A 49 base-pair region of the IRE enhancer directs fast skeletal muscle fiber-type-specific expression of the troponin I (fast) gene

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Abstract

The troponin I fast (TnIfast) gene, a member of a differentially-expressed three-member TnI multigene family is expressed specifically in fast skeletal muscle fibers. The tissue and fiber type specificity of the TnIfast gene is driven by an Intronic Regulatory Element (IRE) in the first intron. The IRE is a 148 bp transcriptional enhancer, that contains four known or suspected cis regulatory elements; E-box, MEF2, CCAC, and CAGG elements. A previous study in this lab suggested that fast-fiber type specificity is driven by elements that reside in the 5'-most 30 bp of the IRE, a region that includes the E-box motif. My initial goal was to further localize the hypothetical fast fiber type specific element(s) within this region. The experimental approach was to make IRE partial deletions and mutations in reporter gene constructs in which IRE derivatives were cloned upstream of an enhancer-dependent TnI fast minimal promoter driving the reporter gene LacZ. The transcriptional activity of these constructs in fast and slow muscle fibers was evaluated by direct gene transfer into adult mouse skeletal muscle followed by histochemical analysis of LacZ reporter expression. My results showed that the E-box was not required for IRE fast-fiber specificity or high-level expression in adult skeletal muscles. Moreover, additional deletion constructs indicated, in contrast with the previous study, that the 5'-most 30 bp segment is not required for fast-fiber-specificity. I was able to show that a 49 bp

IRE segment, not including the 5'-most 30 bp, but including MEF2 and CCAC elements, is sufficient to drive fast-fiber specific expression in adult mouse skeletal muscles. I discuss possible causes for the discordant results between the two studies, and the implications of my findings for the regulatory mechanisms of the IRE.

Une région de 49 paires de bases de l'enhancer IRE regularise la spécificité du gène troponin I (rapide) pour le type de fibre du muscle squelettique

Résume

Le gène troponin I rapide (TnIfast) est un member d'une famille de gènes multiple don't les members s'expriment d'une façons differente celon le type de fibre du muscle squelettique. La spécificité pour le type de tissue et le type de fibre du gène TnIfast est due a un "intronic regulatory element" (IRE) situé dans le premier intron. Le IRE est un enhancer de 148 bp qui contient quatre éléments-cis connus; E-box, MEF2, CCAC et le CAGG elements. Une étude précédente dans notre laboratoire a suggeré que la spécificité du gène troponin I rapide pour le type de fibre est due a des éléments résidant dans les 30 pair de bases situé dans l'éxtrimité gauche du IRE, une région qui contient le site "E-box". Mon but initial était de localise cet élément hypothétique dans ces 30 pairs de bases de l' IRE. Mon approche experimental était de préparer des versions du IRE mutées ou tronquées partiellement. Ces derives de IRE ont été couplés a un gène signal consistant en un promoter TnI fast minimal lié avec le gène LacZ. L'activité transcriptionnelle de ces construits été evalué par transfert de gène *in vivo* dans le muscle squelettique de souris adults. Cela été suivit par

l'analyse histochimique de l'expression du gène signal, le LacZ. Mes resultats ont montre que le E-box n'était pas essentielle pour l'expression ou la spécificité pour les fibre rapide de l' IRE dans les muscle squelettique. De plus, des experiences additionelle ont indique que les 30 bp sont pas essentiel pour la spécificité pour le type de fibre rapide et cela en contradiction avec l'étude précédant. Dans des études additionelles j'ai pu montrer qu'un sègment de 49 bp de l' IRE, inclue les éléments MEF2 et CCAC, est suffisant pour causée l'expression de la spécificité pour le fibre rapide.

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Abbreviations

ATP…….. adenosine triphosphate

β-gal…….. beta galactosidase

bHLH……. basic helix-loop-helix

bp…… base-pairs

DNA…… deoxyribonucleic acid

IRE…….. intronic regulatory element

SURE…….. slow upstream regulatory element

Kb…… kilobasepairs

MCK…… muscle creatine kinase

MEF2…….. myocyte-specific enhancer factor 2

MHC…… myosin heavy chain

MNI……. Montreal Neurological Institute

mRNA……. Messenger ribocucleic acid

OD…… optical density

PBS…….. phosphate buffered saline

PCR……. polymerase chain reaction

TnI…… troponin I

TE……. Tris-EDTA

EDTA…… ethylendiaminetetraacetic acid

U…… unit

V…… volt

I INTRODUCTION

Skeletal muscle fiber type

A unique characteristic of skeletal muscle is its cellular diversity, i.e. skeletal muscles are composed of a large variety of functionally diverse fiber types. This heterogeneity of muscle tissue reflects its high degree of functional specialization and is the basis of its functional plasticity (1).

Using myosin ATPase histochemistry, four major fiber types, one slow and three fast, are currently delineated in skeletal muscles of mammals (2). Slow (or type I) fibers are used for postural tone, contract relatively slowly, are more resistant to fatigue and are specialized for oxidative energy metabolism (1). Fast or (type II) fibers are used for active movements, contract relatively fast, are less resistant to fatigue and have higher levels of glycolytic enzymes. Fast fibers fall into three distinct, metabolically specialized adult fast fiber types: IIA, IIX, IIB (1) ranked in order of increasing contractile speed, glycolytic capacity and fatigability.

During normal behavior slow (type I) fibers are used the most frequently, IIB fibers are used least frequently. IIA fibers have a greater oxidative capacity than IIX or IIB fibers and are used more frequently than IIB fibers. Thus there is a definite relationship between metabolic specialization and activity levels (3). Distinct myosin heavy chain (MHC) isoforms are present in each fiber type (1): MHCI, MHCIIA, MHCIIX, MHCIIB (2). The Vmax ATPase activity for each MHC

isoform is different and accounts for the differing contractile speeds of each fiber type (4).

Not only MHC but many other contractile proteins have multiple fiber-type-specific isoforms (1, 4) and the restricted expression of contractile proteins in specific muscle fiber types is an important aspect of muscle fiber type specialization, a mechanism that is mostly controlled at the level of transcription (5).

Skeletal muscle formation and the role of nerves

Skeletal muscles consist of motor units which may differ considerably in contractile properties and types of usage (6). A motor unit consists of a motor neuron and all the muscle fibers (tens to hundreds) it innervates; all muscle fibers in a given motor unit are of the same fiber type. Muscle has the capacity to adapt by modifying its contractile and metabolic properties in response to different patterns of motoneuron activity (10). Interesting relations between firing pattern and contractile properties appear. First, low within-burst firing frequency characterizes slow motor units, whereas high firing frequency characterizes fast motor units. Second, motor units that are used more often during daily activity are more fatigue-resistant than those used less often. Third, motoneurons fire naturally at frequencies that give maximum control over the tetanic tension developed by the muscle fibers (3).

Though the activity pattern of the innervating motor neuron affects fiber type in the adult, it is now clear that innervation is not required for the initial formation of fast and slow muscle fibers during embryonic and fetal development (8). In mammaliam development there are two broad phases of myogenesis: an early phase, primary myogenesis, in which muscle fiber formation and diversification are largely independent of innervation, and a later phase, secondary myogenesis, in which continued growth and addition of new fibers and refinement of fiber type distribution depends on appropriate motor innervation (8, 63, 64).

With some exceptions primary muscle fibers tend to mature into slow fibers and secondary fibers tend to mature into fast fibers (2). During the period of secondary myogenesis innervation is important for slow fiber development and for the maintenance of slow primary fibers, whereas in the absence of innervation, fast isoform protein expression seems to be "the default program" (2).

Muscle gene regulation

Motor neurons are known to affect muscle growth and fiber type profile by regulating muscle gene expression. This is a basic issue in muscle/nerve biology (9). The restricted expression of contractile proteins in specific muscle types and their regulation in response to neural activity is mostly controlled at the level of

transcription (5). Enhancer and silencer DNA regions containing specific DNA motifs or cis elements have been found to regulate activation of muscle gene promoters (4).

Such regulatory cis-elements have often been identified in cell culture transfection experiments using recombinant DNA constructs in which normal, or mutant, regulatory sequence drive expression of reporter genes. Cell culture transfection studies are appropriate for identifying cis elements that are important in muscle gene activation during early development; the muscle cells produced in cell culture are immature myofibers, rather than fully differentiated adult type fast and slow muscle fibers. The full extent of muscle-type diversification does not occur *in vitro* and thus requires analysis *in vivo*. One experimental approach for mapping the pathways that lead from activity to fiber-type specific transcription has been to use transgenic mice.

To map DNA regulatory sequences conferring fiber-type specificity (10) another, more recently develop approach is somatic gene transfer (by the intramuscular injection/electroporation of DNA constructs into adult muscles, reference 46). This approach is considerably less laborious and expensive than the transgenic mouse approach and is the approach used in my studies.

Fiber type specific muscle genes that have been studied in transgenic mice include myosin light chain 1/3 (11,12), aldolase A (13, 14), TnIslow (15-17), and TnIfast (18-21). Among genes that have been studied by direct gene transfer into adult skeletal muscle are TnIslow (22, 24) and MHCIIB (25).

MyoD family of myogenic regulatory factors

The MyoD family of transcription factors plays an important role in myogenic commitment and muscle fiber differentiation. When expressed in many types of non-myogenic cells, each member of the MyoD family (MyoD, myogenin, myf5, and MRF4) is capable of converting the non-myogenic cells into cells capable of myotube formation and muscle-specific gene expression (8). MyoD, myogenin, myf5, and MRF4 comprise a family of proteins that share a structural motif that includes a highly basic region followed by a structural domain termed the helix-loop-helix (HLH) domain that allow them to form dimers, bind to specific DNA sequences in muscle-specific enhancers, and thus regulate each other's expression and the expression of other muscle-specific proteins such as creatine kinase, acetylcholine receptor, and myosin light chain (8). Like several other basic HLH (bHLH) transcription factor families the DNA sequence element to which MyoD family members bind is the E-box, CANNTG (26). Expression of MyoD family proteins is characteristic of, and entirely restricted to, skeletal muscle (8). It has been suggested that different members of the MyoD family may activate distinct subsets of muscle genes, and may play a role in fiber type specific gene expression (8). MyoD shows preferential expression in fast glycolytic (IIB) fibers while myogenin is preferentially expressed in slow (I) and fast oxidative/glycolytic (IIA) fibers (27). Mice lacking MyoD develop all muscle fiber types, although fiber type proportions may be altered (28). Thus although

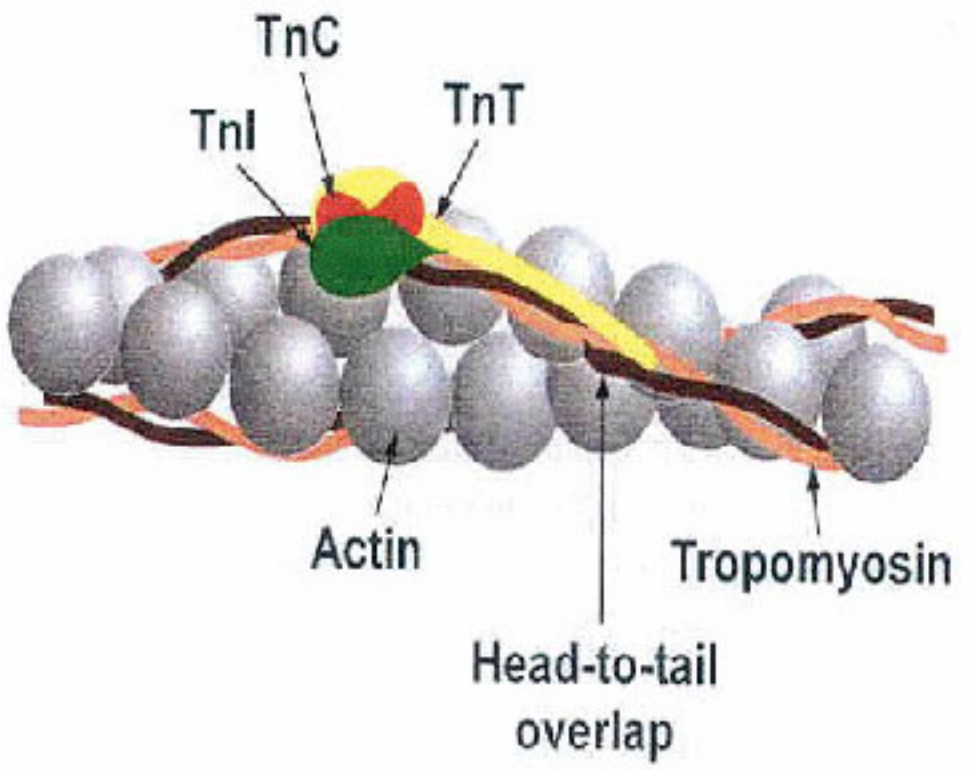
MyoD is not essential for fiber type differentiation, it may contribute to the process.

Troponin I

The work described in this thesis is based on a muscle gene encoding the contractile regulatory protein troponin I (TnI). In vertebrates, TnI is expressed specifically in sarcomeric muscles i.e. skeletal and cardiac muscles. TnI is a part of the troponin complex (Fig. 1) which consists of three subunits: TnI, which is involved in the inhibition of the actomyosin Mg^{2+}-ATPase, TnC, which binds Ca^{2+} and removes TnI inhibition, and TnT, which binds to tropomyosin. In striated muscle (i.e. skeletal and cardiac muscles) contractile proteins are organized in sarcomeres and contraction is regulated via Ca^{2+}-dependent conformation changes in the actin–linked troponin-tropomyosin system (29). In smooth muscle cells, contractile proteins are not organized into sarcomeres and contraction regulation involves Ca^{2+}-dependent phosphorylation of myosin. Troponin is not found in smooth muscles.

Vertebrate TnI isoforms are encoded by three distinct unlinked genes: TnIfast,

Fig. 1. Troponin complex and its interaction with actin filaments. Upon muscle excitation, cytosolic Ca^{2+} levels rise, and Ca^{2+} binds troponin C. This leads to a conformational change whereby TnI releases its hold on the actin filament allowing actin-myosin interaction, and muscle contraction, to take place. Based on fig 4-16 of reference 65.



TnIslow and TnIcardiac (30) that are differentialy expressed in different muscle cell types:

fast skeletal muscle, slow skeletal muscle and cardiomyocytes, respectively (31). Gene regulatory elements, that confer fiber-type specificity for TnIslow and TnIfast, have been identified. The SURE or slow upstream regulatory element is an upstream enhancer that drives the fiber type specific expression of the human (32) and rat (17) TnIslow genes. The FIRE or the IRE is

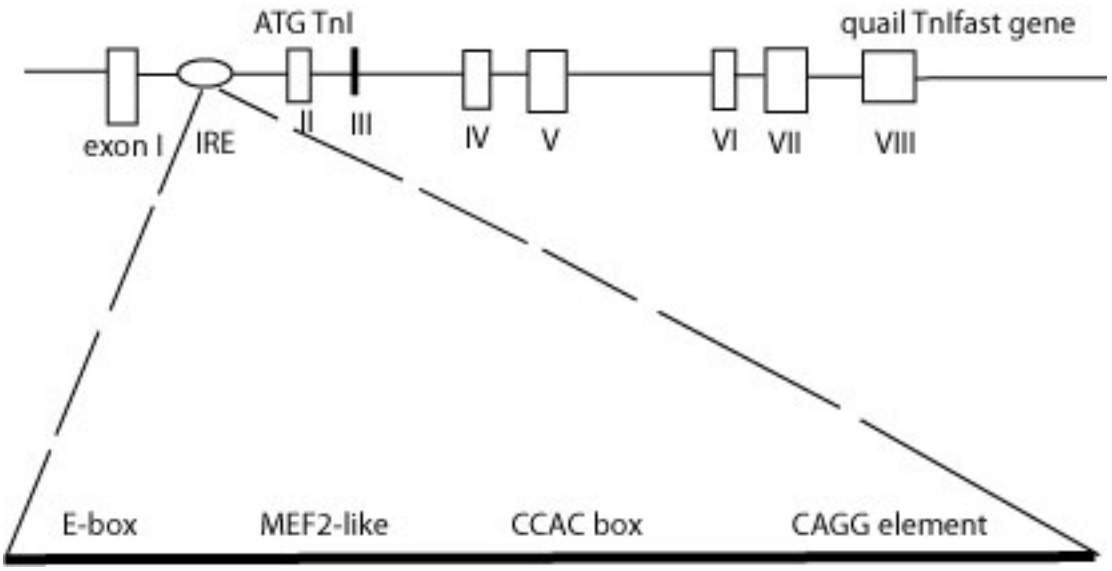
an intronic regulatory element that drives the fast fiber type specific expression of the quail TnIfast gene (33, 20). The main goal in our laboratory is to understand fiber-type specific expression driven by the IRE, by defining the sub-element or elements within the IRE that are responsible for fast fiber type specific expression of the TnIfast gene.

My project is a contribution toward this goal.

TnIfast gene structure and the IRE

The quail TnIfast gene was the first TnI gene to be cloned and characterized (34). It consists of eight exons and seven introns . The first exon encodes only 5'-untranslated mRNA sequence and is separated by a relatively large (1700 bp) intron from the second exon, which contains the translation start codon (35). The 5' organization of the TnIfast gene, including an untranslated first exon followed by a relatively large intron, is common to many known contractile protein genes

Fig. 2. The quail TnIfast gene with its eight exons (roman numerals) and the location of the IRE in the first intron. The order of the four known cis elements on the IRE is indicated in the IRE expanded view.



like α actin (36), cardiac myosin heavy chain (37), and myosin light chain 3 genes (38).

Konieczny et al (39) showed that a cloned quail TnIfast gene, stably transfected into a mouse myogenic cell line, exhibited appropriate developmental activation and quantitive expression during myoblast differentiation.

Further work done in the Hastings lab showed that a quail TnIfast gene construct including the intact gene, 530 bp of 5' flanking DNA and 1.5 kb of 3' flanking DNA was expressed in the skeletal muscles of transgenic mice to levels comparable to that in quail muscle (19).

The quail TnIfast IRE enhancer, located in the first intron of the gene, was originally identified and characterized by Konieczny and coworkers (33), who showed that this enhancer can drive the transcriptional activation of heterologous promoter/reporter gene constructs in differentiating cultures of transfected myoblasts. These gene constructs tested various deletion mutations of the first intron of the gene, which located the IRE within a 148 bp region from bases 634 to 782 (numbered based on the transcription start site).

Subsequent transgenic mouse analysis of a LacZ reporter construct driven by a fragment of the quail TnIfast gene including 530 bp of 5' flanking DNA, the first exon and intron and a part of the second exon showed efficient skeletal muscle-specific expression in fast, but not in slow fibers (18).

In addition, similar fast-fiber-specific expression was observed in transgenic mice

when a heterologous reporter gene, composed of the herpesvirus thymidine kinase (tk) minimal promoter upstream of the LacZ gene, was linked to three copies of the 148 bp IRE enhancer (18). Thus the IRE contains all the DNA sequence information required to direct fast fiber type specific gene expression. An important regulatory element in the TnIslow gene is an upstream enhancer termed SURE, that drives expression in slow muscles in transgenic mice (40). Buonanno and coworkers aligned the slow upstream regulatory element (SURE) of the TnIslow gene and the TnIfast IRE and showed that they share certain common sequences (see Fig. 3) despite their differing location and biological specificities (17).

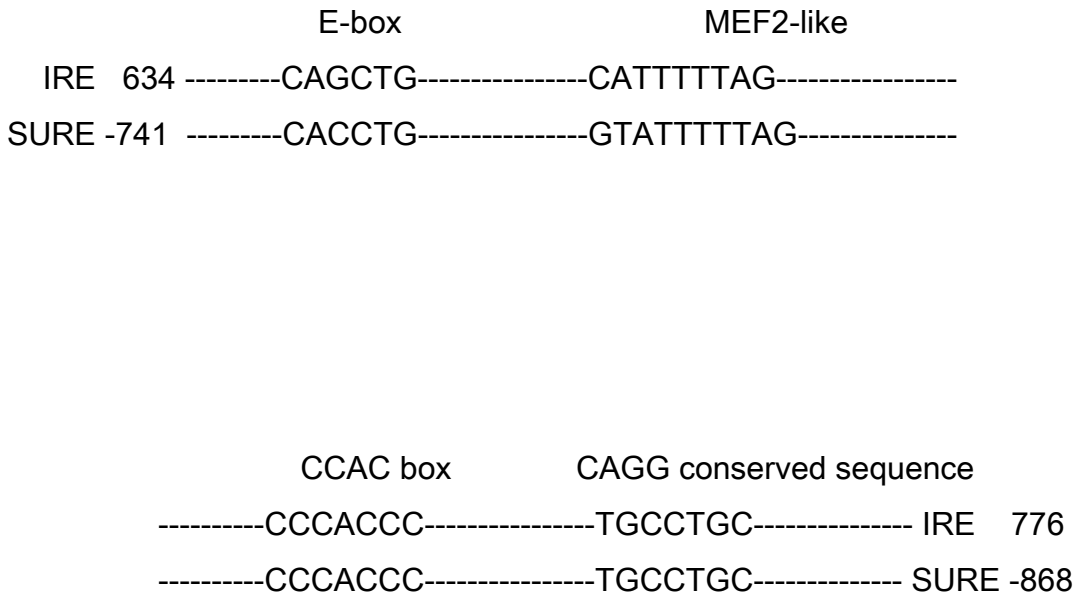
These common elements are:

-**E-box**: A motif CANNTG known to bind homo- or hetero-oligomers of muscle-specific bHLH proteins such as MyoD or myogenin and ubiquitious E-proteins such as E12 and E47.

-**MEF2 like site**: An A/T rich sequence known to bind Mef2 and other MADS-box transcription factors that play roles in controlling myogenesis and morphogenesis of skeletal muscles.

-**CCAC** element: Known to bind Sp1/Sp3 ubiquitious factors, CB40 and winged-helix myocyte nuclear factor-1. The CCAC box has been found in enhancers of the myoglobin, cardiac troponin C (41,42), myogenin (43), and muscle creatine kinase (MCK) genes (44).

Fig. 3 Four cis-elements common to the Intronic Regulatory Element (IRE) of the TnIfast gene and the Slow Upstream Regulatory Element (SURE) of the TnIslow gene (17). Bases are numbered in relation to the transcriptional start sites.



-**CAGG** conserved sequence: Similar to MEF3 site found in many other muscle specific promoters (41, 45) and potential binding site for SIX protein family members. Although the CAGG sequence was conserved but it appears to bear little or no obvious resemblance to this consensus motif. Although the evolutionary relationship of the SURE and IRE enhancers is not clear, these common elements are of particular interest as possibly functionally important cis-regulatory elements.

Research goal

An important long range goal of our laboratory is to elucidate the molecular mechanisms that direct fiber type specific expression of the TnIfast gene. In particular we would like to identify the specific cis-elements within the IRE that are chiefly responsible for fast fiber type specificity (Fig. 2). An initial step in this project was carried out by Angela Kumar, a former graduate student, who prepared two IRE end-deletion constructs. One of these, the right 3/4 (R3/4) construct (668, 782), deleted 30 bases (637 to 667 including the E-box) of the 5' end of the IRE.

The other, termed left 3/4,or L3/4, (638 to 737), deleted 45 bases (from bases 738 to 782) (including the CAGG sequence) of the 3' end of the 148 base IRE (see the schematic below, and Fig. 4).

668 782

5’----------R3/4 IRE----------3’

IRE 5’—[E-box]------[MEF2like]----[CCAC box]-----[CAGG]--3’

5’----------L3/4 IRE----------3’

638 737

When she assessed the gene regulatory capabilities of her constructs by head to tail trimerization and cloning upstream of a minimal TnIfast promoter driving the LacZ (β-galactosidase) reporter gene expression and gene transfer into adult mouse soleus she found that:

1-the L3/4 construct, like the intact IRE, showed preferential expression in fast fibers. This indicated that the 3’-most 45 bases of the IRE do not play an essential role in the fast fiber-type specific expression.

2-the R3/4 construct showed a loss of fast fiber specificity, i.e. it was expressed similarly in both fast and slow fibers.

Based on this result, Kumar concluded that fast-fiber-specificity of the IRE is based on a negative gene regulatory mechanism that represses expression in slow fibers and operates through the left-most 30 bases segment of the IRE (637 to 667), a region including the E-box (66).

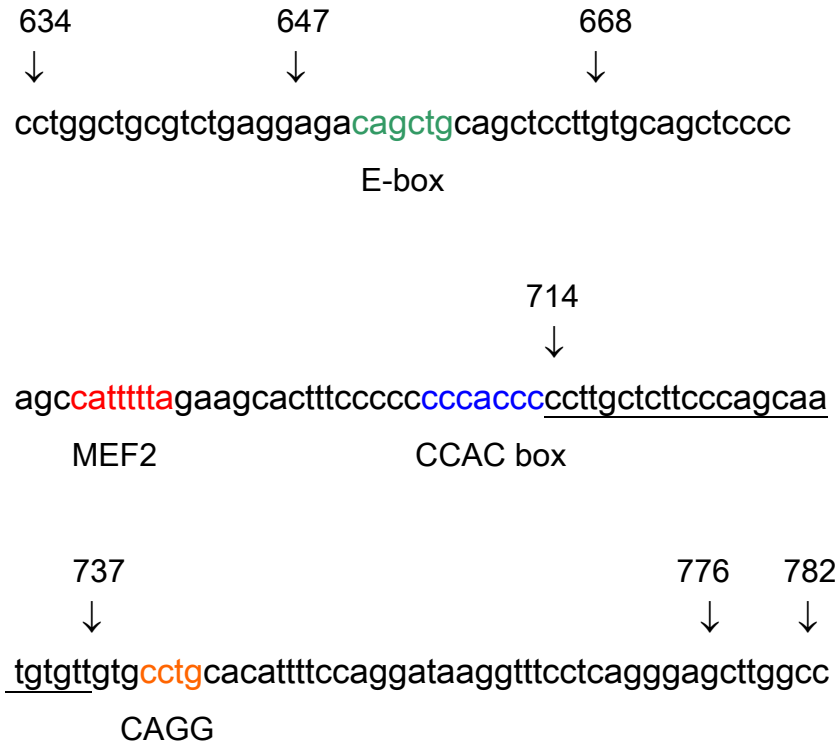
The initial goal of my project was to further analyze the 5’-most 30 bases of the IRE searching for the hypothetical cis-element that represses gene expression in

Fig. 4.

The complete DNA sequence of the IRE enhancer. The four cis elements common to the IRE and the TnIslow SURE are indicated in colors.
The coordinates of end-deletion constructs prepared by Angela Kumar (R3/4, L3/4) and by me (R7/8, Mid1/2, 49IRE) are as follows:

L3/4 634-737
R3/4 668-782
R7/8 647-782
Mid1/2 668-737
49IRE 668-714

The underlined bases indicated the 24 bp thought to have the fast specific cis element according to our hypothesis based on our results and those of Calvo et al (15).



slow fibers. I will describe my findings and conclusions and then outline further studies.

However my experiments have generated results that are clearly inconsistent with the hypothesis of a slow-fiber-repressive element in the 5'-most 30 bases of the IRE. Instead, they suggest that an unlikely event such as the generation of novel sequence at the junctions of the three repeated copies of the R3/4, or at the vector junctions artifactually generated an active element that drove transcription in the slow fibers.

My studies also addressed a distinct hypothesis of fiber-type specificity presented by Calvo et al (15), in their study of TnIslow gene regulation. They reported an experiment in which the 3' half of the IRE (bases 714 to 776) was fused to a piece of the TnIslow SURE that directed a non-fiber-type-specific pan-muscle expression pattern. This fusion construct showed fast muscle specific expression suggesting that the IRE fragment used in the experiment contains an important fast fiber type specific element (see Fig. 19). However my results were not consistent with this hypothesis either. I was able to show that a 49 bp region of the IRE (668 - 714) contains sufficient regulatory information to drive fast fiber type specificity, and this region contains neither the region suggested to direct fast fiber specificity in the Kumar hypothesis, nor that suggested from the work of Calvo et al. The 49 bp fragment is the shortest IRE fragment to date shown to direct fast fiber preferential gene expression, so my studies have identified a small target region for further work in this area.

My study also revealed that the TnIfast IRE enhancer E-box is not required for enhancer activity in adult mouse skeletal muscle, in contrast with previous transfection studies that showed it to be critical for gene activation in differentiating mouse myoblast cultures (46).

II MATERIALS AND METHODS

In order to locate the cis elements responsible for fast fiber type specific expression I have prepared several IRE-based reporter gene constructs. These included a site-directed base-substitution mutation construct and several end-deletion constructs.

The general approach for construct-preparation was based on previous experiments done by Angela Kumar (66). In overview, this involved cloning head-to-tail trimers of the IRE derivative into a plasmid vector, and then introducing into sequence-validated clones a reporter gene made up of the minimal TnIfast gene promoter (-198, +22) linked to an E.coli β-galactosidase (LacZ) protein-coding sequence.

IRE mutant constructs

I have made 4 IRE based constructs as outlined below (see Fig. 5):

1- IRE43: This consists of the intact 148 bp IRE (634 - 782) with a mutant E-box, based on the IRE43 mutation of Konieczny et al (47), which was shown to severely reduce the activity of the IRE in differentiating myoblast cultures. This mutation was:

IRE wild type E-box: GAGACAGCTGCAGC .

IRE mutant E-box: GAGACAGCAAAGC .

This mutation consisted of substituting AA for TGC.

2- R7/8: This consists of a deletion construct lacking the 5'-most 13 bases (634 - 646) of the IRE.

3-Mid 1/2: This consists of a 76 bp-long construct that represents the middle part of the IRE from bases 668 - 737.

4- 49IRE: This consists of a 49 bp-long IRE construct, from 668 - 714.

Construct preparation strategy

The same plan was used to prepare all constructs. I will present a summary of the overall procedure before explaining each step in detail.

Fig. 5 *Overview of IRE mutant constructs generated for the present studies.*

IRE 5'---[E-Box]-------[MEF2-like]-------[CCAC]------[CAGG]--3'
CAGCTG

IRE43 5'---[mut E-Box]------[MEF2-like]-------[CCAC]------[CAGG]—3'
CAGCAA

R7/8 5'[E-Box]-------[MEF2-like]-------[CCAC]------[CAGG]--3'

Mid1/2 5'----[MEF2-like]-------[CCAC]---3'

49IRE 5'----[MEF2-like]-------[CCAC]3'

Monomeric mutant DNA molecules were produced either by PCR amplification or by oligonucleotide synthesis. Monomers were ligated to create multimers, and head-to-tail tandem trimers were recovered. Trimers were cloned into the *Sal*I site of pBluescript IISK+ (Stratagene).

Subsequently the TnIfast minimal promoter (-198, +22) linked to the reporter gene LacZ, was cloned into trimer-containing plasmids. Finally the IRE-trimer/TnIfast promoter/LacZ reporter cassette was removed from the pBluescript IISK+ vector and introduced into the vector pSP72, in order to obtain better DNA yields.

The following sections give details for each step.

Monomer preparation

Longer monomer IRE derivatives (IRE43, R7/8) were prepared by PCR amplification, and shorter derivatives (Mid1/2, 49IRE) were prepared by annealing complementary synthetic oligonucleotides.

1- PCR approach

Fig. 6 shows the locations of the primers and oligonucleotides used to generate the constructs presented in this thesis. Primers and oligonucleotides sequences are shown in Fig. 7, which highlights *Sal*I and *Xho*I sites introduced in order to carry out the trimerization strategy.

IRE43

IRE43 was PCR amplified using primers 9043 and 9046 (Fig. 7) from TnI43E1b (47), a construct provided by S. Konieczny in which E-box at positions (651 - 656) had been mutated from CAGCTG to CAGCAA The IRE43 mutation was previously shown to severely reduce the activity of the IRE in myoblast culture (47).

R7/8

R7/8 monomers were prepared by PCR amplification from the construct TnILacZ1B(18), which contains 5' upstream sequence of the quail TnIfast gene, as well as exon I, the IRE-containing intron I and part of exon II. Primers used to amplify R7/8 were 9046 and 9097.

PCR reactions (110 ul final volume) contained 2 ng/ul of each primer, 20 ng (total) template DNA, 1X Stratagene cloned Pfu polymerase buffer, 20 U/ul Pfu polymerase (MBI), 0.2 mM each dNTP (dATP, dCTP, dGTP, dTTP).

Reaction mixtures were overlaid with 50 ul mineral oil and subjected to 13 cycles of: denaturing at 94 C for 30 sec, annealing at 60 C for 30 sec, and extension at 72 C for 1 min (extended to 12 min in the last cycle) in a Perkin Elmer Cetus thermal cycler.

Fig. 6 scheme for the PCR approach and the synthetic oligonucleotide approach indicating the locations of the primers and oligonucleotides used to generate the different constructs presented.

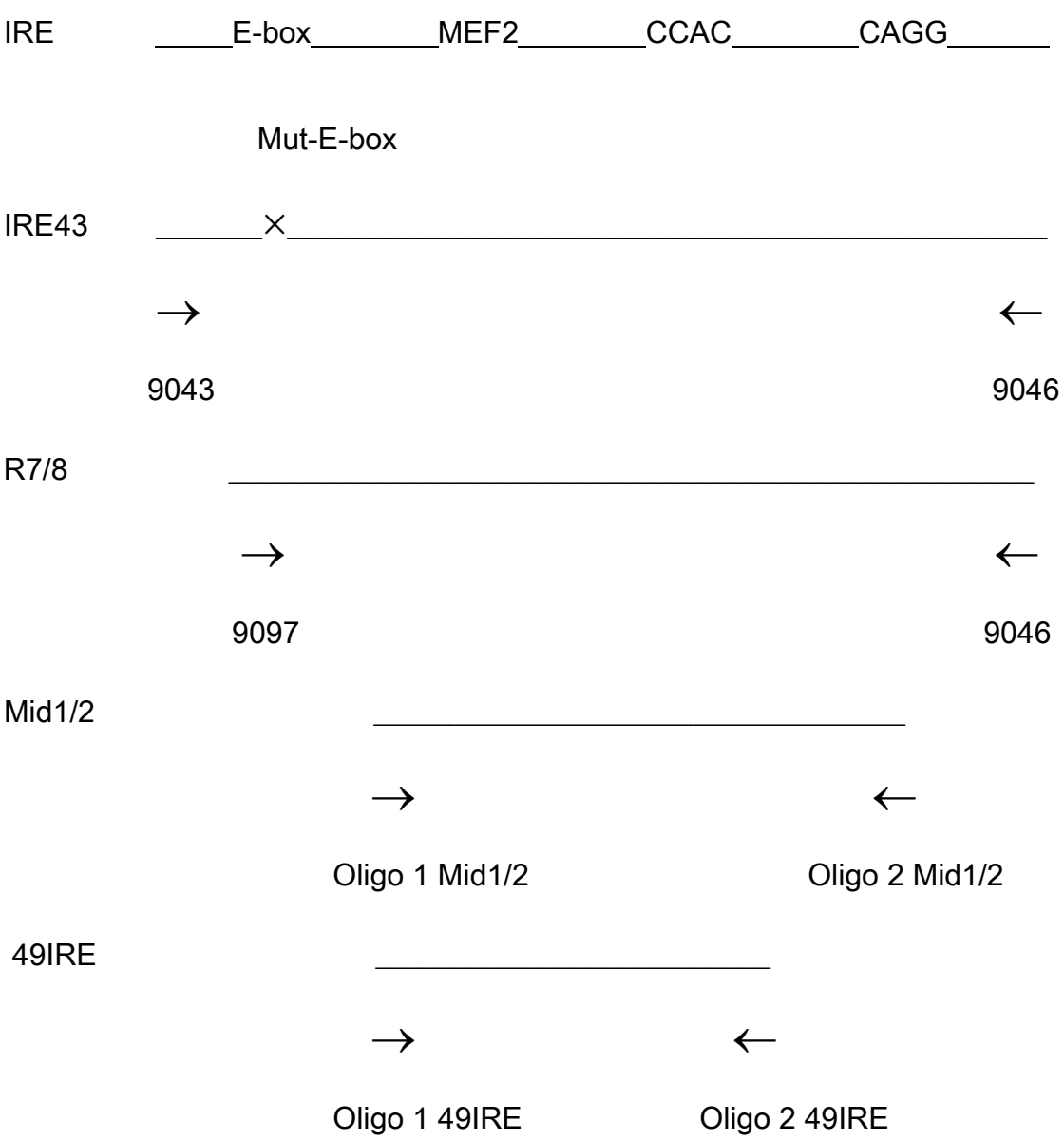


Fig. 7. PCR primers used to amplify the IRE43 and R7/8 monomers and complementary synthetic oligonulcleotides pairs used to prepare Mid1/2 and 49IRE monomers.

*Sal*I

5’ CTCA**GTCGAC**GGCTGCGTCTGAGGAGA primer 9043 (rightward)

5’CTA**CTCGAG**GCCAAGCTCCCTGAGGAA primer 9046 (leftward)

*Xho*I

IRE43 primer

---

*Sal*I

5’ CTC**GTCGAC**GAGACAGCTGCAGCTCCT primer 9097 (rightward)

5’CTA**CTCGAG**GCCAAGCTCCCTGAGGAA primer 9046 (leftward)

*Xho*I

R7/8 primers

---

*Sal*I

5’**TCGAC**TGTGCAGCTCCCCAGCCATTTTTAGAAGCACTTTCCCCCCCCACC

CCCTTGCTCTTCCCAGCAATGTGTT**C** **Oligo 1 Mid1/2**

*Xho*I

5’**TCGAG**AACACATTGCTGGGAAGAGCAAGGGGGTGGGGGGGGAAAGTGCT

TCTAAAAATGGCTGGGGCGCTGCACA**G** **Oligo 2 Mid1/2**

Mid1/2 Oligos

---

*Sal*I

5’**TCGAC**TGTGCAGCTCCCCAGCCATTTTTAGAAGCACTTTCCCCCCCCACC

CC**C** **Oligo 1 49IRE**

*Xho*I

5’**TCGAG**GGGGTGGGGGGGGAAAGTGCTTCTAAAAATGGCTGGGGAGCTGC

ACA**G** **Oligo 2 49IRE**

49IRE Oligos

Oil was removed by gliding the aqueous phase drop along a Parafilm surface, to which the oil adheres. PCR products were purified by electrophoresis on a 1% agarose gel in Tris-acetate-EDTA buffer (7) containing 0.1 ug/ml ethidium bromide and specific DNA bands were cut from the gel under ultraviolet transillumination and were recovered using the Sephaglas BandPrep Kit (Amersham Biosciences).

2- Synthetic oligonucleotide approach

49IRE and Mid1/2 constructs monomers were prepared by total synthesis as complementary single stranded oligonucleotides at IDT (www.idtdna.com). Synthetic oligonucleotides included 5'-phosphates and were designed so that when complementary oligonucleotides were annealed, the resulting double-stranded products contained single stranded overhangs at the end identical to those left by digestion with restriction enzymes *Sal*I and *Xho*I.

To prepare double-stranded monomers, the single-stranded complementary oligonucleotides were dissolved separately in STE Buffer (10 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA) at a concentration of 0.4 – 4.0 mg/ml. The individual complementary oligonucleotides were mixed in approximately equimolar amounts (1 ug of each oligonucleotide) in 50 ul STE buffer, heated to 94 C for 20 mins in a water bath and gradually cooled to room temperature over several hours by unplugging the water bath. The resulting double-stranded product was stored at –4C or –20 C.

Production of head-to-tail tandem trimers

PCR-amplified monomers were digested with both *Sal*I and *Xho*I to create ligatable sticky ends. The 250 ul digestion reaction in Orange+ buffer (MBI Fermentas) included ~8ng/ul of DNA and 0.2 U/ul of each enzyme and was incubated at 37 C for 1 h.

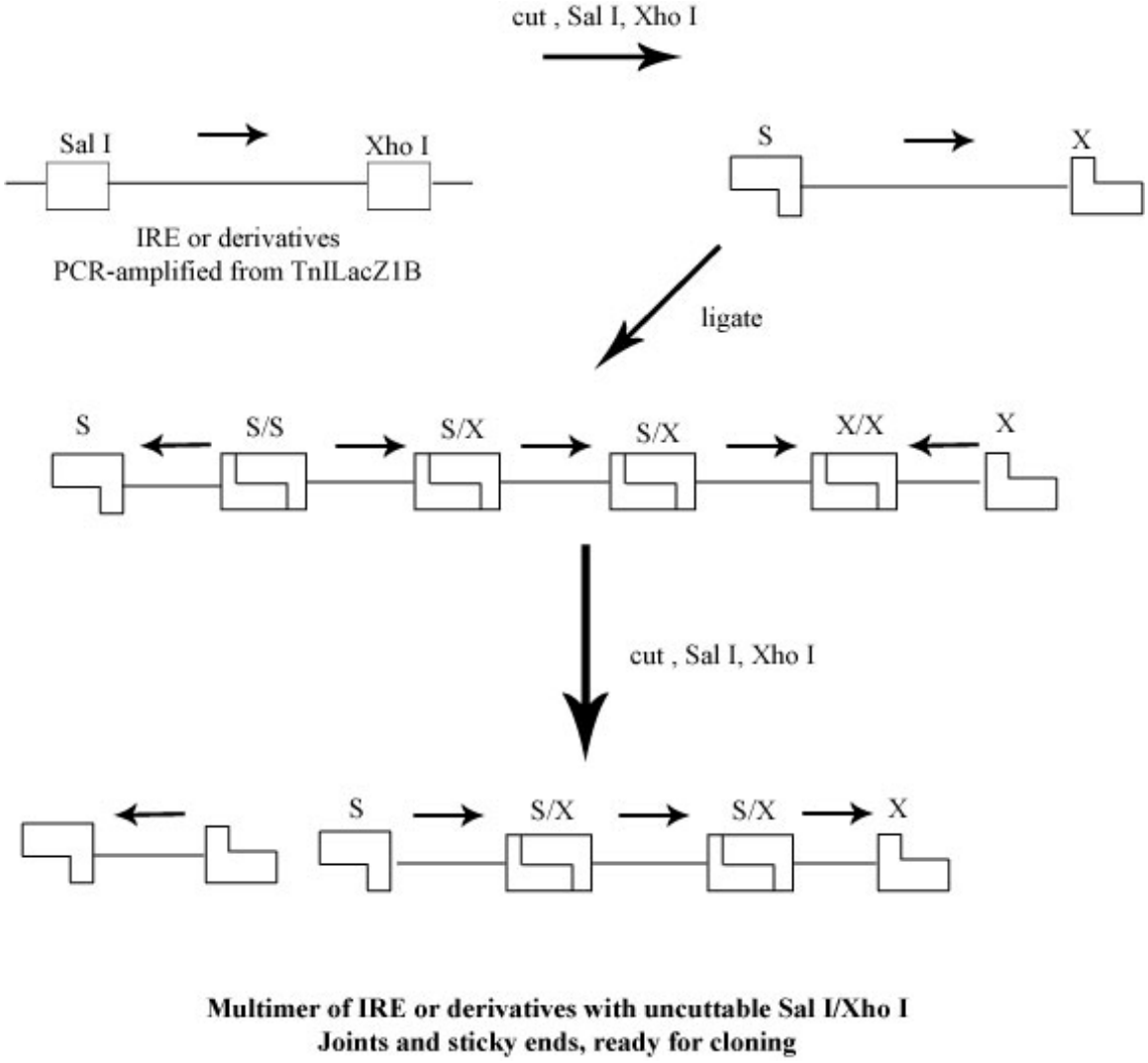
Monomers were recovered following agarose gel electrophoresis using the Sephaglas Bandprep kit (Pharmacia).

Monomers prepared from in vitro synthesized oligonucleotides did not require this initial digestion step because they were designed to carry ligation-ready *Sal*I/*Xho*I sticky ends.

Monomers were ligated in 10 ul reaction containing ~ 0.5 ug DNA, 1X T4 DNA ligase buffer (MBI, Fermentas), 0.2 U/ul T4 DNA ligase (MBI, Fermentas), at 12 C overnight. The ligation products were then redigested with *Sal*I and *Xho*I to cleave head-to-head and tail-to-tail joints, as follows: DNA was purified after ligation by ethanol precipitation using 2.5 volume of 95% ethanol and 0.1 M NaCl and incubation for 20 min at –20 C followed by 10 min centrifugation. The DNA pellet was air-dried and resuspended in 30 ul water which was then made up to a 50 ul *Sal*I/*Xho*I digest in 1X Orange+ buffer (MBI Fermentas)

Fig. 8 Scheme for production of head-to-tail multimers from PCR-amplified IRE fragments. Monomer PCR products were cleaved on the left ends with *Sal*I, and on the right ends with *Xho*I and multimerized, in random orientations by DNA ligase. Ligation products were re-digested with *Sal*I and *Xho*I to produce multimers containing only un-cleavable *Sal*I/*Xho*I (i.e. head-to-tail) joints. Mid1/2 and 49IRE monomers prepared from complementary synthetic oligonucleotides were designed to have ends corresponding to *Sal*I and *Xho*I cleavage products, so no restriction enzyme digestion was required prior to the initial ligation step.

Those constructs were ligated directly to create multimers and were then subjected to *Sal*I and *Xho*I digestion to eliminate head-to-head or tail-to-tail joints. Figure modified from ref. 66.



containing 0.2 U/ul of each enzyme and incubated at 37 C for 1 h. Tandem head to-tail repeats joined by uncuttable *SalI/Xho*I fusion sites were then subjected to gel electrophoresis and trimer bands (3xIRE43, 3xR7/8, 3xMid1/2, 3x49IRE) were cut out and recovered with the Sephaglas Bandprep kit (Fig. 8). Trimers were chosen because this was the highest number of repeats obtained in reasonable yields.

Cloning

My overall strategy was first to clone IRE-based constructs into the vector pBluescript II SK+ (Stratagene), and then to introduce the TnIfast minimal promoter/β-gal reporter gene. This strategy permitted the dual use of the blue/white selection scheme based on the α complementing fragment of E. coli β-galactosidase encoded by the vector pBluescript II SK+. The initial insertion of triplicated IRE fragments interrupted the β-gal α fragment coding sequence, permitting selection of insert-containing clones as white colonies against a background of blue colonies reflecting reconstructed vector (but see below for exceptional behavior of some constructs). Subsequent insertion of the intact β-gal coding sequence from gCTnIf(-198,+22)Z (66) resulted in detectable β-gal expression permitting selection of blue colonies against a background of white colonies representing parental plasmids that had not acquired the β-gal reporter insert.

Cloning trimerized IRE mutants in pBluescript II SK+

The head-to-tail trimer IRE derivatives containing *Sal*I and *Xho*I-cut ends were cloned into *Sal*I-cut vector pBluescript II SK+ (Fig. 9). One ng of vector and 5 ng of insert were combined in a 15 ul reaction containing 0.2 U of T4 DNA ligase (Fermentas) and 1X T4 DNA ligase buffer (Fermentas) and incubated overnight at 12 C.

Ligation products were introduced into competent tetracycline-resistant E.coli cells (XL1 blue, Stratagene) by electrotransformation. After transformation, cells were plated on L-agar plates contains tetracycline/ampicillin and incubated at 37 C overnight. In general, white colonies were picked for further analysis, but we discovered that the blue/white selection did not work as expected for some inserts. IRE-derived trimer-containing plasmids were named pb(3xIRE43), pb(3xR7/8), pb(3xMid1/2) and pb(3x49IRE). In the cases of pb(3xIRE43) and pb(3xR7/8), insert-containing plasmids were recovered from white colonies, as expected. However, in the case of pb(3xMid1/2) and pb(3x49IRE) very few white colonies were produced leading us to suspect that insert-containing plasmids might generate blue rather than white colonies. This raised a problem because reconstruction of the single-cut vector used generated many blue colonies. To overcome the vector background problem for these constructs, *Sal*I-cut pBluescript II SK+ was first dephosphorylated using shrimp alkaline phosphatase (SAP) (MBI, Fermentas). This greatly decreased the vector background and

allowed us to confirm that in fact plasmids carrying the 3xMid1/2 and 3x49IRE inserts gave rise to blue, not white colonies. This was a surprising finding, especially for the 3xMid1/2 insert, which contains a TAA stop codon in each of the three possible reading frames. Perhaps there is some cross-suppression of TAA stop codons by the amber (TAG) suppressor tRNA mutation that is present in E.coli XL1blue, the host cell for transformations.

Introduction of the (TnIfast promoter + β-galactosidase) cassette

In a second cloning step I introduced a minimal TnIfast promoter (bases -198 to +22) linked to the β-gal gene. This was recovered as a *Hind*III/*EcoR*I fragment from plasmid gCTnIf(-198,+22)Z (produced by P.Hallauer) and was ligated to *Hind*III and *Eco*RI sites of the enhancer-containing vectors (Fig. 10). These constructs were named: gCTnIf(-198, +22) (3x IRE43)Z, gC TnIf(-198,+22) (3xR7/8)Z, gCTnIf(-198,+22) (3xMid1/2)Z, gCTnIf(-198,+22) (3x49IRE)Z.
Blue β-gal containing colonies were picked and constructs were verified by sequencing using T3 and T7 sequencing primers, at the *Hind*III and *Eco*RI joints into which the promoter/β-gal cassette had been cloned. Sequencing was done at Sheldon Biotechnology Centre, McGill University.

Cloning into pSP72 vector

I found that the above-described plasmids based on pBluescript II SK+ did not give sufficiently high yields of isolated plasmid DNA using the Qiagen Maxi Prep procedure. In order to increase yields each enhancer+ promoter+β-gal gene insert was recloned into the plasmid verctor pSP72 (New England biolabs). Enhancer+ promoter+β-gal gene cassettes were cut out of the pBluescript II SK+ vector with *Xho*I and *EcoR*I and were agarose gel isolated and purified with Sephaglas (Pharmacia).

The cassettes were then ligated into *Xho*I and *EcoR*I-cut gel-purified pSP72 vector (Fig. 11) in 10ul reaction mixtures containing 10 ng each of insert and vector, 1X T4 DNA ligase buffer and 0.2U T4 DNA ligase (Fermentas) incubated overnight in 12 C. Blue colonies were picked and the constructs were named: gCTnlf(-198, +22) (3x IRE43)72Z, gCTnlf(-198, +22) (3xR7/8)72Z, gCTnlf(-198, +22) (3xMid1/2)72Z, gCTnlf(-198, +22) (3x49IRE)72Z.

Plasmid DNA injection/electroporation into mouse soleus muscle

Plasmid DNAs for direct gene transfer into mouse soleus muscle were prepared by theQiagen Endotoxin-free Maxi Prepkit. Prior to injection/electroporation, DNA was ethanol precipitated and resuspended at 1mg/ml in sterile phosphate buffered saline (PBS: 150 mg/ml K_2HPO4, 144 mg/ml NaCl and 26 mg/ml NaH_2PO4-H2O). CD1 mice aged 7-10 weeks were anaesthetized with 0.1 ml/10g body weight of 5% chloral hydrate solution injected intraperitoneally.

Fig. 9 Presentation of the first cloning, trimers of IRE enhancer-based trimers cloned in pBluescript II SK+ vector. pb (3x IRE-based fragment).

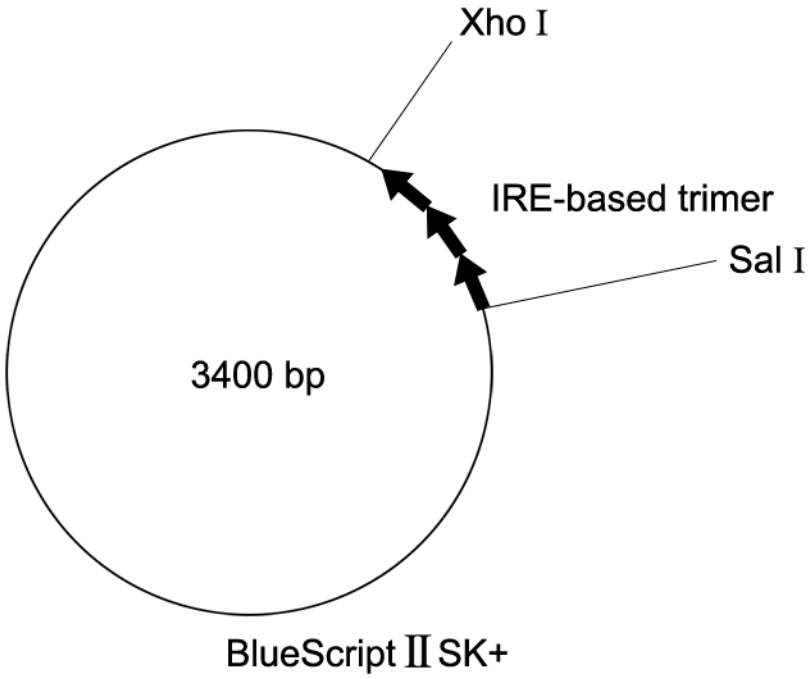


Fig. 10 Introduction of the (TnIfast promoter + β-galactosidase) cassette. gC(-198,+22)(3xIRE-based fragment)Z.

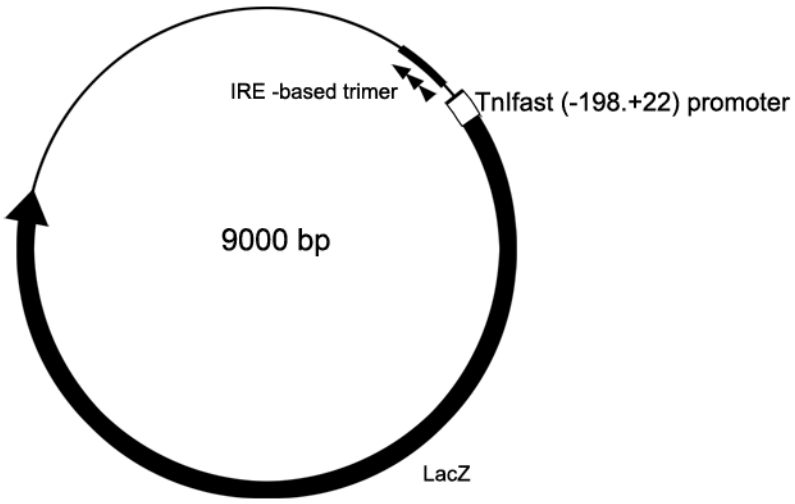


Fig. 11 Cloning into pSP72 vector.
gC(-198,+22)(3xIRE-based fragment)72Z.

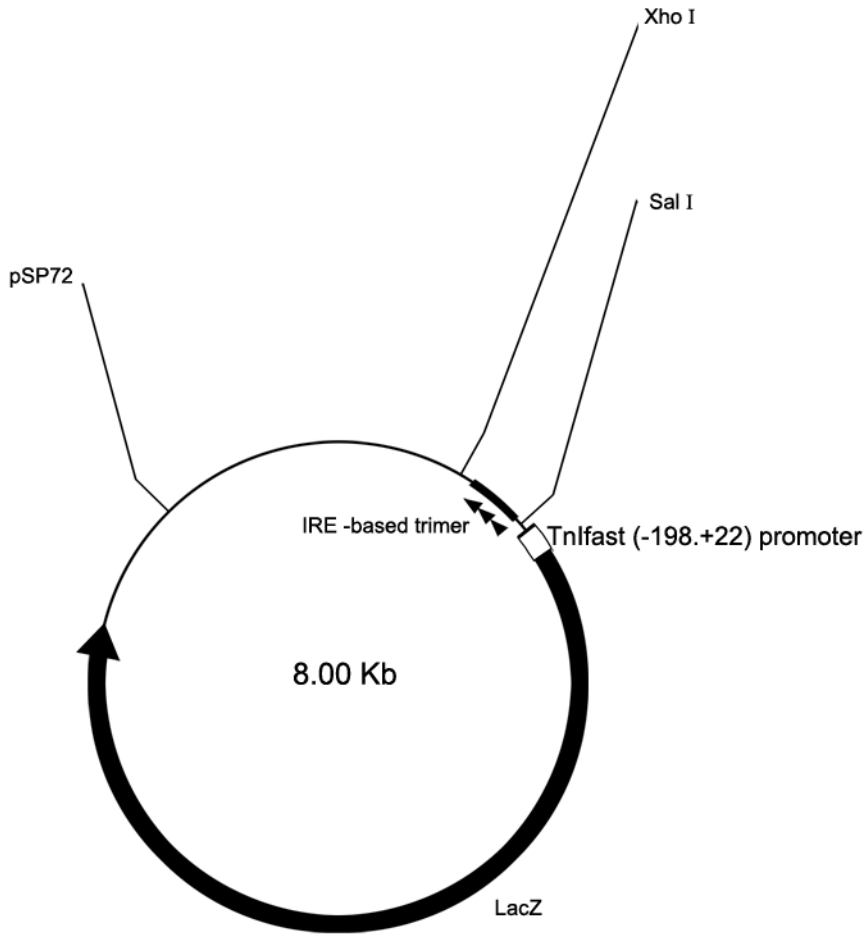


Table 1 shows a complete list of all plasmids constructs:

Constructs in this thesis	Description
pb(3xIRE43)	Trimer of IRE43 in pBluescript II SK+
pb(3xR7/8)	Trimer of R7/8 in pBluescript II SK+
pb(3xMid1/2)	Trimer of Mid1/2 in pBluescript II SK+
pb(3x49IRE)	Trimer of 49IRE in pBluescript II SK+
gC(-198,+22)(3xIRE43)Z	Minimal TnI fast promoter and 3XIRE43 as enhancer driving the reporter gene β-gal.
gC(-198,+22)(3xR7/8)Z	Minimal TnI fast promoter and 3XR7/8 as enhancer driving the reporter gene β-gal.
gC(-198,+22)(3xMid1/2)Z	Minimal TnI fast promoter and 3XMid1/2 as enhancer driving the reporter gene β-gal.
gC(-198,+22)(3x49IRE)Z	Minimal TnI fast promoter and 3X49IRE as enhancer driving the reporter gene β-gal.
gC(-198,+22)(3xIRE43)72Z or IRE43	Minimal TnI fast promoter and 3XIRE43 as enhancer driving the reporter gene β-gal. all cloned in pSP72 vector.
gC(-198,+22)(3xR7/8)72Z or R7/8	Minimal TnI fast promoter and 3XR7/8 as enhancer driving the reporter gene β-gal. all cloned in pSP72 vector.

gC(-198,+22)(3xMid1/2)72Z or Mid1/2	Minimal TnI fast promoter and 3XMid1/2 as enhancer driving the reporter gene β-gal. all cloned in pSP72 vector.
gC(-198,+22)(3x49IRE)72Z or 49IRE	Minimal TnI fast promoter and 3X49IRE as enhancer driving the reporter gene β-gal. all cloned in pSP72 vector.

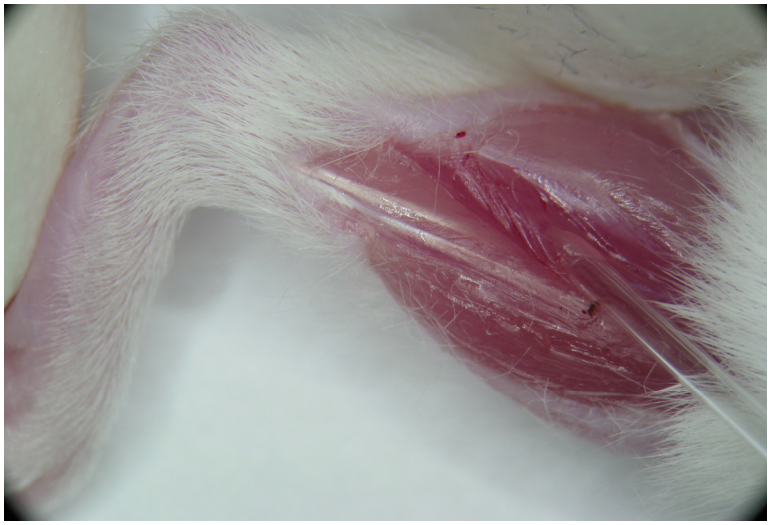
Soleus muscles were exposed by skin incision and blunt dissection. The soleus is a small muscle lying behind the gastrocnemieus muscle. The soleus muscle was chosen because it contains approximately equal numbers of fast and slow fibers. Exposed soleus muscles were injected using a drawn glass pipet (Fig. 12) with ~5 ul plasmid DNA solution (~5 ug DNA). After intramuscular DNA injection, needle electrodes (0.5 cm diameter) connected to an electroporator (BTX electro square porator ECM 830) were then applied on either side of the injected soleus. The electroporator settings were as follows: 160V / 0.5 cm distance between electrodes, 8 pulses of 20 ms each, square wave, 1 Hertz, mode low voltage and polarity unipolar. The mice were then returned to their cages for 14 days before euthenasia and harvesting the muscles by dissection.

Fig. 12 Mouse leg after exposing the soleus muscle.

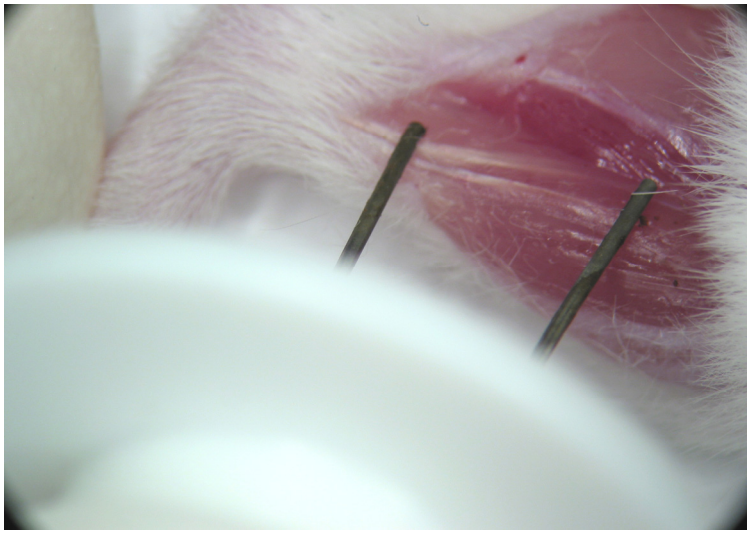
A. Intramuscular injection of the construct into mouse soleus muscle.

B. Electroporation of the injected muscle.

A.



B .



Processing of muscle tissue

Reporter gene expression was examined by preparing serial cryosections of the transduced soleus muscles, histochemical staining for β-gal enzymatic activity, and determining slow versus fast fiber type by immunohistochemistry for fast and slow myosin isoforms.

Muscles were individually frozen by immersion (for 20 s) in isopentane brought to the temperature of liquid nitrogen. Muscles were stored at –70 C in 1.5 ml microcentrifuge tubes filled with isopentane.

Muscle cross-sections, 10 um thick, were cut on a cryostat (Microm, HM 500 M). Several sets of serial sections were collected on cover slips at various points along the length of the muscles. Transgene β-gal reporter expression was visualized by X-gal staining as described by Sanes et al (68), sections were fixed in 0.25% glutaraldehyde in water for 3 min and were washed several times in water. Excess water was lightly removed from the cover slip with an absorbant tissue paper. Coverslips, face up in a weigh boat inside a Petri dish whose bottom was layered with moist paper towel, were overlaid with a solution made up of 1.6 mg/ml X-gal, 5mM ferrocyanide, 5mM ferricyanide, and 2mM $MgCl_2$ in PBS, and left for 24 h in the covered Petri dish at room temperature. The sections were rinsed in water several times before mounting them on a microscope slide with Immu-Mount solution (Microscopy Aquatex, EMD Chemicals Inc.).

Muscle fiber-types were determined by imunostaining unfixed sections with monoclonal antibodies specific for particular myosin heavy chain isoforms, as described by Schiaffino et al (67). Primary antibody BA-D5 or SC-71 (to identify types I and IIA myosin heavy chains respectively) was overlaid on the sections for 1 h at room temperature. After three PBS washes, biotinylated secondary antibodies against IgM (for primary BA-D5) or IgG (for primary SC-71) (Vector Laboratories, Inc.) were overlaid for 30 min at room temperature. Then, following three PBS washes, the sections were overlaid with avidin-coupled horseradish peroxidase (Vector Laboratories, Inc.) for 30 min followed by three 2 min PBS washes and a 5 min incubation in diamino benzidine solution (Lab Vision Corp.). The sections were then washed several times with water and mounted as with X-gal stains. Type IIX fibers do not react with either of these antibodies, hence fibers not stained for type I and IIA were classified as IIX.

III RESULTS

Experimental approach

The main goal of our laboratory is to understand the fiber type specific expression of the TnIfast gene by identifying the element or elements that are responsible for this specific expression within its IRE enhancer.

Earlier studies in the laboratory were based on the use of transgenic mice as an assay system for the gene regulatory capability of the IRE. However, the development of methods for direct gene transfer into adult muscle fibers by intramuscular injection/electroporation has provided a far less laborious and expensive approach (46).

In our laboratory, extensive studies have shown that, following direct gene transfer into adult mouse muscle by this technique, the intact IRE drives fast fiber preferential expression of reporter constructs in which a minimal TnIfast promoter is linked to the E coli LacZ β-galactosidase gene (66, and P. Hallauer, unpublished data). The soleus muscle is well-suited for analysis of fast-versus-slow fiber type specificity because it consists of approximately equal numbers of fast and slow fibers.

The studies I report in this thesis were based on this technique. In these studies gene expression was monitored at the level of individual muscle fibers by microscopic histochemical analysis of reporter β-gal gene expression by X-gal histochemistry of muscle cross-sections. Muscle fibers detectably expressing the reporter gene and showing the characteristic blue X-gal stain were identified by visual inspection, and the level of β-gal activity was assessed by quantitative analysis of muscle fiber optical densities by micro-densitometric analysis. Fiber types of individual muscle fibers were determined by immunohistochemical analysis of serial sections with antibodies specific for slow (type I) or fast (type IIA) isoforms of myosin heavy chain (Fig. 13).

From these data I derived two measures of fiber type preferential expression. In the first, I assessed whether the distribution of muscle fiber X-gal optical densities differed between fast and slow fibers. Optical densities were determined for all muscle fibers in a patch of ~ 100 contiguous fibers. The Mann-Whitney U-test was used to assess the probability that the distribution of staining intensities in the fast and slow fiber subsets could represent samplings from the same population of optical density distributions. When that probability is small, then the null hypothesis that there is no difference in the behavior of the reporter gene in the fast and slow fibers is rejected, and fiber type preferential expression is established. This test assesses whether the observed differences between fast and slow fibers are significant but does not provide any quantitative measure of the relative expression levels in fast and slow fibers. For this purpose we have developed a measure termed the relative specificity factor. We count the numbers of fast and slow fibers in a contiguous patch of ~ 100 fibers and calculate the proportions of the total number that are fast or slow. We also measure the optical densities of all fibers in that patch that are visibly stained. Then the separate optical density measures are added together to yield a " total optical density" and the fraction of this total that is present in fast and slow fibers is calculated. From the fraction of the total optical density presentin fast fibers we

Fig. 13

Serial cross sections of a mouse soleus muscle injected/electroporated with 3xIRE43 14 days previously.

A.) Section treated with anti-type I (slow) myosin heavy chain antibody (BA-D5) for slow fiber type identification.

B.) Section treated with anti-typeIIA (fast) myosin heavy chain antibody (SC-71) for type IIA fast fiber type identification.

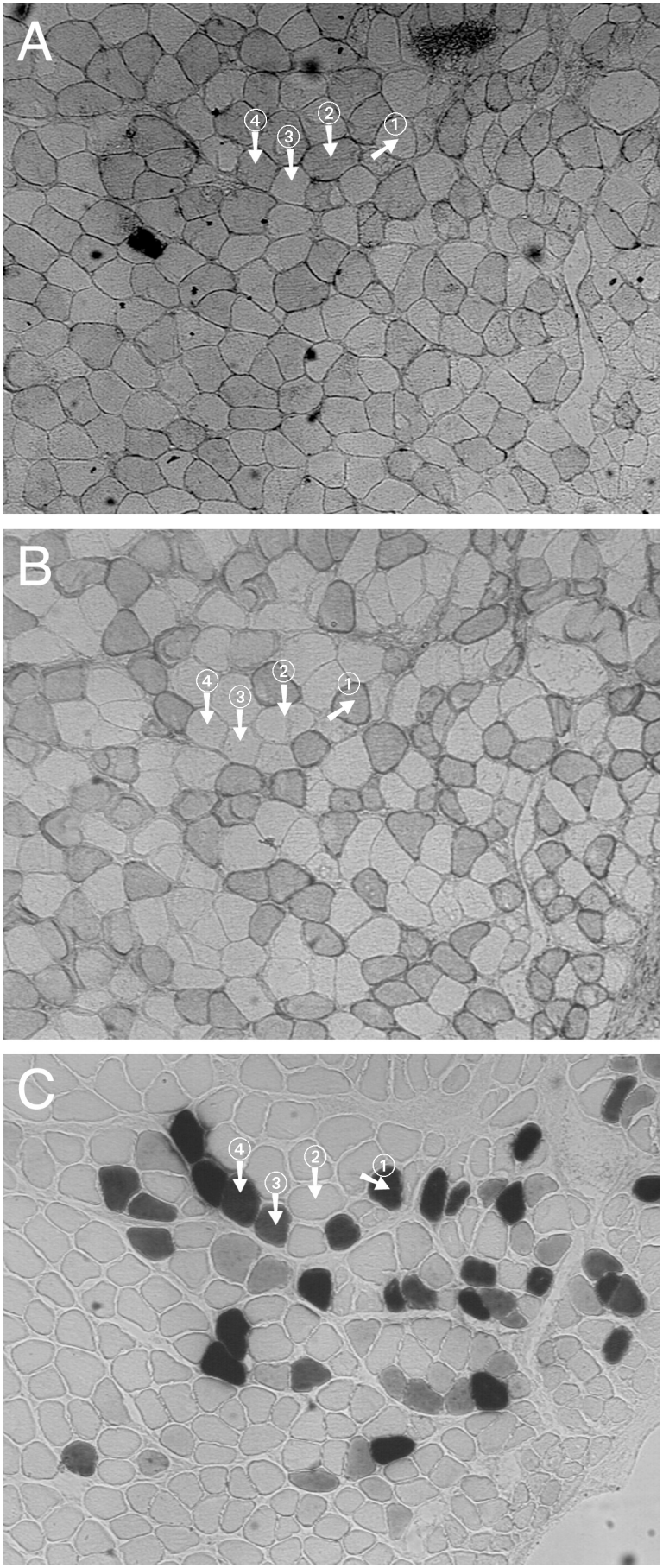
C.) Section stained for beta-galactosidase reporter gene activity to identify fibers that express the construct injected.

The numbered arrows indicate: 1- A type IIA fast fiber, stained with SC-71 (panel B) and not with BA-D5 (panel A), that expresses the construct injected as shown by its staining for b-gal (panel C).

2- A slow type I fiber, stained with BA-D5 (panel A) and not with SC-71 (panel B), that dosent expresses the construct injected as shown by its non staining for b-gal (panel C).

3- A type IIX fast fiber, stained neither with SC-71 (panel B) nor with BA-D5 (panel A), that expresses the construct injected as shown by its staining for b-gal panel C).

4- A slow type I fiber, stained with BA-D5 (panel A) and not with SC-71 (panel B), that expresses the construct injected as shown by its staining for β-gal (panel C).


A
4
3
2
1
B
4
3
2
1
C
4
3
2
1

subtract the fraction of the total number of fibers in the patch that are fast. When the fraction of the total optical density in fast fibers is greater than the fractions of fibers that are fast, this indicates fast fiber preferential expression. To correct for variation in the fiber type compositions from muscle to muscle, or from patch to patch, the measure is normalized as follows:

$$\text{Relative specificity factor} \quad \frac{F(OD)fast - F(fibers)fast}{1.0 - F(fibers)fast}$$

F(OD)fast is the fraction of the total optical density that is in fast fibers.

F(fibers)fast is the fraction of fibers that are fast.

Note that regardless of the fiber type composition of any patch of muscle fibers, if all of the optical density is in fast fibers the relative specificity factor is 1.00. This corresponds to complete fast fiber specificity. Conversely if there is preferential expression in slow fibers then the relative specificity factor is a negative number. Finally, if there is no preferential expression in fast or slow fibers. The relative specificity factor is 0. In this thesis for each construct tested I report the Mann-Whitney statistic establishing whether fast and slow fibers differ, and the relative specificity factor, which assess the extent of any preferential expression.

The plasmid pRSVZ, in which lacZ expression is driven by the Rous sarcoma virus long terminal repeat shows effective expression in both fast and slow fibers (P. Hallauer unpublished data). This establishes that both fast and slow fiber types are efficiently transduced by the injection/electroporation method. PRSVZ consistently gives relative specificity factor near zero (see for example Fig. 18). Most TnIfast constructs tested, even those with positive relative specificity factor showing preferential expression in fast fibers, showed some expression in slow fibers. Expression of a fast-preferential construct in some slow fibers could reflect unusually high levels of transferred DNA in those fibers; the amount of DNA taken up varies from fiber to fiber. Alternatively, because we know that TnIfast constructs taken up by regenerating muscle fibers show dysregulated expression in both fast and slow fibers (21), expression of a fast-preferential construct in some slow fibers could reflect muscle damage and regeneration occurring as a result of the injection/electroporation procedure.

Technical aspects of construct preparation

All of our constructs were based on head-to-tail trimers of the IRE or derivatives linked to a TnIfast gene minimal promoter (-198, +22) / β-galactosidase reporter gene cassette. This same construct strategy was used previously by Angela Kumar (66). Trimers were used because the " strength" of DNA regulatory elements can often be augmented by the production of serial multimers (58), and we wished to be able to detect residual enhancer activity even in deletion

constructs that might lack elements important for high-level quantitative expression.

As described in materials and methods, triplicated IRE derivatives were produced either by PCR amplification from the native IRE DNA sequence or by annealing complementary synthetic oligonucleotides. Head-to tail multimers were prepared using DNA ligase and restriction enzyme digestion and following agarose gel electrophoresis of multimers, the band corresponding to trimers was recovered and cloned into a plasmid vector. I found that only one of the two possible orientation was obtained, the same one present in the constructs previously made by Angela Kumar (66). The other orientation was not found in several repeated attempts. Perhaps these inserts in the missing orientation may have been toxic, or may have failed to inactivate the α-complementing fragment of β-gal encoded by the pBluescript II SK+ vector resulting in blue rather than white colonies and loss through discard as presumed vector background.

In all constructs the enhancer is in the opposite orientation with respect to the transcriptional direction by comparision to the native gene. The IRE like many enhancers has been shown to activate transcription when present in either orientation (33).The β-gal (lacZ) gene was selected as the reporter gene for all tested constructs. The advantages of this reporter were: 1-It offered the possibility of blue color selection in the cloning stage when inserted into enhancer-containing vectors. 2-It provided a way of visualizing transgene expression by X-gal staining of muscle tissue cross-sections.

Cassettes containing the TnIfast minimal promoter (-198, +22) plus the lacZ gene were cloned into the enhancer multimer-containing pBluescript II SK vectors. Angela Kumar's prior studies had shown that the TnIfast promoter/ β-galactosidase constructs showed minimal activity on its own, but depended on the presence of a functional enhancer to drive detectable expression in adult mouse muscle (66).

In vivo direct gene transfer studies

Search for hypothetical slow fiber repressive element

My experiments were initially focused on the 30 bp region of IRE from coordinate positions 637 to 667 (Fig. 4). Angela Kumar had prepared an IRE derivative (3xR3/4) lacking this 30 bp segment and found that it drove expression of a TnIfast minimal promoter/ β-gal reporter gene in slow as well as in fast fibers. This result implies the existence of an element within this 30 bp region that normally functioned to repress IRE activity in slow fibers. My first experiments were designed to further localize this hypothetical repressive element. Because the E-box was the only known/suspected element within the 30 bp region of interest my first experiment was to mutate the E-box. If the E-box was responsible for repressing IRE activity in slow fibers, we would predict that mutating the E-box would eliminate or markedly reduce the fast-preferential expression driven by the IRE. To test this, the construct IRE43 was prepared and

introduced into mouse soleus muscle by injection/electroporation. The IRE43 is an E-box mutation construct based on the IRE43 construct of Konieczny et al that had been shown to severly reduce the ability of the IRE to activate gene expression in differentiating mouse myoblast cell culture (47).

I found that IRE43 showed fast fiber type preferential expression ($p= 0.001$), Mann Whitney U-test (Fig. 14). The relative specificity factor for IRE43 was 0.49 (Fig. 18) similar to that of the intact TnIlacZ1 reporter transgene (0.46) (Data for TnILacZ1B was kindly provided by Dr. Patricia L. Hallauer).

This high level fast preferential expression of the IRE43 construct indicated that the E-box is not the hypothetical slow repressive element responsible for fast fiber-type specificity of the IRE. It further implies that differing mechanisms regulate TnIfast gene expression in adult muscle fibers and in muscle cell culture as the IRE43 mutation was previously shown to severely reduce the ability of the IRE to drive expression in muscle cell culture (47). I observed that expression levels of IRE43 in adult mouse soleus muscle were not notably reduced by comparsion with constructs carrying the intact IRE.

Fig. 14

Distributions of β-galactosidase IRE43 histochemical optical density and muscle fibers, among fast and slow fiber types following injection/electroporation into adult mouse soleus muscle.

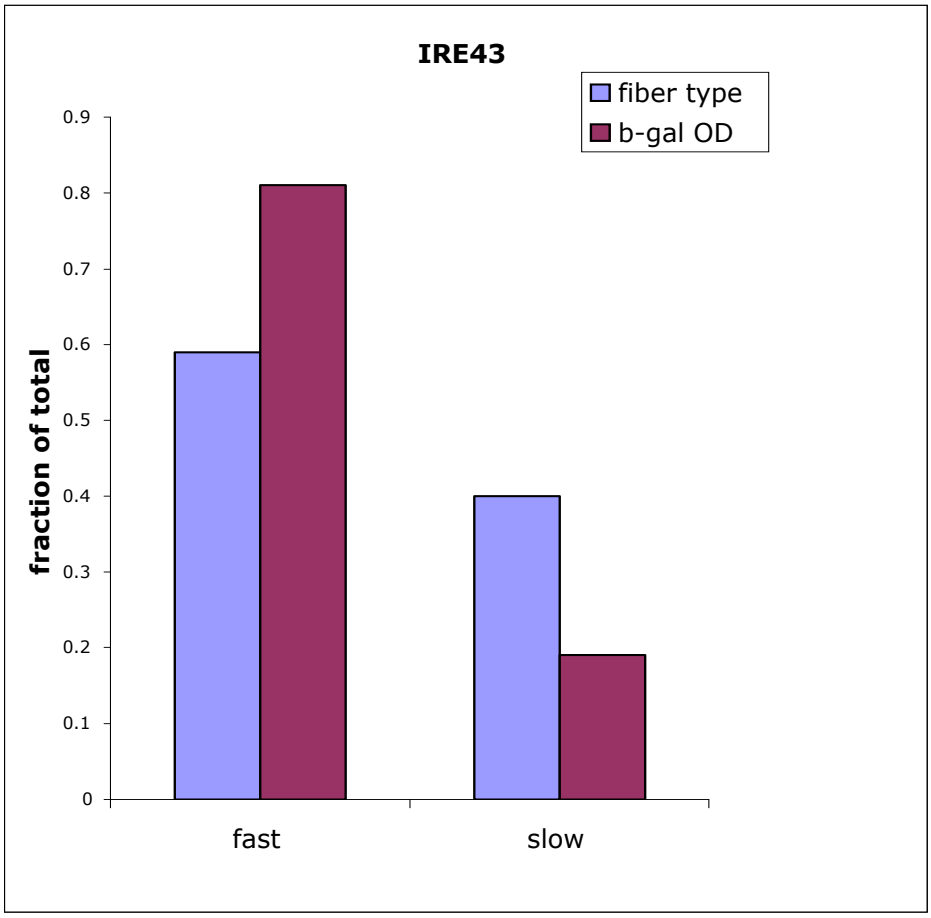


Fig. 15

Distributions of β-galactosidase R7/8 histochemical optical density and muscle fibers, among fast and slow fiber types following injection/electroporation into adult mouse soleus muscle.

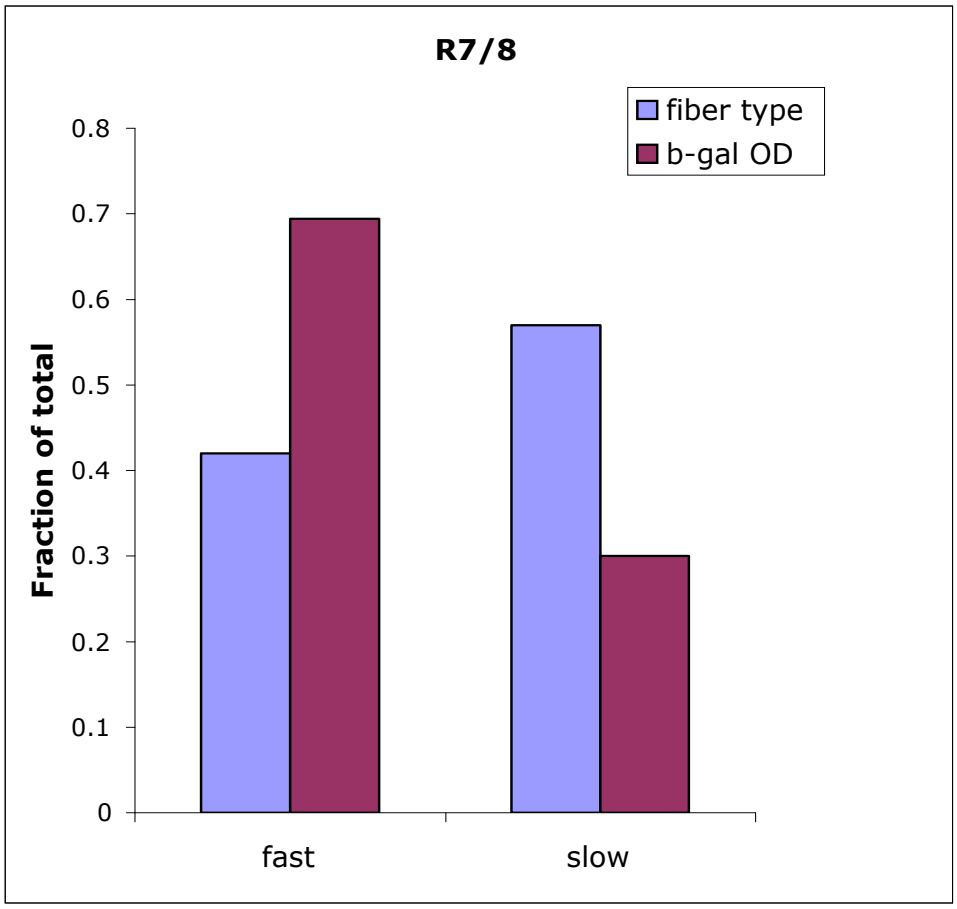


Fig. 16

Distributions of β-galactosidase Mid1/2 histochemical optical density and muscle fibers, among fast and slow fiber types following injection/electroporation into adult mouse soleus muscle.

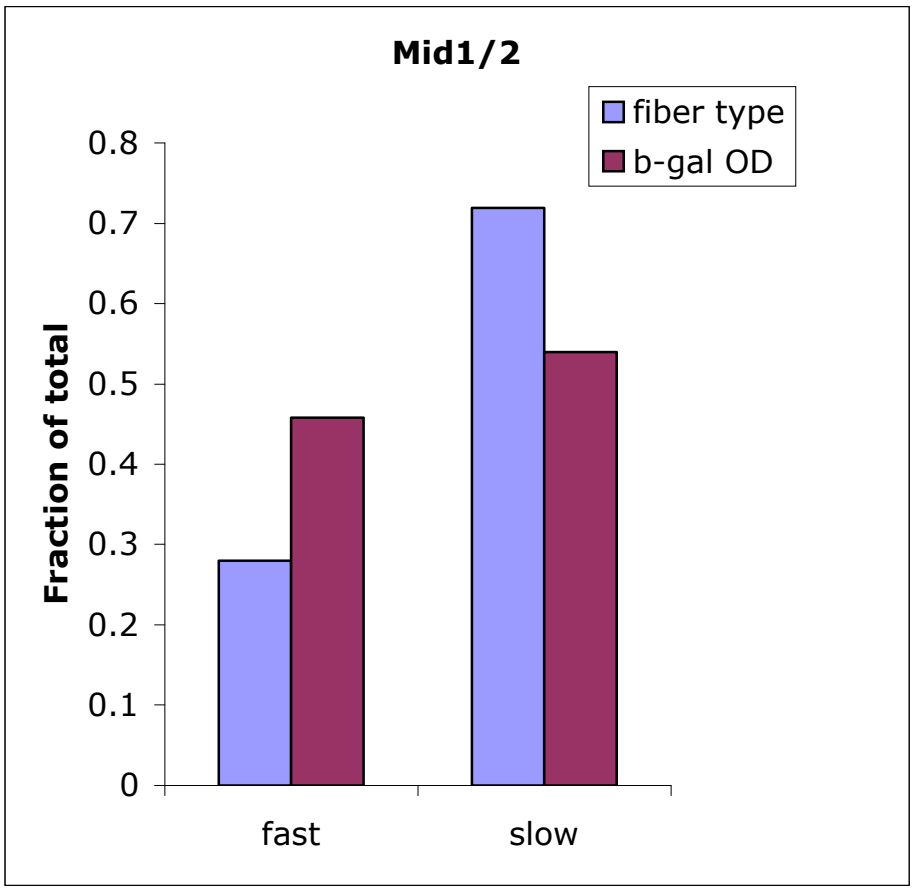
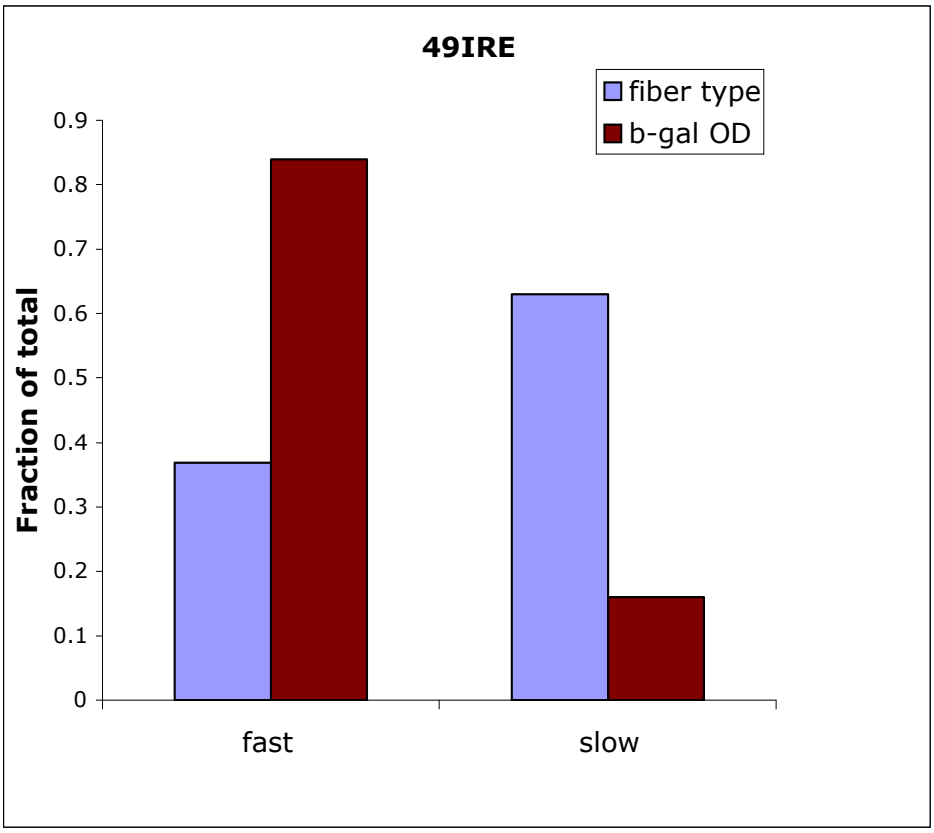


Fig. 17

Distributions of β-galactosidase 49IRE histochemical optical density and muscle fibers, among fast and slow fiber types following injection/electroporation into adult mouse soleus muscle.



The 5'-most 30 bp (637 to 667) of the IRE do not include a repressive regulatory element

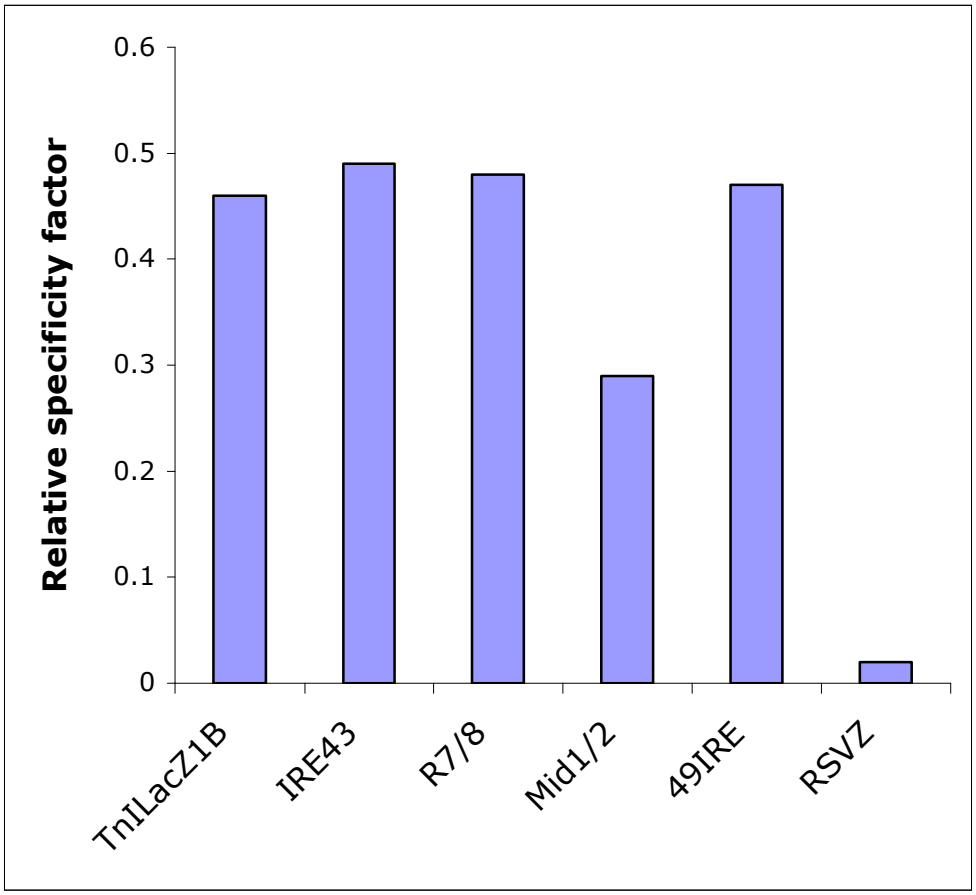
In a further attempt to localize the hypothetical slow repressive regulatory element in the 5'-most 30 bp of the IRE, I prepared the R7/8 construct, a deletion construct that lacks the 5'-most 13 bp of the IRE but which retains the E-box. When analyzed by direct gene transfer into adult mouse soleus muscle, this construct showed fast fiber-type preferential expression ($p = 0.0066$ Mann-Whitney U-test) (Fig. 15), with a relative specificity factor of 0.48 (Fig. 18), similar to that of the intact TnILacZ1 reporter gene.

Thus, the hypothetical slow fiber repressive element did not reside in, or overlap with, bases 1-13 of the IRE.

Given that the IRE43 and 3xR7/8 constructs together mutated/deleted a significant fraction of the 5'-most 30 bp segment of the IRE, I began to consider a possible alternative explanation for Kumar's observation of expression of the R3/4 construct in both fast and slow fibers. This expression pattern could represent, not the loss of a slow-fiber-repressive element vis-à-vis the full length IRE, but the accidental creation of a new element that actively drove "artifactual" transcription in slow fibers. Such a novel element could in principle be generated at the end-to-end joints of repeat units in the 3xR3/4 construct, or at their junctions with vector DNA as these joints represent novel sequences. To test this

Fig. 18

Fiber type expression of the constructs IRE43, R7/8, Mid1/2 and 49IRE. Showing fast fiber type specific expression as compaired to the fast specific construct TnILacZ1B (the data for TnILacZ1B was provided by Dr. Patricia Hallauer), and to the non specific construct RSVZ.



alternative “artifactual element” hypothesis I, prepared the Mid1/2 construct which, like the R7/8 construct lacks the 5’-most 30 bp of the IRE. According to the slow-fiber hypothesis of Kumar’s theory, such a construct should give expression in fast and slow fibers. In addition to IRE bases 1-30 (637-667), Mid1/2 also lacked bases 738 to 782, the 3’-most 45 bp. Thus upon trimerization this would generatejoints that are different than those of Kumar’s R3/4. If the artifactual element hypothesis is correct and a slow active element was created accidentally at the joints of a trimer in 3xR3/4 then our construct would have different joints and would not be expected to contain the artifactual element, and would show fast-fiber preferential expression. When testing this construct in mice, I found that the Mid1/2 construct gives fast fiber-type preferential expression (p=0.006 Mann-Whitney U-test) (Fig. 16) with a relative specificity factor 0.30 (Fig. 18) just slightly less than TnILacZ1B (Fig. 18). Results with 49IRE (see below) also indicate that 5’ most 30 bases can be removed without affecting fast preferential expression.

The marked fast fiber preferential expression of Mid1/2 is not consistent with the hypothesis that the 5’-most 30 bp of the IRE include a slow repressive element but it is consistenet with the artifactual element theory. Therefore I did not pursue any further the initial goal of mapping the hypothetical slow fiber repressive element.

Addressing the hypothesis of Calvo et al

Whereas the studies of Kumar had suggested that fast specificity of the IRE might reside near the 5' end of the IRE, independent studies by Calvo et al suggested that a different region was important in directing fast-fiber-type-specific expression. Calvo et al (15), in their studies of the TnIslow upstream enhancer SURE, reported that a 62 bp fragment of the IRE, positions 714 to 776, could, when transferred to a fragment of the SURE that drove "pan muscle" expression in both fast and slow fibers, impose fast muscle-specific expression (Fig. 19). Conversely, adding the corresponding segment of the SURE lead to slow muscle-specific expression. These results indicated that elements directing fast fiber preferential expression reside within IRE bases 714 to 776. In comparision with Calvo et al's fast fiber specific IRE/SURE hybrid enhancer, my Mid1/2 construct, which also shows fast fiber preferential expression, overlaps only by 24 bp (from 714 to 737) (Fig. 4). Thus it seemed possible that a key element driving fast-preferential expression might reside in this 24 bp segment. To test this hypothesis I prepared the 49IRE deletion construct, a 49 bp-long IRE construct (from 668 to 714) which corresponded to Mid1/2 from which the 24 bp segment in question had been deleted.

According to our hypothesis the 49IRE construct was expected to give fast and slow fiber-type expression i.e. was expected to show loss of specificity. Alternatively the 49IRE construct might be inactive if the 24 bp segment deleted contained an important site for activity.

The 49IRE construct was tested, like our previous constructs, by direct gene transfer in mouse soleus muscle and was found to be active and to show fast-fiber type preferential expression ($p= 0.003$ Mann-Whitney U-test) (Fig. 17), with a relative specificity factor of 0.47 (Fig. 18), similar to relative specificty factor of the construct TnILacZ1.

This result is difficult to reconcile with Buonnano's hypothesis that fast fiber specificity of the IRE resides in the segment 714 to 737 (15). The 49 bp segment present in 49IRE (from 668 to 714) is the shortest fragment from the IRE that has been shown to drive fast fiber-type preferential expression in adult mouse muscle.

IV DISCUSSION

The long-range goal of our laboratory is to understand the mechanisms by which the IRE enhancer of the TnIfast gene drives fast-fiber-preferential gene expression. Two mechanistic hypotheses have been advanced in previous studies. Angela Kumar, a previous student in the lab, made the 3xR3/4 construct, and based on its non fiber-type-preferential expression in fast and slow fibers in direct gene transfer experiments suggested that fast fiber specificity of the IRE was based on a slow fiber specific repression mechanism that operated through a site contained within, or overlapping, the IRE segment 637 to 667. In contrast, Calvo et al, in transgenic mouse studies based on hybrid TnIfast (IRE) and

TnIslow (SURE) enhancers suggested that fast-specificity of the IRE operated through a mechanism based on the IRE segment 714 to 776.

I designed and carried out experiments to test the validity of these hypotheses. As summarized below, my results are entirely inconsistent with the Kumar hypothesis and prove that region 637 to 667 is not essential for fast fiber preferential expression. I suggest an alternative explanation for her 3xR3/4 construct results. My results are also inconsistent with the simplest form of the Buonnano hypothesis, although a modestly more complex form was not ruled out. I suggest further experiments to probe the hypothesis. My studies showed that fast fiber preferential expression is driven by a 49 bp segment of the IRE, the shortest segment that has yet been shown to drive fast-preferential expression. This region includes two of three elements, MEF2-like and CCAC that were previously shown to be important for high level expression in differentiating myoblast cell cultures. The third such element, the E-box, I show by several ways plays no essential role in expression in adult muscle. I discuss the role of E-box-binding factors in muscle gene regulation and gene regulatory differences between muscle cell cultures and mature adult muscle in vivo.

Evidence inconsistent with the 638 - 667 slow-fiber-repressor hypothesis of Kumar

Angela Kumar's studies of L3/4 and R3/4 indicated that fast-fiber-specificity of the IRE is based on a negative gene regulatory mechanism that represses expression in slow fibers and operates through the 5'most 30 bp segment of the IRE (638 to 667). My initial studies were aimed at localizing the hypothetical slow fiber repressor element more specifically by mutating/deleting regions within that 30 bp segment. I made two such constructs: IRE43, which mutated the E-box at positions 651-656, and the end-deletion construct R7/8, which deleted nucleotides 638-646. Both of these constructs showed fast-fiber-preferential expression suggesting either that the repressor site resides elsewhere within the 638-667 region, or that the hypothesis was incorrect.

Because the slow fiber repressor hypothesis was based on the behavior of a single construct, the 3xR3/4 construct, I decided to probe the validity of the hypothesis by making an additional construct that would also lack the hypothetical slow fiber repressive element and thus would be predicted to be expressed in a non-fiber type specific manner in fast and slow fibers. To ensure that the hypothetical element would be missing, I made an IRE derivative that had exactly the same 5' 30 bp deletion as 3xR3/4 had. To make the new construct, termed Mid1/2, different from 3xR3/4, I also made a deletion at the 3' end of the IRE, residues 738-782. This corresponded to the IRE region deleted in Kumar's other construct 3xL3/4. Because Kumar had found that 3xL3/4 shows active and fast-fiber-preferential expression similar to the intact IRE and had thus concluded that region 738-782 did not contain any essential elements, it was

expected that my deletion of this region as in the Mid1/2 construct would not have a major impact. Thus, if the hypothesis of a slow fiber repressive element residing within positions 637-667 is correct, we expect Mid1/2 to be expressed in a non-fiber-type specific manner in fast and slow fibers.

However, if the hypothesis is incorrect, and there is in fact no slow fiber repressive element in region 637-667, we would expect the Mid1/2 construct to show fast fiber preferential expression. My results showed strong fast-preferential expression. This results rules out the slow fiber repressive element hypothesis. It cannot be argued that the activity of the hypothetical repressor also required the presence of elements in the 3' region 738-782 because Angela's L3/4 construct, which also lacks this region, shows fast-fiber-preferential expression. We do not know the reason for the non-fiber-type specific expression of the R3/4 construct in fast and slow fibers observed by Kumar. However, one possible explanation is that rather than the R3/4 construct having lost a negative element that represses expression in slow fibers, it may have artifactually gained a positively-acting element that drives expression in slow (and perhaps in fast) fibers.

Such a novel element could in principle be generated at the end-to-end joints of repeat units in the 3xR3/4 construct, or at their junctions with vector DNA, as these joints represent novel sequences.

The Mid 1/2 construct lacks both the 5’-most 30 bp and the 3’-most 45 bp of the IRE. Thus the left end of the monomer unit was identical to that of R3/4, but the right end was different. Therefore in the 3x setting all junctions would differ from

those of R3/4 except that between the vector and the left end. Thus, unless it resided at this particular junction, the artifactual element would not be present in 3xMid1/2. The fast fiber preferential expression of 3xMid1/2 could reflect the loss of the hypothetical artifactual element driving expression in slow fibers.

Addressing the hypothesis of Calvo et al

Because my results were clearly inconsistent with Kumar's hypothesis of a slow-fiber-repressive element in the left quarter of the IRE we explored the alternative hypothesis of IRE fiber type preferential expression raised by Calvo et al (15). The studies of Calvo et al were focused principally on TnIslow gene regulation controlled by the SURE enhancer. They were able to dissect the TnIslow SURE into two functionally distinct regions. One region, -807 to -741 (corresponds approximately to IRE 638 to 713) is sufficient to confer pan-muscle specificity, i.e. expression in fast and slow fibers, in transgenic mice and thus contains regulatory sequences necessary for general muscle specificity. Slow fiber specificity resided in the remainder of SURE, region –868 to -807 (Fig. 19). Moreover, Calvo et al also did an experiment in which the corresponding section of the IRE (right most ~1/2 IRE (714, 776) 62 bp), was added to the pan-muscle active –807 to -741 SURE DNA fragment. The resulting chimeric enhancer drove fast-fiber preferential expression. They concluded that this right most 62 bp fragment of the IRE contains the elements that confer fast specificity of the IRE.

I noted that there is a 24 bp overlap between the 62 bp IRE segment used by Clavo et al (15) and my Mid1/2 construct (Fig. 20). Thus, if one element is chiefly responsible for fast fiber specificity it would be predicted to reside within those 24 bases. I designed the 49IRE construct to address this hypothesis. The 49IRE is a right-end shortened version of the Mid1/2, that lacks the 24 bp candidate fast-fiber regulatory region. When I tested thisconstruct in the direct gene transfer assay in mouse soleus muscle I found that it was active and showed fast-preferential expression.

This result is inconsistent with the hypothesis that the deleted 24 bp segment contains key regulatory elements directing fast fiber specificity.

If there is, as Calvo et al suggest, an element within the right most 62 bp of the IRE that drives fast fiber preferential expression, then our results indicate that this element is functionally redundant with other elements present within the 49 bp of the 49IRE construct. Further clarification of the fast fiber specificity element hypothesized by Calvo et al will require its precise localization in a different

Fig. 19

Some of TnI SURE reporter constructs used in Calvo et al study. Wildtype SURE (-868 to -74) and all its derivatives were placed upstream of the -95 TnI basal promoter driving luciferase. The SURE deletion (SURE-807) terminates upstream of the CACC box and contains three of the four motifs conserved between SURE and IRE (CACC, MEF2, and E-box) The FIRE-SURE enhancer was generated by fusing the 5' half of IRE (from 776 to 714) to the 3'half of SURE (from -807 to -741), resulting in a chimeric enhancer with a preserved spatial organization of all four conserved motifs.

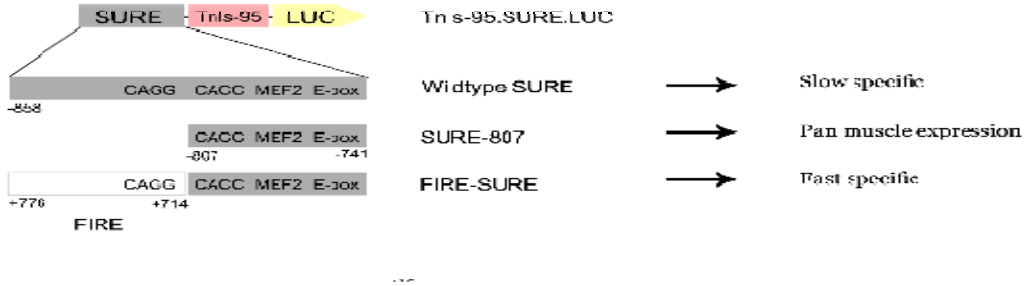


Fig. 20 IRE sequence, the four known cis elements are indicated in colours
The stars indicate where Mid1/2 start and end.
The triangles indicate where Calvo et al IRE part started and ended.
And the bases in the box indicate the 24 bases in common.

context in which its function is not redundant, e.g. in the chimeric SURE/IRE element of Calvo et al.

A 49 bp segment of the IRE is sufficient to drive fast-fiber preferential gene expression

The 49-bp 49IRE construct is the smallest IRE fragment that has been shown to direct fast fiber preferential expression. This DNA segment includes two of the four SURE/IRE “conserved” elements noted by Nakayama et al (17) i.e. the MEF2-like site and the CCAC site, but the E-box and CAGG element are not present. The CAGG element is also absent from my Mid1/2 construct and Angela Kumar’s L3/4, both of which drive active fast-fiber-preferential expression in adult muscle direct gene transfer experiments so its non-essential nature is substantiated by multiple constructs. In apparent contrast the corresponding CAGG box of the TnIslow SURE enhancer was found to be essential for high level expression in adult muscles in transgenic mice (16).

The E-box is missing from 49IRE and from Mid1/2, and was targeted by site directed mutagenesis in my IRE43 construct, all of which drive fast-fiber-preferential expression. Thus its nonessential nature is also supported by multiple constructs.

Previous studies have shown that E-box is necessary for TnIfast IRE activity in

differentiating mouse myoblast cultures (47). Thus our data indicate that although the E-box is important for IRE activity in muscle cultures, it is not important for enhancer activity in adult skeletal muscle. This implies that differing mechanisms regulate TnIfast gene expression in adult muscle fibers and in muscle cell culture.

E-box independent expression in adult muscle has also been established for another fast-fiber-type-specific enhance/promoter, the pM promoter of the aldolase A gene (48) although Wheeler et al found that mutation of the E-box within a proximal region of the mouse MHCIIB promoter reduced the activity of that promoter by 90 fold when injected into adult rat muscle (52).

An interesting case of E-box-dependent gene expression in adult muscle concerns the gene encoding the acetylcoline receptor clustering protein RAPSYN. Natural human mutations in two different E boxes in the rapsyn gene upstream DNA have been identified and are associated with reduced transcriptional activity in adult muscle and with congenital myasthenia gravis (49).

There have also been suggestions that E-boxes can inhibit various promoters. For example, Yan et al (50) studying mutation of the intragenic E-box in the myoglobin promoter found that the E-box acted as a negative regulatory element that repressed myoglobin expression in adult muscle.

The differing importance of the E-box for expression of diverse adult muscle genes in the above described data reflects the complexity of gene regulatory

mechanisms even among genes expressed in the same tissue, muscle. Such complexity including regulation through a multitude of combinatorial factors may be a biological necessity because if all muscle genes were regulated by the same transcription factor, it would eliminate the ability of the muscle fiber to differentially control multiple genes over diverse conditions and ultimately reduce the ability of the fiber to maintain its plasticity (51).

My studies localize the elements necessary for fast preferential expression of the IRE to a 49-bp segment including MEF2 and CACC elements.

Other genes and future research direction

Elucidating the molecular mechanisms that confer fiber type specificity on skeletal muscle genes is a very active research area.

Published literature suggests that muscle fiber type gene expression is regulated by multiple signalling pathways and transcription factors rather than a single 'master' switch or signalling pathway (51). Much more is known about slow fiber-type specific expression than fast fiber type specific expression. Data from multiple investigations have indicated that the Ca^{2+}-regulated protein phosphatase calcineurin (CnA) may play a role in slow fiber type gene expression (54).

Chin et al concluded that slow-fiber specific transcription appeared to be mediated by a combinatorial mechanism involving CnA-mediated effects on NFAT and MEF2 proteins (54). A connection of MEF2 activation with CnA

signalling was established by Wu et al (58) who proposed that MEF2 serves as a nodal point in the molecular signaling pathway by which motor nerve activity controls distinctive programs of gene expression in myofibers.

Additional, entirely distinct mechanisms may drive slow fiber specific expression in other genes. Recent data suggest a role for GTF3 (General Transcription Factor 3) as a regulator of slow TnI expression during early stages of muscle development. GTF3 expression is neither muscle nor fiber type specific. Calvo et al (15) showed that transcription from TnI SURE is repressed by GTF3 when overexpressed in electroporated adult soleus muscles. Hardeman et al (55) present data supporting a role for hMusTRD1α1 (a human homologue of GTF3) as a repressor of slow fiber specific genes in fast fibers by preventing MEF2-mediated transcriptional activation.

In contrast to slow fiber type determination, very little is known about the molecular mechanisms which operate to establish and maintain the fast fiber phenotype in adult skeletal muscle. The first evidence of a transcriptional pathway controlling the fast twitch glycolytic phenotype of adult skeletal muscle was recently reported by Grifone et al (56) who showed that transcription factor Six1 and its partner Eya1 are enriched in the nuclei of fast-twitch fibers and that forced expression of these proteins in slow twitch muscle can activate genes of the fast contractile apparatus. Their data indicate that Six1 and Eya1 are able to act in a synergistic fashion to drive the transformation of slow-twitch oxidative

fibers toward a fast-twitch glycolytic phenotype even in the presence of persistent slow motoneuron innervation.

The Six protein family of homeodomain transcription factors bind to MEF3 sites which include a CAGG box and MEF3/CAGG sites are important in adult muscle expression from the aldolase pM enhancer/promoter in transgenic mice (53). In this respect the TnIfast IRE enhancer in which the CAGG element appear to be unnecessary differs from the aldolase pM enhancer/promoter. The only known cis elements in the 49 bp segment of the IRE that my results show is enough to confer fast fiber type preferential expression are MEF2 and CCAC box. Interestingly, MEF2 and CCAC have been implicated in slow fiber gene expression by Esser et al (1999) who found that the CCAC site and the MEF2 site were both necessary for proper activation of the MLC2 (myosin light chain 2) slow promoter (57). In addition, these elements are frequently found in enhancers for muscle genes not expressed in a fiber type specific fashion, such as the α-actin (59, 60) and MRF-4 (62) genes, so their functional capabilities have not yet been completely defined.

We have not yet identified the fast fiber specific regulatory element in the IRE but my work narrows down the region of the IRE that is enough to drive fast fiber type specificity to 49 bp (from 668 to 714), a segment that contains both MEF2 and CCAC sites.

According to the studies presented above MEF2 and CCAC have been implicated in other muscle genes muscle-specific transcription and thus these sites make intersting mutational targets for any future study on the IRE.

REFERENCES

1. Pette D., Staron R. S. 1990. Cellular and molecular diversities of mammalian skeletal muscle fibers. Physiol. Biochem. Pharmacol. 116: 1-76.

2. Pette D., Staron R. S. 1997. Mammalian skeletal muscle fiber transitions. Int Rev of Cytology 170: 143-223.

3. Henning R., Lomo T. 1985. Firing patterns of motor units in normal rats. Nature 314: 164-66.

4. Schiaffino S., Reggiani C. 1996. Molecular diversity of myofibrillar proteins: gene regulation and functional significance. Physiological Reviews 76: 371-423.

5. Buonanno A, Rosenthal N. 1996. Transcriptional control of muscle plasticity. Dev Genet. 19: 95-107.

6. Burke R. E., Engel A. G., Franzini-Armestrong C. The structure and function of motor units. Myology basic and clinical, third edition, volume 1, McGraw-Hill. Toronto, 2004 p 104-118.

7. Sambrook J., Frisch E. F., Maniatis T. 1989. Molecular cloning a laboratory manual. Second edition. Book 1. Cold Spring Harbor Laboratory Press.

8. Miller J. B. 1991. Myoblasts, myosins, MyoDs, and the diversification of muscle fibers. Neuromuscular Disorders 1: 7-17.

9. Schiaffino S., Murgia M., Serrano A. L., Calabria E., Pallafacchina G. 1999. How is muscle phenotype controlled by nerve activity. Ital J Neural Sci 20: 409-412.

10. Buonanno A, Fields D. R. 1999. Gene regulation by patterned electrical activity during neural and skeletal muscle development . Current Opinion in Neurobiology, 9: 110-120.

11. Kelly R, Alonso S, Tajbakhsh S, Cossu G, Buckingham. 1995. Myosin light chain 3F regulatory sequences confer regionalized cardiac and skeletal muscle expression in transgenic mice. J Cell Biol 129: 383-96.

12. Donoghue M. J, Alvarez J. D, Merlie J. P, Sanes J. R. 1991. Fiber type and position-dependant expression of a myosin light chain-CAT transgene detected with a novel histochemical stain for CAT. J Cell Biol 115: 423-34.

13. Spitz F, Demeurie J, Sabourin J. C, Kahan A, Daegelen D, Marie P. 1998. A binding site for nuclear receptors is required for the differential expression of the aldolase A fast-twitch muscle promoter in body and head muscles. J Biol Chem 273: 561-67.

14. Spitz F, Vasconelos Z. A, Chatelet F, Demignon J, Kahn A, Mira J. C., Daegelen D. 1998. Proximal sequences of the aldolase A fast muscle specific promoter direct nerve and activity-dependent expression in transgenic mice. J Biol Chem 273: 14975-81.

15. Calvo S, Vullhorst D, Venpally P, Cheng J, Buonnano A. 2001. Molecular dissection of DNA sequences and factors involved in slow muscle-specific transcription. Mol Cell Biol 21: 8490-8503.

16. Calvo S, Venpally P, Cheng J, Buonnano A. 1999. Fiber type specific expression of the troponin I slow gene is regulated by multiple elements. Mol Cell Biol. 19: 515-25.

17. Nakayama M, Staffer J, Banerjee-Basu S, Buonanno A. 1996. Common core sequence are found in skeletal muscle slow-and fast fiber-type specific regulatory element. Mol Cell Biol 16: 2408-17.

18. Hallauer P. L, Bradshaw H. L., Hastings K. 1993. Complex fiber-type-specific expression of fast skeletal muscle troponin I gene constructs in transgenic mice. Development 119: 691-701.

19. Hallauer P. L, Hastings K., Peterson A. 1988. Fast skeletal muscle-specific expression of quail troponin I gene in transgenic mice. Mol Cell Biol 8: 5072-5079.

20. Hallauer P. L, Hastings K. 2002. Multistep development regulation during skeletal muscle fiber type differentiation. Devel Dynam 224: 422-31.

21. Hallauer P. L, Hastings K. 2002. Co-regulation of fast contractile protein transgene and glycolytic enzyme expression in mouse skeletal muscle. Cell Physiology 282: 113-24.

22. Corin S. J, Levitt L. K, O'Mahoney J. V, Jova J. E, Hardeman E. C, Wade R. 1995. Delineation of a slow-twitch-myofiber- specific transcription element by using in vivo somatic gene transfer. Proc Natl Acad Sci USA 92: 6185-9.

23. Lin H, Yutzey K. E., Konieczny S. F. 1991. Muscle-specific expression of the troponin I gene requires interactions between helix-loop-helix muscle regulatory factors and ubiquitious transcription factors. Mol Cell Biol 11: 267-280.

24. O'Mahoney J. V, Guven K. L, Lin J, Joya J. E, Robinson C. S, Wade R. P, Hardeman E. C. 1998. Identification of a novel slow muscle-fiber enhancer binding protein, MusTRD1. Mol Cell Biol 18: 6641-52.

25. Wheeler M. T, Snyder E. C, Patterson M. N, Swoap S. J. 1999. An E-box within the MHC11B gene is bound by MyoD and is required for gene expression in fast muscle. Physiol 276: 1069-78.

26. Firulli A. B, Olson E. N. 1997. Modular regulation of muscle gene transcription: a mechanism for muscle cell diversity. Trends in Genetics 13: 364-69.

27. Hughes S. M, Taylor J. M, Tapscott S. J, Gurley C. M, Carter W. J, Peterson C. A. 1993. Selective acumulation of MyoD and Myogenin mRNAs in fast and slow adult skeletal muscle is controlled by innervation and hormones. Development 118: 1137-47.

28. Hughes S. M, Koishi K, Rudnicki M, Maggs A. M. 1997. MyoD protein is differentially accumulated in fast and slow skeletal muscle fibers and required for normal fiber type balance in rodents. Mechanisms of Development 61: 151-63.

29. Farah C. S, Reinach F. C. 1995. The troponin complex and regulation of muscle contraction. FASEB J 9: 755-767.

30. Wilkinson M. J., Grand R. J. A. 1987. Comparison of amino acid sequences of troponin I from different striated muscles. Nature (London) 271: 30-35.

31. Dhoot G. K, Perry S. V. 1979. Distribution of polymorphic forms of troponin components and tropomyosin in skeletal muscle. Nature 278: 714-18.

32. Corin S. J., Levitt L. K, O'Mahoney J. V, Hardeman E. C, Wade R. 1995. Delineation of a slow-twitch-myofiber-specific transcriptional element by using in vivo somatic gene transfer. Proc Natl Acad Sci USA 92: 6185-9.

33. Yutzey K. E, Kline R. L, Konieczny S. E. 1989. An internal regulatory element controls troponin I gene expression. Mol Cell Biol 9: 1397-1405.

34. Hastings K.E.M., Emerson C.P jr. 1982. Proc. Natal. Acad. Sci. USA 79: 1153-1157.

35. Baldwin A., Kitter E., Emerson C. 1985. Developmental Biology 82: 8080-8084.

36. Zakut R., Shani M., Givol D., Newman S., Yaffe D, Nudel U. 1982. Nucleotide sequence of the rat skeletal muscle actin gene. Nature(London) 298: 857-859.

37. Mahdavi V., Chambers A.P, Nadal-Ginard B. 1984. Cardiac α - and ß - myosin heavy chain genes are organized in tandem. Proc. Natl. Acad. Sci. USA 81: 2626-2630.

38. Nabeshima Y., Fujii-Kuriyama, Muramatsu M, Ogata K. 1984. Alternative transcription and two modes of splicing result in two myosin light chains from one gene. Nature (London) 308: 333-338.

39. Konieczny S, Emerson C. 1987. Complex regulatoion of the muscle-specific contractile protein (troponin I) gene. Mol. Cell. Biol. 7: 3065-3075.

40. Banerjee-Basu S, Buonanno A. 1993. Cis-acting sequences of the rat troponin I slow gene confer tissue- and development-specific transcription in cultured muscle cells as well as fiber type specificity in transgenic mice. Mol. Cell. Biol. 13: 7019-702.

41. Parmacek M. S, Ip H. S, Jung F, Shen T, Martin J. F, Vora A. J, Olsen E. N, Leiben J. M. 1994. A novel myogenic regulatory circuit controls slow/cardiac troponin C transcription in skeletal muscle. Mol Cell Biol. 14: 1870-1885.

42. Schreier T, Kedes L, Gahlman R. 1990. Cloning, structural analysis and expression of the human slow-twitch skeletal muscle cardiac troponin C gene. J Biol Chem 265: 21247-21253.

43. Edmondson D. G, Olson E. N. 1989. A gene with homology to the myc similarity region of Myo D1 is expressed during myogenisis and is sufficient to activate the muscle differentiation program. Genes Dev 3: 628-640.

44. Horlick R. A, Benfield P. A. 1989. The upstream muscle specific enhancer of the rat muscle creatine kinase gene is composed of multiple elements. Mol Cell Biol. 9: 2396-2413.

45. Hidaka K, Yamamoto I, Arai Y, Mukai T. 1993. The MEF3 motif is required for MEF2 mediated skeletal muscle-specific induction of the rat aldolase A gene. Mol Cell Biol. 13: 6469-78.

46. Bertrand A., Ngo-Muller V., Hentzen D, Concordet J. P., Daegelen D., Tuil D. 2003. Muscle electrotransfer as a tool for studying muscle fiber-specific and nerve-dependent activity of promoters. Am J Physiol Cell Physiol 285: C1071-C1081.

47. Yutzey K. E, Konieczny S. F. 1992. Different E-box regulatory sequences are functionally distinct when placed within the context of the troponin I enhancer. Nucleic Acids Research. 20: 5105-5113.

48. Spitz F., Salminen M., Demignon J., Kahn A., Daegelen D, Maire P. 1997. A combination of MEF3 and NFI proteins activates transcription in a subset of fast-twitch muscles. Mol Cell Biol. 17: 656–666.

49. Ohno K., Sadeh M., Blatt I., Brengman J. M., Engel A. G. 2003. E-box mutations in the RAPSN promoter region in eight cases with congenital myasthenic syndrome. Hum. Mol. Genet. 12: 739-748.

50. Yan, Z., Serrano, A. L., Schiaffino, S., Bassel-duby. 2001. Regulatory elements governing transcription in specialized myofiber subtypes. J Biol Chem 276: 17361-17366.

51. Spangenburg E. E, Booth F. W. 2003. Molecular regulation of individual skeletal muscle fiber types. Acta Phisiol Scand 178: 413-424.

52. Wheeler M. T., Snyder E. C., Patterson M. N, Swoap S. J. 1999. An E-box within the MHC IIb gene is bound by MyoD and is required for gene expression in fast muscle. Am J Physiol Cell Physiol. 276: C1069-C1078.

53. Salminen M., López S., Maire P., Kahn A., Daegelen D. 1996. Fast-muscle-specific DNA-protein interactions occurring in vivo at the human aldolase A pM promoter are necessary for correct promoter activity in transgenic mice. Mol Cell Biol. 16: 76–85.

54. Chin E. R., Olson E.N., Richardson J. A. 1998. A calcineurin-dependent ranscriptional pathway controls skeletal muscle fiber type. Genes& Development 12: 1780-1786.

55. Polly P., Haddadi L. M., Hardeman E. C. 2003. hMusTRD1α1 represses MEF2 activation of the troponin I slow enhancer. J Biol Chem 278: 36603-10.

56. Grifone R, Lacelf C, Spitz F, Lopez S, Concordet J. P, Maire P. 2004. Six1 and Eya1 expression can reprogram adult muscle from the slow-twitch phenotype into the fast-twitch phenotype. Mol Cell Biol, 24: 6253-6267.

57. Esser K, Nelson T, Lupa-Kimball V, Blough E. 1999. The CCAC box and the myocyte enhancer factor-2 sites within the myosin light chain 2 slow promoter cooperate in regulating nerve-specific transcription in skeletal muscle. J Biol Chem 274: 12 095-12 102.

58. Wu H, Naya F. J, McKinsey T.A. 2000. MEF2 responds to multiple calcium-regulated signals in the control of skeletal muscle fiber type. EMBO J 19: 1963-1973.

59. Biesiada E., Hamamori Y, Kedes L, Sartorelli V. 1999. Myogenic basic helix-loop-helix proteins and Sp1 interact as components of a multi-protein transcriptional complex required for activity of the human cardiac alpha-actin promoter. Mol. Cell. Biol. 19: 2577-2584.

60. Sartorelli V., Webster K. A, Kedes L. 1990. Muscle-specific expression of the cardiac alpha-actin gene requires MyoD1, CArG-box binding factor, and Sp1. Genes Dev. 4: 1811-1822.

61. Amacher S. L, Buskin J. N, Hauschka S. D. 1993. Multiple regulatory elements contribute differentially to muscle creatine kinase enhancer activity in skeletal and cardiac muscle. Mol. Cell.Biol. 13: 2753-2764.

62. Naidu P. S, Ludolph D. C, To R. Q, Hinterberger T. J, Koniecezny S. F. 1995. Myogenin and MEF2 function synergistically to activate the MRF4 promoter during myogenises. Mol. Cell. Biol. 15: 2707-2718.

63. Condon K., Silberstein L., Blau H. M., Thompson W. J. 1990. Differentiation of fiber types in aneural musculature of the prenatal rat hindlimb. Developmental Biology 138: 275-295.

64. Condon K., Silberstein L, Blau H. M., Thompson W. J. 1990. Development of muscle fiber types in the prenatal rat hindlimb. Developmental biology 138: 256-274.

65. Matsumoto F., Makino K,. Maeda K., Patzelt H., Fujiwara S. 2004. Conformational changes of troponin C within the thin filaments detected by neutron scattering. J. Mol. Biol. 342: 1209-1221.

66. Kumar A. 2003. Fast skeletal muscle fiber-type-specificity of the troponin I (fast) gene IRE enhancer resides in a 30 base-pair region. M.Sc. thesis, Dept. of Biology, McGill University.

67. Schiaffino S., Gorza L., Sartore S., Saggin L., Ausoni S., Vianello M., Gundersen K., Lomo T. (1989). Three myosin heavy chain isoforms in type 2 skeletal muscle fibres. J. Musc. Res. Cell Motil. 10: 197-205.

68. Sanes J. R., Rubenstein J. L. R., Nicholas J. F. (1986). Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. EMBO J. 5: 3133-3142.