissue distribution of both GATA-2 and NFAT-2. However GATA-2 was found to be highly expressed in uterine smooth muscle, a novel finding suggesting that the previously reported segregation of the GATA family into two functionally distinct groups (Patient and McGhee, 2002) may not be as distinct as was previously thought. Furthermore, developmental expression patterns of GATA-2 coincide with a drop in

emMyHC expression (Sun *et al.*, 2003) suggesting a role in the regulation of this gene. Though only a preliminary study, the reporter studies using the cloned emMyHC regulatory region in conjunction with the GATA-2 expression construct seemed to support the idea that GATA-2 inhibits emMyHC expression. The wider significance of these results is discussed in this Chapter, together with possible avenues for future research arising from the findings.

## 6.2 THE ROLE OF GATA-2

The evidence for a negative, regulatory role for GATA-2 in the control of emMyHC expression remains only an initial observation, and some questions remain unanswered. First of all, as discussed in Chapter Five, a more detailed study generating more data for statistical analysis is required. Assuming that the initial observations hold, however, one of the uncertainties is whether GATA-2 is binding directly to emMyHC upstream regulatory elements, or acting indirectly through the activation of another signalling intermediate. Evidence for a direct interaction is provided by the putative GATA-2 binding site in the pEGFP/5.25 kb reporter construct. There is also a precedent in cardiac development for the control of MyHC gene expression by members of the GATA family. GATA-4 has been demonstrated to bind directly to the slow/ $\beta$  MyHC regulatory region (Wang *et al.*, 1998). Further confirmation of direct binding would need to be attained through other methods, perhaps the easiest method being the generation of reporter constructs that specifically lacked the -4kb GATA-2 binding site. Another possibility is the mobility shift, or band-shift, assay (Fried and Crothers, 1981; Garner and Revzin, 1981). This technique exploits the difference in mobility of the transcription factor or DNA fragments, compared to bound DNA/protein complexes, during gel electrophoresis.

Another question is the identity of other transcription factors, if any, that might be co-operating with GATA-2 in the putative regulatory role. The particular outcome of generic signal transduction or transcription factor activity is mediated by interactions between different pathways. In examples such as pathways downstream of IGF-1 signalling (Singleton and Feldman, 2001), or calcineurin signalling (Crabtree, 1999), specificity is often conferred at the level of combinatorial activation of gene expression

by transcription complexes. The association between GATA family members and NFAT is well studied (Molkentin *et al.*, 1998) and has been reported in GATA-2 and NFAT-2 in particular (Sakuma *et al.*, 2003). However, GATA has also been reported as a co-factor for other factors such as MEF-2 (Morin *et al.*, 2000). Consensus binding sites for both NFAT and MEF-2 have been uncovered in the emMyHC/5.2 kb construct. However, GATA factors can also self associate as functional homodimers (Crossley *et al.*, 1995), or as heterodimers with different GATA family members (Charron *et al.*, 1999). If it could be determined which of these transcriptional co-factors are operating in conjunction with GATA in the regulation of emMyHC, this could determine the upstream pathways that are involved and help further pick apart the molecular mechanisms for developmental emMyHC down-regulation.

### 6.2.1 FIBRE TYPE SWITCH

The studies into the function of the porcine GATA-2 found no evidence in support of, or refuting, a role for GATA-2 in mediating the fast to slow switch in fibre-type. This was largely due to the mismatch between the *in vitro* differentiation model and the set of RT-PCR primer and probe sets for quantifying MyHC family expression. While the C2C12 cells proved a useful differentiation model, the MyHC primers and probes available to the project were porcine specific. Unfortunately there was insufficient time left to the project work to obtain murine primer and probe sets, and the use of a cell line as opposed to unaltered myoblast cells would have posed other problems may have given a false indication of the *in vivo* situation. Resolving the issue of non-myoblast contamination of the primary porcine muscle isolates and obtaining differentiated myotubes would rescue the primary cell culture as a useful model for RT-PCR studies. As demonstrated in the study by Da Costa *et al.* (2002), the use of quantitative real-time RT-PCR to pinpoint changes in MyHC expression is a useful means to observe changes in fibre phenotype in the pig.

One avenue of research that might be explored is the relationship of GATA-2 with proliferator-activated receptor  $\gamma$ -coactivator-1 $\alpha$  (PGC-1 $\alpha$ ). As discussed in Section 1.3.4.3, PGC-1 $\alpha$  has been described as a positive regulator of the slow-fibre phenotype (Lin *et al.* 2002). At the time of writing, there has been no report of an interaction between GATA-2 and PGC-1 $\alpha$ , either in muscle or any other tissue. There is an

indirect connection, however, in that both PGC-1 $\alpha$  and GATA factors are associated with the regulation of peroxisome proliferator-activated receptors (PPARs) in other tissues, the PPAR $\gamma$  isoform in particular. The PPARs are lipid-activated nuclear receptors that transcriptionally regulate genes involved in a variety of metabolic processes (reviewed in Berger and Moller, 2002). PGC-1 $\alpha$  is a co-activator of transcription with PPAR $\gamma$  in adipose tissue (Puigserver *et al.* 1998), while the GATA factors act upstream of PPAR $\gamma$ , regulating its transcription. GATA-2 and -3 down-regulate adipocyte PPAR $\gamma$  expression (Tong *et al.* 2000), while in contrast GATA-6 promotes PPAR $\gamma$  expression in vascular smooth muscle (Abe *et al.* 2003), though it is not known if a similar interaction between GATA-2 and the PPARs exists in skeletal muscle. The relationships, if any, between GATA-2 activity and either PGC-1 $\alpha$  or the PPARs might be a useful subject for further studies investigating a role for GATA-2 in the regulation of muscle fibre type and metabolism.

### **6.2.2 DEVELOPMENT AND REGENERATION**

To a certain extent, the process of muscle regeneration recapitulates the process of embryonic development (Parker *et al.*, 2003), with a number of features common to the two. For example, the c-met receptor mediates myoblast migration in both satellite-cell regeneration and embryogenesis (Tatsumi *et al.*, 1998; Buckingham *et al.*, 2003). With regard to MyHC expression, a similar pattern of activation of developmental isoforms is observed in the formation of myotubes during regeneration, cell culture in addition to prenatal myogenesis (Weydert *et al.*, 1987; Da Costa *et al.*, 2003; LaFramboise *et al.*, 2003). If the effect of GATA-2 on cmMyHC expression in development is genuine, as the expression data suggests, one question that might then be asked is whether GATA-2 performs the same function in satellite cell differentiation.

As already discussed, there are previous reports of a role for GATA-2 in aspects of muscle biology mediated by satellite cell activation and differentiation, such as regeneration (Sakuma *et al.* 2003) and hypertrophy (Paul and Rosenthal, 2002). Sakuma *et al.* (2003) report a gradual increase in GATA-2 co-precipitation with NFAT-2 following muscle injury. It might be useful to determine whether this is similarly associated with a decrease in cmMyHC expression, as in the proposed interaction in development. Such experiments necessitate an *in vivo* model, though given the numbers

of animals that a time course study might involve and the required lack of genetic variability, a rodent model might be a better choice over the pig for such studies. Similarly, observing the relative levels of GATA-2 and emMyHC following hypertrophic stimuli, such as IGF-1, could determine whether the relationship is common to differentiating satellite cells and developing muscle.

### 6.3 NFAT DISTRIBUTION

The studies into the porcine NFAT-2 orthologue remain incomplete without the full transcript. The only two findings gleaned from this study on the porcine NFAT-2 orthologue were the 3' sequence information data and the expression data. Without any functional studies, the conclusions that can be drawn from the results of this study remain speculative. Firstly, the sequence data recovered indicates that the isoform in question is a distinct form from the other porcine NFAT-2 isoform isolated from the pig, the NFATmac isolated from porcine macrophages (Miskin *et al.*, 1998). Both isoforms are closest in 3' transcript structure to the human NFAT-2b isoform (Serfling *et al.*, 2004). The length of the 5' UTR of either porcine isoform seems different enough from the NFAT-2a and -2c isoforms to suggest a significantly different family organisation in the pig, though this is impossible to verify without the 5' sequence data for the transcript represented by NFAT clone-3.

The expression patterns of the NFAT-2 isoform are very different from that of the porcine GATA-2, suggesting a different role in function. While GATA-2 showed distinct upregulation late in development, NFAT-2 remained at a fairly constant level throughout development, at low levels compared with the adult. If there is a relationship between GATA-2 expression patterns and the possible regulatory role over emMyHC, then the differences would suggest that this particular NFAT-2 isoform is not acting as a co-factor for GATA-2 in the control of emMyHC. If the problems encountered in the NFAT cloning could be resolved, perhaps through through optimisation of different RACE techniques, the isolation of a full-length porcine NFAT-2 would go a long way to answering these questions.

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