In compliance with the
Canadian Privacy Legislation
some supporting forms
may have been removed from
this dissertation.

While these forms may be included
in the document page count,
their removal does not represent
any loss of content from the dissertation.

Fast skeletal muscle fiber-type-specificity of the troponin I (fast) gene IRE enhancer resides in a 30 base-pair region

Angela Kumar

Department of Biology

McGill University

February 2003

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the M.Sc. degree.

Your file Votre référence
ISBN: 0-612-88235-7
Our file Notre référence
ISBN: 0-612-88235-7

Fast skeletal muscle fiber-type-specificity of the troponin I (fast) gene IRE enhancer resides in a 30 base-pair region

Abstract

Troponin I (TnI), like many striated muscle contractile proteins, consists of multiple isoforms encoded by a multigene family whose members are differentially expressed in the different striated muscle cell types. Two TnI genes, TnIfast and TnIslow, are expressed in skeletal muscle the former in fast muscle fibers, the latter in slow fibers. The tissue- and fiber-type-specificities of theTnI fast and slow genes are driven by transcriptional enhancer elements: a Slow Upstream Regulatory Element (SURE) upstream of the TnIslow gene and a fast Intronic Regulatory Element (IRE) within the first intron of the TnIfast gene. Within the 144 bp IRE, there are 4 known cis elements, and the aim of this work was to initiate the studies to map the element(s) that are chiefly responsible for directing the fast-fiber-specificity of IRE-driven gene expression. This was approached by making IRE end-deletion constructs lacking either the left-most or right-most IRE cis-element. These IRE derivatives were coupled to a reporter gene consisting of a minimal (enhancer-dependent) TnIfast promoter linked to *E. coli* β-galactosidase coding sequences. The transcriptional activity of these constructs was first evaluated in cell culture transfection experiments, and then by *in vivo* gene transfer into adult mouse skeletal muscles. The conclusion of these experiments was that fast-fiber-specificity of IRE-driven gene expression resides in the left-most 30bp of the IRE., a region including an E-box binding site for myogenic regulatory factors of the MyoD family.

La spécificité du gène troponin I (rapide) pour le type de fibre du muscle squelettique est localisé dans une région de 30 paires de bases de l'enhancer IRE.

Résumé

Troponin I (TnI), comme beaucoup de protéines contractiles des muscles striés, consiste en isoformes multiples codés par une famille de gènes multiples dont les membres sont différentiellement exprimés dans les différents types de cellules du muscle strié. Deux gènes TnI, TnIfast et TnIslow, sont exprimés dans les fibres musculaires squelettiques rapides et lentes, respectivement. Les gènes TnIfast et TnIslow sont activés transcriptionellement par des enhancers responsables de leur spécificité pour un type de fibre: le "Slow Upstream Regulatory Element" (SURE) qui se trouve en amont du gène TnIslow, et le "fast Intronic Regulatory Element" (IRE), dans le premier intron du gène TnIfast. Dans les 144 pb du IRE, se trouvent 4 éléments-cis connus et le but de ce travail était d'amorcer les études pour dresser la carte de l'élément(s) qui est principalement responsable de diriger la spécificité de l'expression du gène dans les fibres rapides. Pour ceci nous avons mis au point des versions du IRE tronquées de l'élément-cis de l'extrémité droite ou gauche, respectivement. Ces dérivés du IRE ont été couplés à un gène signal consistant en un promoteur TnIfast minimal lié avec le gène ß-galactosidase d'*E. coli* . L'activité transcriptionnelle de ces construits a été d'abord évalué à l'aide d'expériences de transfection chez des cellules en culture et ensuite par transfert de gène *in vivo* dans le muscle squelettique de souris adultes. La conclusion de ces expériences était que la spécificité des fibres rapides réside dans l'extrêmité gauche du IRE, dans une

région de 30pb qui comprend un site "E-box" nécessaire pour les facteurs de régulation myogéniques de la famille MyoD.

Acknowledgements

I would like to sincerely thank Dr Ken Hastings, my MSc thesis supervisor for his dedication in guiding me throughout the course of my research, for advising me on the course of my experiments and for his invaluable assistance in the writing of this thesis. I would like to also thank Dr Patricia Hallauer for sharing with me her expertise in molecular biology research techniques, for assisting me in the execution of direct gene transfer experiments in mice, and in the data retrieval and analysis for these experiments, without which the timely completion of this thesis would not have been possible. Thanks also to Brendan Battersby for his advice on PCR technique and to Claude Guerin for his help in cell culture experiments. I also thank Dr Renald Gilbert and Steve Prescott for teaching me the novel direct gene transfer with electroporation technique. I would also like to thank Balthazar Lauzon and Florin Sasarman for editing the French translation of the Abstract for this thesis.

List of Tables and Figures

Table/Figure **Page**

Table of Contents

Abbreviations

ATP.....Adenosine triphosphate
β-gal.....beta galactosidase
bHLH.....basic helix-loop-helix
bp.....basepairs
CAT.....chloramphenicol acetyl transferase
DNA.....deoxyribonucleic acid
DMEM.....Dulbecco's Modified Eagle Medium
EDTA.....ethylenediaminetetraacetic acid
h.....hour
HBS..... Ham's HEPES buffered saline
IRE.....Intronic Regulatory Element
Kb.....kilobasepairs
L.....liter
MCK.....Muscle Creatine Kinase
MEF2..... Myocyte-specific enhancer factor 2
MHC.....myosin heavy chain
mg.....milligram
ml.....milliliter
mM.....millimolar
MNI.....Montreal Neurological Institute
MRF.....myogenic regulatory factors
mRNA.....messenger ribonucleic acid
OD.....optical density
ONPG.....ortho-nitrophenyl-β-D-galactopyranoside
PBS..... phosphate buffered saline
PCR.....polymerase chain reaction
TnI.....troponin I
Ca++.....Ca++
sec.....second
SURE.....slow upstream regulatory element
TE..... tris-EDTA
µl.....microliter
µm.....micrometer
U.....unit
V.....volt
Vmax.....maximum velocity

I INTRODUCTION

Skeletal muscle fiber type

Skeletal muscle fibers fall into several biochemically and physiologically distinct classes, or fiber types. Slow (type I) fibers contract relatively slowly and are specialized for oxidative energy metabolism and are used mostly for postural tone. Fast (type II) muscle fibers have faster contraction speeds and higher levels of glycolytic enzymes than do slow fibers and are used in active behaviour. Three distinct, metabolically specialized adult fast fiber types exist: IIA, IIX, IIB (1) ranked in order of increasing glycolytic capacity and contractile speed. There is a relationship between on the one hand, contraction speed and metabolic specialization, and on the other hand activity levels; IIB fibers are used infrequently, IIA more frequently, and slow oxidative (I) more frequently

still (2).

Each fiber type contains a distinct myosin heavy chain (MHC) isoform (1): MHCI, MHCIIA, MHCIIX and MHCIIB (3). The globular head of each myosin heavy chain molecule contains a catalytic site for ATP hydrolysis. The V_{max} rate of myosin ATPase activity is different for each MHC isoform, and this accounts for the differing contractile speeds of each fiber type (4). Many other contractile proteins also consist of multiple fiber-type-specific isoforms, although in most cases fewer isoforms exist, e.g. one fast isoform expressed in all fiber types, one slow isoform and in some cases, a distinct cardiac isoform (1,4). Thus, differential expression of genes encoding contractile protein gene family members is an important aspect of muscle fiber type specialization.

Skeletal muscle differentiation

Skeletal muscle fibers are multinucleate syncitia formed by the fusion of uninucleate myoblasts. Muscle-specific contractile protein gene expression is activated during myoblast fusion (3). In mammals, muscle fiber formation occurs in two developmental waves termed primary and secondary. Fast and slow adult fiber types originate from both primary and secondary fibers, although primary muscle fibers have a strong predilection to mature into slow fibers, and secondary fibers tend to mature into fast fibers (3).

Innervation plays an important role in determining whether a fiber develops as a fast or slow type. A motor unit consists of a motor neuron and all of the muscle fibers (tens to hundreds) it innervates. It has been found that all fibers in a given motor unit are of the same fiber type (5). Slow fiber development may depend on innervation, whereas in the absence of innervation, fast myofibrillar protein expression seems to be "the default program." (3). Cross-reinnervation of a fast-twitch muscle with a slow nerve or a slow-twitch muscle with a fast nerve can make the fast muscle slower or the slow muscle faster, by altering fiber-type-specific gene expression profiles (3).

Some aspects of muscle differentiation can be studied in a simplified cell culture setting. Undifferentiated myoblasts can be isolated from embryonic or perinatal muscle and be induced to differentiate by forced withdrawal from the cell cycle. In myoblast cell culture this is achieved by replacing high-serum growth medium with low-serum fusion medium.The myoblasts then stop growing and start fusing and differentiating into multinucleate myofibers. Several continuous myoblast cell lines have been generated that are capable of myoblast fusion and muscle gene activation upon mitogen depletion (6). Muscle cell culture is thus a useful system for studies of gene regulation during myoblast differentiation.

Muscle gene regulation

Most contractile protein genes are regulated transcriptionally (4). 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 sequences drive expression of reporter genes. Cell culture transfection studies are appropriate for identifying cis elements that are important in muscle gene activation during myoblast fusion. However, the muscle cells produced in cell culture are immature myofibers, rather than fully differentiated adult type fast and slow muscle fibers. Thus cell culture transfection studies do not reveal information regarding the possible role of these, or other, elements in fiber-type-specific gene expression. Only intact animals generate mature adult fast and slow muscle fiber types. Gene regulatory studies of fiber type specificity therefore require the use of either transgenic mice or somatic cell direct gene transfer by intramuscular injection of plasmid DNA (6a). Genes that have been studied by direct gene transfer into adult skeletal muscleinclude TnIslow (26, 28) and MHCIIB (28a). Among the muscle genes that have been studied in transgenic mice are the myosin light chain 1/3 (7,8), aldolase A (9-12), TnI slow (13-15) and TnIfast (16-19).

MyoD family of myogenic regulatory factors

The MyoD family of transcription factors plays an important role in myogenic commitment and muscle fiber differentiation. The four members of this family are MyoD, myogenin, myf5 and MRF4 (6). These proteins have in common a basic helix-loop-helix (bHLH) structural motif that allows them to form heterodimers with other proteins having this motif and to bind to DNA (6). Like several other bHLH transcription factor families the DNA sequence element to which MyoD family proteins bind is the E-box, CANNTG (20). Skeletal muscle genes are

activated by direct binding of these factors to the E box, or indirectly by myogenic transcription factors such as MEF2, that are themselves regulated by bHLH proteins (20). Expression of MyoD family proteins is characteristic of, and entirely restricted to, skeletal muscle. MyoD expression is activated prior to myoblast differentiation and ectopic expression studies show that it plays a key role in myoblast determination. MyoD and myf5 are expressed embryonically (20). MRF4 is transiently expressed in early development and later becomes upregulated in differentiated muscle fibers so that eventually it becomes the predominant bHLH protein in adult skeletal muscle (20). Myogenin gene expression is markedly upregulated during myoblast differentiation, and acts as a molecular switch to activate expression of other muscle genes such as contractile protein genes (20). 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 (6). MyoD shows preferential expression in fast glycolytic (IIB) fibers while myogenin is preferentially expressed in slow (I) and fast oxidative/glycolytic (IIA) fibers (21). This suggests the possibility that differential expression of myoD and myogenin contribute to glycolytic versus oxidative fiber type differentiation. Mice lacking MyoD develop all muscle fiber types, although fiber type proportions may be altered in these mice (22). Specifically, some fast muscles show a quantitative reduction in the number of fast IIB fibers, and/or an increase in slow type I and type IIA fibers. Also, the slow soleus muscle shows a loss of slow fibers with an increase in type IIA and type IIX fibers (22). Thus although MyoD is not essential for fiber type differentiation, it may contribute to the process. Moreover, a possible explanation for the development of fast glycolytic fibers in mice lacking MyoD is that upregulation of myf-5 expression occurs and may compensate for the lack of MyoD (23). The observation of a shift in fiber type proportions in mice lacking MyoD, especially the loss of type IIB fibers in fast

muscle suggests that this compensatory role of myf-5 for MyoD is not complete (22).

Troponin I

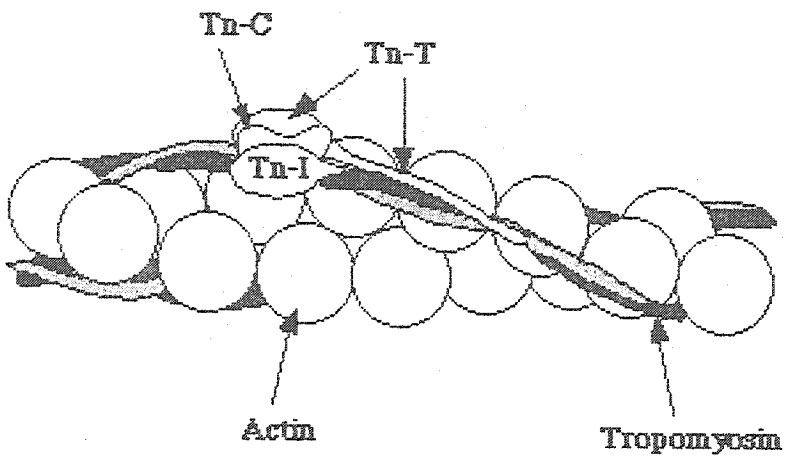
The work described in this thesis is based on a muscle gene encoding the contractile regulatory protein troponin I (TnI). Troponin I as part of the troponin complex (see Fig 1), together with tropomyosin, plays a role in preventing myosin heads from binding actin filaments, thus inhibiting contraction in relaxed muscle. Upon muscle excitation, cytoplasmic Ca++ levels rise and Ca++ binds to troponin C, leading to altered TnI:actin interactions that result in a reversal of the TnI-based inhibition, so that contraction can occur (24).

The TnI gene family has three members: TnIfast, TnIslow and TnIcardiac, which are differentially expressed (25, 25a). TnIcardiac is expressed exclusively in cardiomyocytes. TnIfast and TnIslow are expressed in skeletal muscle, specifically in fast and slow fibers, respectively (25). Recently progress has been made in defining gene regulatory elements that confer fiber-type specificity to TnIslow and TnIfast genes. An upstream enhancer element (SURE) which confers expression to slow-twitch muscles has been identified in the human (26) and rat (15) TnIslow genes. A fast intronic regulatory element (IRE) has been found in the quail TnIfast gene which directs transcription of this gene in fast-twitch muscles (15, 18, 29). These same elements direct transcriptional activation during myoblast differentiation in cell culture transfection studies (14, 27). These slow- and fast-fiber-specific enhancers are about 150 basepairs in length and contain several cis-elements that act as binding sites for distinct transcription factors. Slow fiber specificity of the TnIslow enhancer has been attributed to an element termed USE B1 (28) or BLE (13), but the TnIfast IRE cis-elements that direct fast-fiber-specificity are unknown. A long-range goal of research in our laboratory, to which my project was directed, is to identify the subelements of the TnIfast IRE enhancer that chiefly direct its fast-fiber specificity.

The quail TnIfast IRE was originally identified and characterized by Konieczny and coworkers (29), who showed that this enhancer can drive the transcriptional activation of heterologous promoter/ reporter gene constructs (tk-CAT) in differentiating cultures of transfected myoblasts. The IRE is located within the gene's first intron from bases +634 to +781. Further studies showed the existence of three functionally important cis-elements within the IRE; an E-box sequence (CANNTG) to which muscle-specific transcription factors of the MyoD family bind, and two additional sites, Site I and Site II, which footprinting studies identified as binding sites for ubiquitous, unknown factors. Mutation of the E-box (+650 to +663) reduced the enhancer activity of the IRE in muscle cell culture by 95%. Mutation of Site 1 (+665 to +692) reduced enhancer activity by 80-90%, as did mutation of Site 11 (+700 to +759). This showed that the presence of all three sites is important to ensure full enhancer activity, at least in the in vitro cell culture setting (27). These studies form an important background but they do not directly address the functions of the IRE in adult fast and slow muscle fibers.

The behaviour of quail TnIfast gene constructs in transgenic mice has been studied in the Hastings laboratory. The intact quail TnIfast gene, with 530 bp of 5' flanking DNA, was shown to direct high-level fast-fiber-specific expression of quail TnIfast mRNA in transgenic mouse skeletal muscle (and with no expression in tissues other than skeletal muscle) (17). A β-gal reporter construct including the first exon and intron (including the IRE) and 530 bp of upstream DNA, but in which all TnIfast protein coding sequences were replaced by β-gal encoding DNA, also showed fast-fiber-specific expression (16).

Figure 1. Troponin complex and its interaction with actin filaments. Upon muscle excitation, cytosolic Ca^{++} levels rise, and Ca^{++} 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.


Tn-C
Tn-T
Tn-I
Actin
Tropomyosin

Moreover constructs containing 3 copies of a 150 bp segment of the first intron containing the IRE, linked to a heterologous promoter/ reporter gene (herpes virus tk/B-gal) also showed fast-fiber-specific expression (18). Thus DNA cis-elements that direct fast-fiber-specific gene expression reside within the IRE.

In work done in the Buonanno, Wade and Hardeman laboratories, deletion analysis of the rat and human TnIslow genes in transgenic mice showed that sequences residing from –500 to –1900 are necessary for expression in slow muscle (26, 28, 30). Upon sequencing, a 128 bp sequence was discovered to be highly homologous to the human TnI slow gene, and addition of this 128bp sequence to the inactive –500 TnIslow promoter was found to confer slow-fiber-type expression. In contrast, addition of the quail TnIfast IRE to the same promoter conferred fast-fiber-enriched transcription in transgenic mice (15).

Interestingly, despite their differing locations (5'- flanking versus intron) and biological specificities, SURE and IRE have been found to share certain common sequences, i.e an E-Box, a MEF2-like site (overlapping with Site I), a CCAC Box (in the centre of Site II), and a novel CAGG conserved sequence (Fig 2). A TnIslow construct harbouring a mutation in this CAGG sequence was shown to completely abolish transcription of the SURE in cell culture and transgenic mice (14).

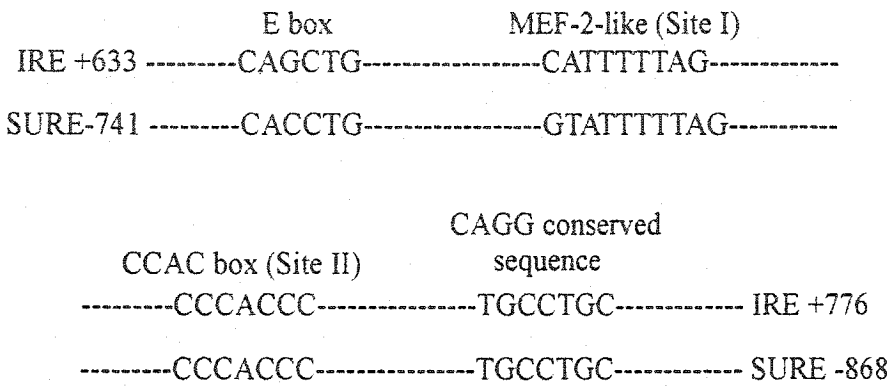
Of the four conserved sequences, the E-Box and MEF2-like sequence have been previously well-described in other muscle genes, whereas relatively little is known about the function of the CCAC Box and the CAGG sequence. E Boxes are binding sites for MyoD family trans factors, and the MEF2 site binds MEF2 factors which are expressed in muscle (although unlike the MyoD family are not absolutely specific for skeletal muscle but are also expressed in neurons (31) and other cell types (32, 33).) It has been shown that MEF2 synergizes with the MyoD family factors in activating muscle gene expression, although the exact mechanism

is unknown (34). The CCAC box has been found in enhancers of the myoglobin, cardiac troponin C (35, 36), myogenin (37), and muscle creatine kinase (MCK) genes (38). This site binds ubiquitous factors including Sp1, and must be present in combination with the MEF2 like sequence as well as the E-Box for high-level expression of the TnIfast gene(27). The function of the CAGG sequence is yet unknown, although similar so-called MEF3 sites have been identified in some muscle promoters (35, 39).

Research Goals

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 cis-elements in the IRE enhancer that are chiefly responsible for fast fiber type specificity. My project was to initiate such studies by preparing IRE end-deletion constructs lacking either the left-most or right-most elements, and characterizing the gene expression capabilities. It was expected that deletion derivatives lacking cis-elements would be reduced in activity compared with wild-type IRE. In order to increase the probability of obtaining detectable expression from such weakened enhancers, we adopted the approach of multimerizing elements. This approach has been shown to increase levels of expression but would not be expected to change patterns of expression (40). We planned to initially characterize enhancer activity in muscle cell culture transfection studies. In principle, direct gene transfer studies in adult muscle could have been envisaged as an approach to characterize gene expression patterns in adult fast and slow muscle fibers. However previous analysis indicated that fast versus slow fiber type specificity of TnI gene constructs was perturbed by muscle fiber regeneration associated with the experimental procedure (19), so that conventional direct gene transfer was not applicable to studies of TnIfast fiber type specificity.

Figure 2. 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. Bases are numbered in relation to the transcriptional start sites. (Note that the CAGG motif reads CCTG on the strand shown).


E box
MEF-2-like (Site I)
IRE +633 ---------CAGCTG----------CATTTTTAG----------
SURE-741 ---------CACCTG----------GTATTTTTAG----------
CAGG conserved
sequence
CCAC box (Site II)
---------CCCACCC----------TGCCTGC---------- IRE +776
---------CCCACCC----------TGCCTGC---------- SURE -868

However, recently, new more efficient techniques of direct gene transfer into adult muscle have been developed based on electroporation. McMahon et al have recently shown that the transduction efficiency of plasmid gene transfer into skeletal muscle can be significantly improved by the application of an electrical field to the muscle following injection of plasmid DNA (30). One of my goals was to assess the applicability of this technique to the study of fast-fiber-type-specific gene expression. My studies showed that indeed IRE-driven constructs showed fast-fiber-specific TnIfast gene expression when introduced into mouse soleus muscle fibers. Moreover, testing the end-deletion constructs revealed that fast-fiber-specificity of the IRE is based on a negative gene regulatory mechanism operating through the left-most 30bp segment of the IRE, a region including the E box.

II MATERIALS and METHODS

Overview

The transcriptional regulatory capabilities of wildtype or end-deleted IRE enhancers, produced by PCR amplification, were assessed by cloning them into plasmids containing the minimal enhancer-dependent promoter of the TnIfast gene linked to a β-gal reporter gene, and transferring the plasmids into cultured cells by transfection or into adult muscle fibers by intramuscular injection/ electroporation, and monitoring reporter β-gal expression by biochemical assay of cell homogenates or by histochemical stain of muscle tissue sections. We produced plasmid constructs containing directly repeated copies of the IRE-derived enhancer to be tested because we expected the end-deleted IRE enhancers to be weakened,

and multimerization was thought to be a way to augment their residual transcriptional regulatory activity.

Plasmid Constructs

Production of TnIfast multimeric enhancer elements via PCR amplification from the quail TnIfast gene

Wildtype or end-deleted IRE enhancers were produced by PCR amplification from the construct TnILacZ1B, which contains 5' upstream sequence of the quail TnIfast gene, as well as exon 1, the IRE-containing intron 1 and part of exon II (Fig 3). Primer pairs were designed to amplify either the entire 144bp IRE, or the right-most 114 bp (the "right ¾" or R3/4 segment) or the left-most 101 bp (the "left ¾" or L3/4 segment). PCR primer design included the introduction of Sal 1 (rightward primers) or Xho 1 (leftward primers) restriction sites at the 5'- ends; these sites were used to create tandem head-to-tail trimers (see Fig 4). Primers were synthesized by Cortec Labs. PCR reactions (50ul final volume) contained 2 µg/ml primers, 1x PCR buffer (Pharmacia Biotech), 1.5mM $MgCl_2$ (MBI) 0.2mM dNTPs (Pharmacia Biotech), 0.2µg/ml TnILacZ1B template DNA and 20U/ml Taq DNA polymerase (Pharmacia Biotech). Reaction mixtures were overlaid with 50ul mineral oil and subjected to 10 cycles of: denaturing at 94°C for 30 secs, annealing at 60°C for 30 secs, and extension at 72°C for 1 min (extended to 12 min in the last cycle) in a Perkin Elmer Cetus thermal cycler. PCR amplified DNA elements were phenol/ chloroform extracted and ethanol precipitated. They were then cut with restriction enzymes Sal I and Xho I to create sticky ends, and re-purified by agarose gel electrophoresis and recovery on Sephaglas (Pharmacia Biotech). These fragments were then ligated to create multimers. In 10µl ligation reaction mixtures

Figure 3. Scheme for PCR amplification of monomer units of end-deletion sequences left3/4 (L3/4), right3/4 (R3/4) and the full-length IRE. The template for PCR amplification was the plasmid TnILacZ1B containing upstream genomic DNA from the quail TnIfast gene including the IRE-containing first intron. PCR primers are identified by number (see Fig 4 for sequences).

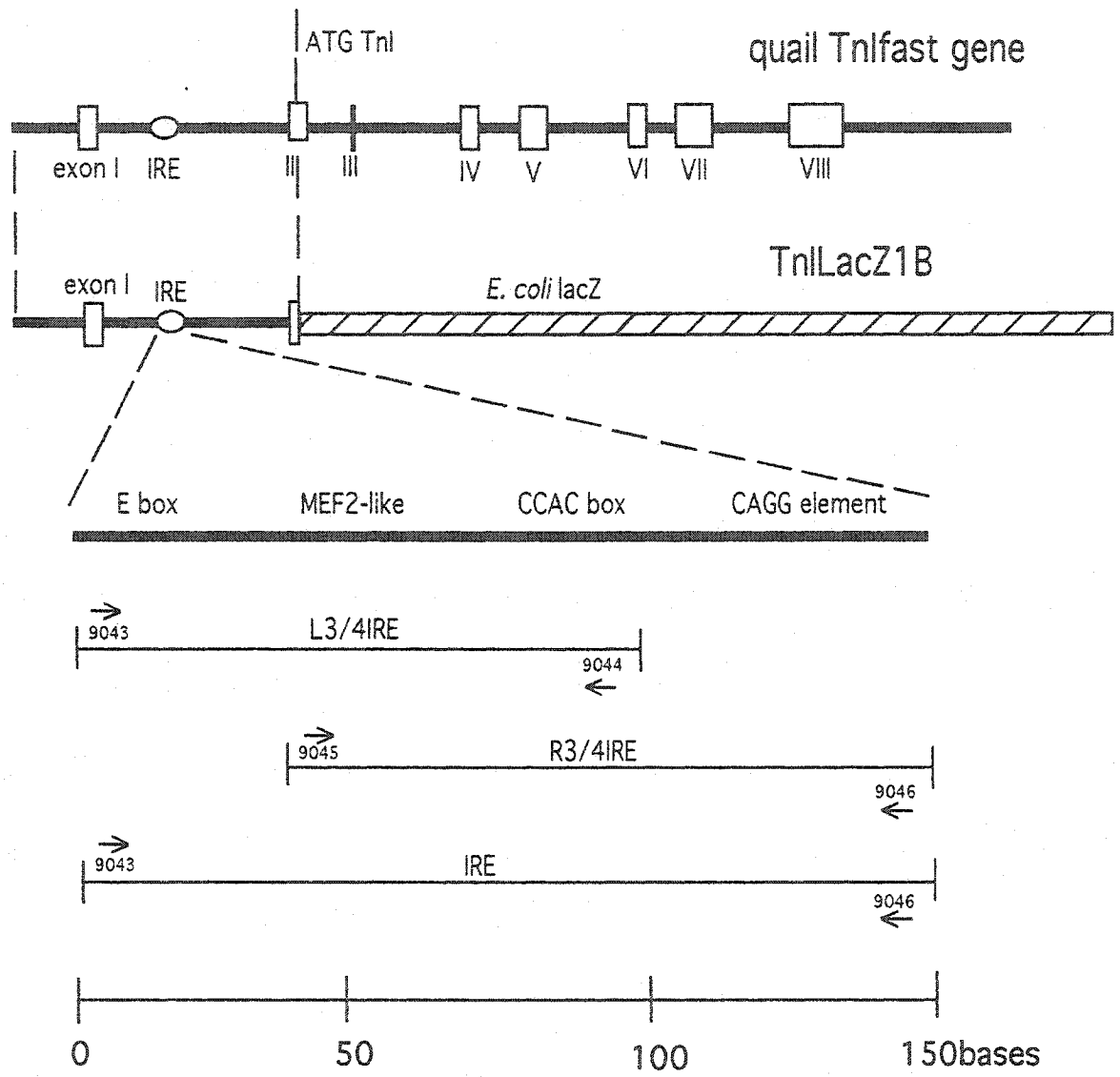

ATG TnI
quail TnIfast gene
exon I
IRE
II
III
IV
V
VI
VII
VIII
TnILacZ1B
exon I
IRE
E. coli lacZ
E box
MEF2-like
CCAC box
CAGG element
9043
L3/4IRE
9044
9045
R3/4IRE
9046
9043
IRE
9046
0
50
100
150bases

Figure 4. PCR primers used to amplify the L3/4 and R3/4 end-deletion fragments and the full IRE, from construct TnILacZ1B. Rightward and leftward refer to the orientation of the IRE as depicted in Figs 2 and 3, i.e., rightward is in the same direction as transcription.

Sal I
5' CTA**GTCGAC**GGCTGCGTCTGAGGAGACA primer 9043 (rightward)

5'CTA**CTCGAG**AACACACATTGCTGGGAAGAG primer 9044 (leftward)
Xho I

Left ¾ primers

Sal I
5' CTC**GTCGAC**TGTGCAGCTCCCCAGCCAT primer 9045 (rightward)

5'CTA**CTCGAG**GCCAAGCTCCCTGAGGAA primer 9046 (leftward)
Xho I

Right ¾ primers

Sal I
5' CTCA**GTCGAC**GGCTGCGTCTGAGGAGA primer 9043 (rightward)

5'CTA**CTCGAG**GCCAAGCTCCCTGAGGAA primer 9046 (leftward)
Xho I

IRE primers

containing ~0.5µg DNA, 1X One Phor All Plus buffer (Pharmacia Biotech), 1mM ATP and 0.2U/µl T4 DNA ligase (MBI), incubated at 16°C overnight. The ligation reaction products were then redigested with Sal I and XhoI to cleave head-to-head and tail-to tail joints. Only tandem head-to-tail repeats, joined by uncuttable Sal I+Xho I joints would survive this digestion (see Fig 5). Products were subjected to gel electrophoresis and three-mer bands (3 x L3/4 IRE, 3 x R3/4 IRE and 3 x IRE) were cut out and recovered with Sephaglas (see Fig 6). Three-mers were chosen because they contained the highest number of repeats obtained in reasonable yields. The three-mer IRE-derived enhancer fragments were then cloned into Sal I - cut vector pBlueScript II SK+. Cloned inserts were completely sequenced on both strands at Mobix Labs, McMaster University, Hamilton , Ontario, Canada using T3 and T7 sequencing primers. In several molecules examined, one of the three IRE element repeats had a single base change, presumably reflecting synthesis errors by Taq DNA polymerase. However for each enhancer construct, i.e., 3xIRE, 3xL3/4 and 3xR3/4, a clone was obtained with exactly the predicted structure and sequence. These sequence-verified clones were used for subsequent work.

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

Our overall strategy was first to clone IRE enhancer elements into the basic vector, and then to introduce the TnIfast promoter/β-gal reporter gene. This strategy permitted dual use of the blue/white selection scheme based on the α complementing fragment of the *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 white insert-containing clones against a background of blue colonies reflecting reconstructed

Figure 5. 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.

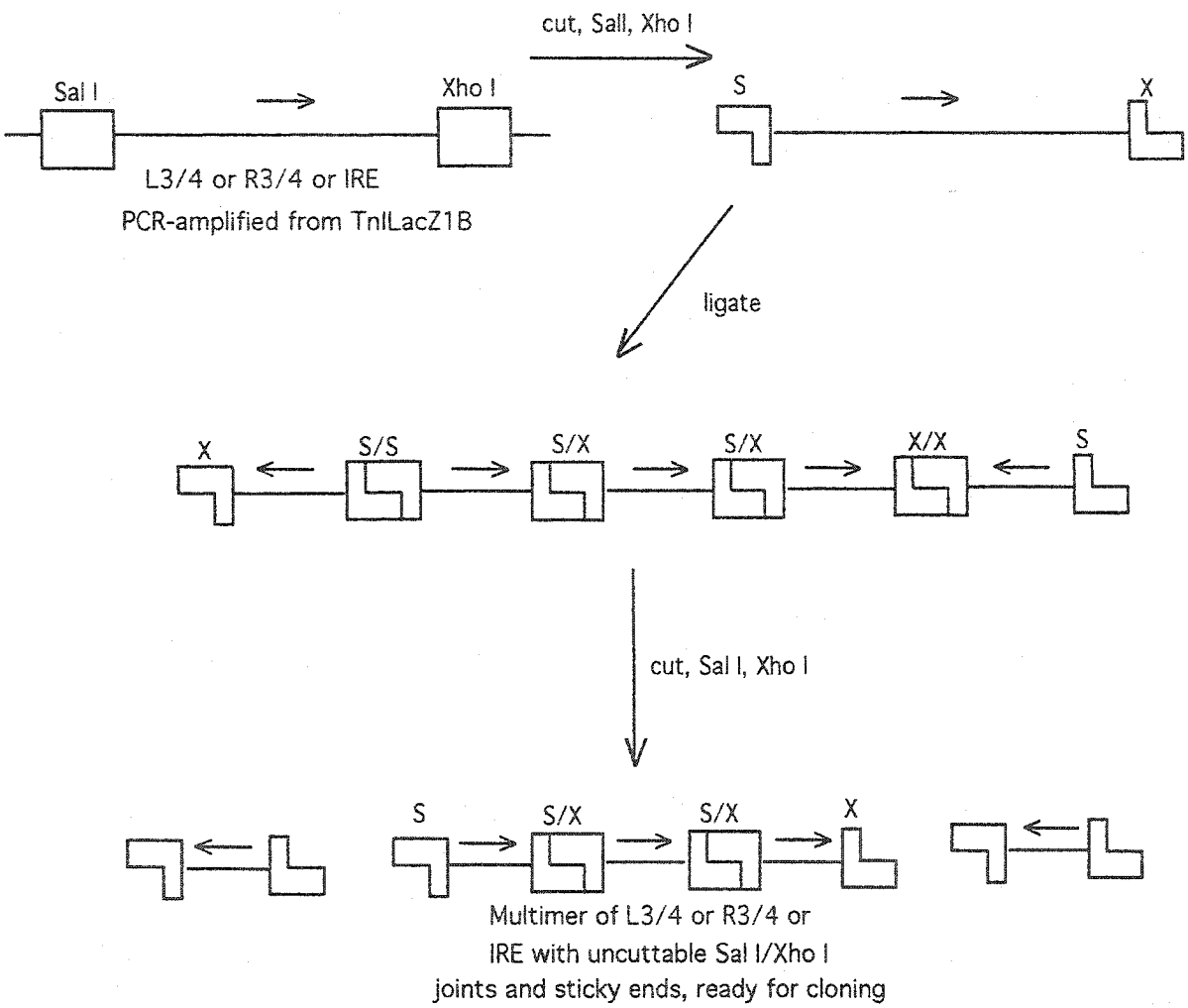
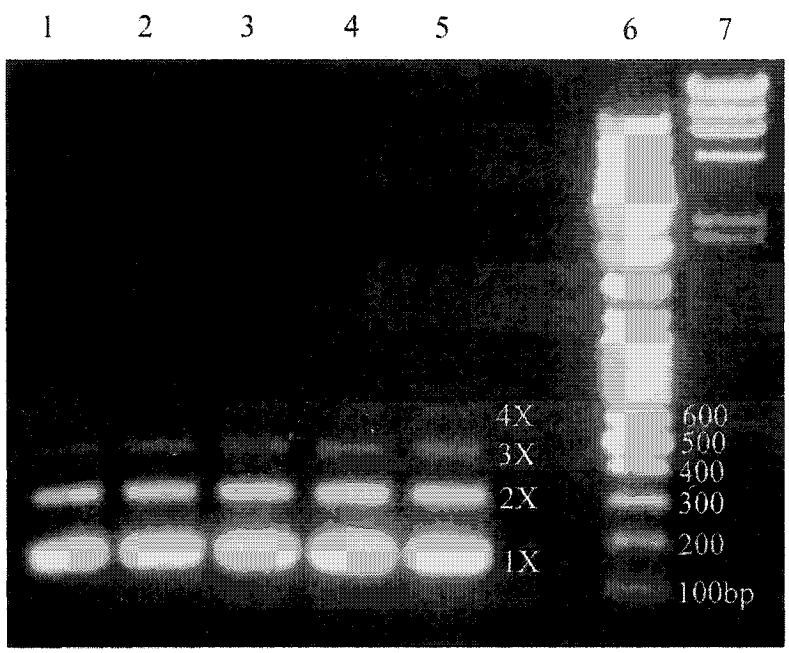

cut, SalI, Xho I
Sal I
Xho I
S
X
L3/4 or R3/4 or IRE
PCR-amplified from TnlLacZ1B
ligate
X
S/S
S/X
S/X
X/X
S
cut, Sal I, Xho I
S
S/X
S/X
X
Multimer of L3/4 or R3/4 or
IRE with uncuttable Sal I/Xho I
joints and sticky ends, ready for cloning

Figure 6. Agarose gel electrophoresis of full-length IRE enhancer element head-to-tail multimers. Monomer PCR products were digested with Sal I and Xho I, multimerized with DNA ligase, and redigested with Sal I and Xho I. Lanes 1-5 contain equal aliquots of this re-digested product. Monomers and head-to-tail multimers of various sizes were present in a high enough concentration to be visualized by ethidium bromide fluorescence under UV light. These are marked 1x, 2x, 3x and 4x. Lane 6 contains DNA size standards which confirm the expected sizes of the multimers, eg 1x=150bp, 2x=300bp, 3x=450bp and 4x=600bp. Lane 7 contains a Hind III restriction digest of phage λ DNA, used for comparative estimation of IRE multimer DNA quantities. The 3x multimer bands were recovered for cloning and eventual production of (-198)IRE and (-530)IRE constructs. L3/4 and R3/4 end-deletion segments were treated similarly, including recovery of 3x head-to-tail multimers for cloning.


1
2
3
4
5
6
7
4X
3X
2X
1X
600
500
400
300
200
100bp

vector. Subsequent insertion of the intact β-gal coding sequence from pRSVZ permitted section of blue clones against a background of white colonies representing enhancer multimer containing plasmids that did not acquire the β-gal reporter construct. Two series of TnIfast promoter/β-gal constructs were prepared based on two TnIfast promoter fragment lengths, long and short. The short TnIfast promoter, (bases -198 to +22 of the TnIfast gene) linked to the β-gal gene (Sma I-EcoRI fragment of pRSVZ) was subcloned from the plasmid gCTnIf (-198:+22)Z (produced by P. Hallauer) into the HindIII and EcoRI sites of the enhancer-containing vectors. Another set of (long promoter-containing) constructs was made by subcloning the long TnIfast promoter (bases -530 to +22 of the TnIfast gene) followed by the β-gal gene (Sma I-EcoRI fragment of pRSVZ) from the plasmid gCTnIf (-530:+22)Z (produced by P. Hallauer) into the HindIII and EcoRI sites of the enhancer-containing vectors. Two enhancerless controls (one with the short TnIfast promoter and one with the long promoter) were also made by subcloning the promoter+β-gal cassettes as above into pBluescript II SK+ vector (not containing enhancer elements). All the β-gal-containing recombinant clones were then sequence verified with T3 and T7 sequencing primers, at the Hind III and EcoRI joints into which the promoter/β-gal cassettes had been cloned, by Mobix Labs, McMaster University, Hamilton , Ontario, Canada.

I found that these plasmids did not give sufficiently high yields of isolated plasmid DNA using the Qiagen Midi Prep procedure. This difficulty was overcome by subcloning each enhancer+promoter+β-gal gene insert into the plasmid vector pSP72. Enhancer+promoter+β-gal gene cassettes were cut out of the pBluescript II SK+ vectors with Xho I and Eco RI and were agarose gel isolated and purified by Sephaglas. The cassettes were then ligated into Xho I and Eco RI– cut and gel-purified pSP72 vector in 10μl reaction mixtures

containing ~30ng each of vector and insert DNA, 1X One Phor All Plus buffer (Pharmacia Biotech), 1mM ATP and 0.2U/μl T4 DNA ligase (MBI) incubated at 16°C overnight. The plasmids gCTnIf (198:+22)Z and gCTnIf (-530:+22)Z could then be used as the new enhancerless controls, as they were already in the pSP72 vector. Plasmid maps are shown in Figures 7-15. Table 1 shows a complete list of the final constructs.

Construct in this thesis	Official laboratory name	Description
Z72	Z72	Enhancerless, promoterless control: lacZ coding sequence in pSP72 vector
(-198)control	gCTnIf(-198:+22)Z	Enhancerless control(-198) TnIfast promoter driving lacZ
(-530)control	gCTnIf(-530:+22)Z	Enhancerless control(-530) TnIfast promoter driving lacZ
(-198)L3/4	gCTnIf(198:+22)Z(3xL3/4IRE) R72	3 x left3/4IRE enhancer plus (-198) TnIfast promoter driving lacZ
(-530)L3/4	gCTnIf(-530:+22)Z(3XL3/4 IRE)R72	3 x left3/4IRE enhancer plus (-530) TnIfast promoter driving lacZ
(-198)R3/4	gCTnIf(-198:+22)Z(3xR3/4 IRE)R72	3 x right3/4IRE enhancer plus (-198) TnIfast promoter driving lacZ
(-530)R3/4	gCTnIf(-530:+22)Z(3xR3/4 IRE)R72	3 x right3/4IRE enhancer plus (-530) TnIfast promoter driving lacZ
(-198)IRE	gCTnIf(-198:+22)Z(3XIRE)R72	3 x IRE enhancer plus (-198) TnIfast promoter driving lacZ
(-530)IRE	gCTnIf(-530:+22)Z(3XIRE)R72	3 x IRE enhancer plus (-530) TnIfast promoter driving lacZ

Table 1. A list of the suite of plasmids created by cloning trimer IRE-derived enhancer fragments upstream of either the (-198) or (-530) TnIfast promoter driving β-gal. All clones are in the vector pSP72.

Figures 7-15. Plasmid DNA construct maps.

Figure 7. Enhancerless, promoterless control Z72

Figure 8. Enhancerless (-198)control

Figure 9. Enhancerless (-530)control

Figure 10. (-198)L3/4

Figure 11. (-530)L3/4

Figure 12. (-198)R3/4

Figure 13. (-530)R3/4

Figure 14. (-198)IRE

Figure 15. (-530)IRE

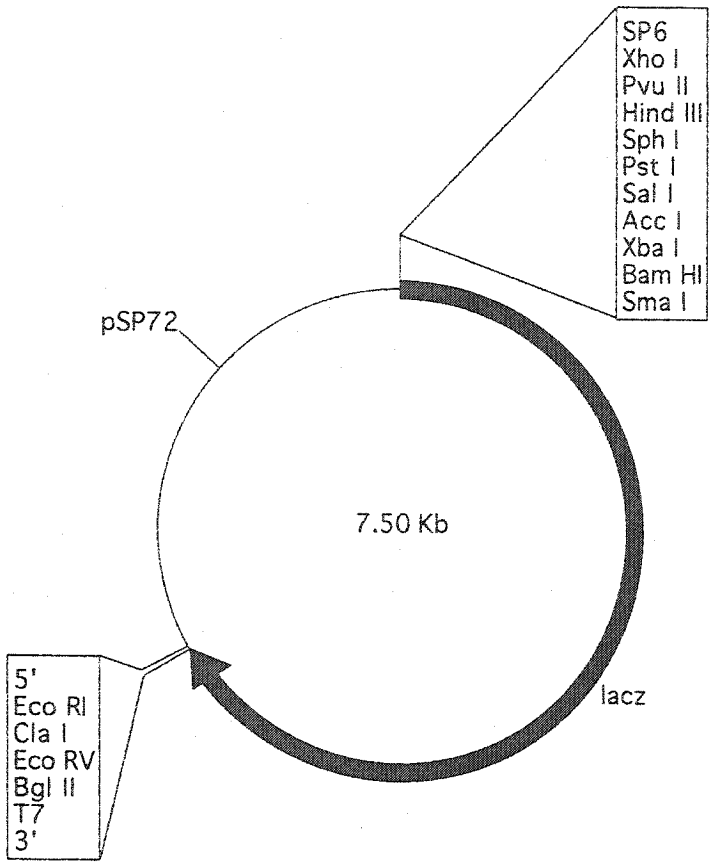

SP6
Xho I
Pvu II
Hind III
Sph I
Pst I
Sal I
Acc I
Xba I
Bam HI
Sma I
pSP72
7.50 Kb
lacz
5'
Eco RI
Cla I
Eco RV
Bgl II
T7
3'

Figure 7

(-198)control

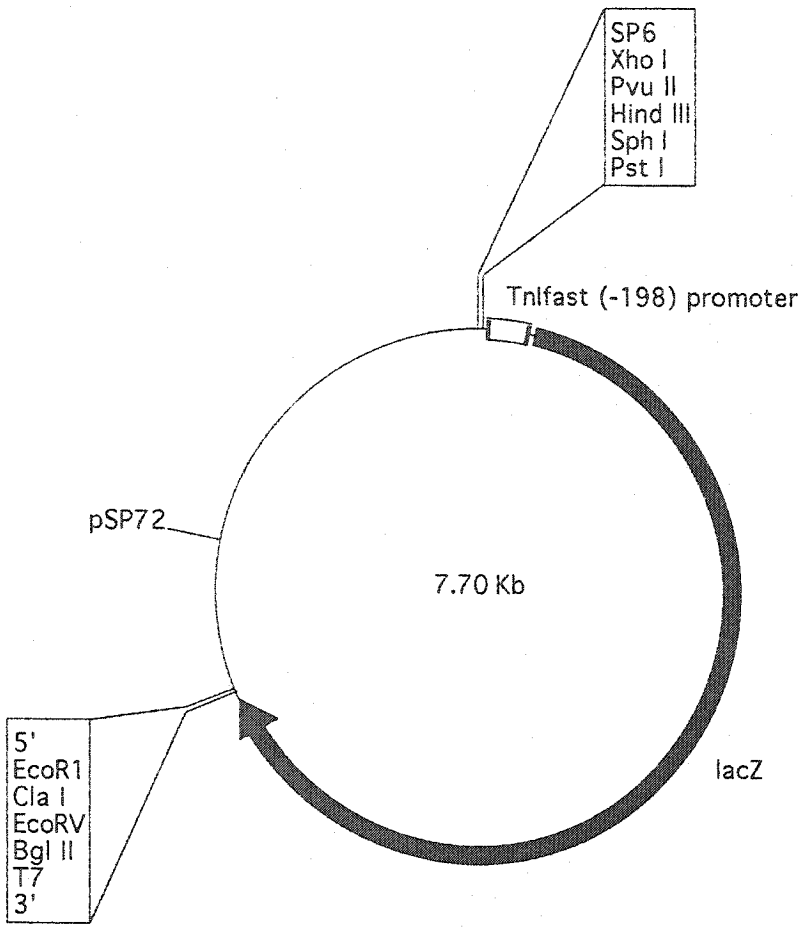


Figure 8

(-530)control

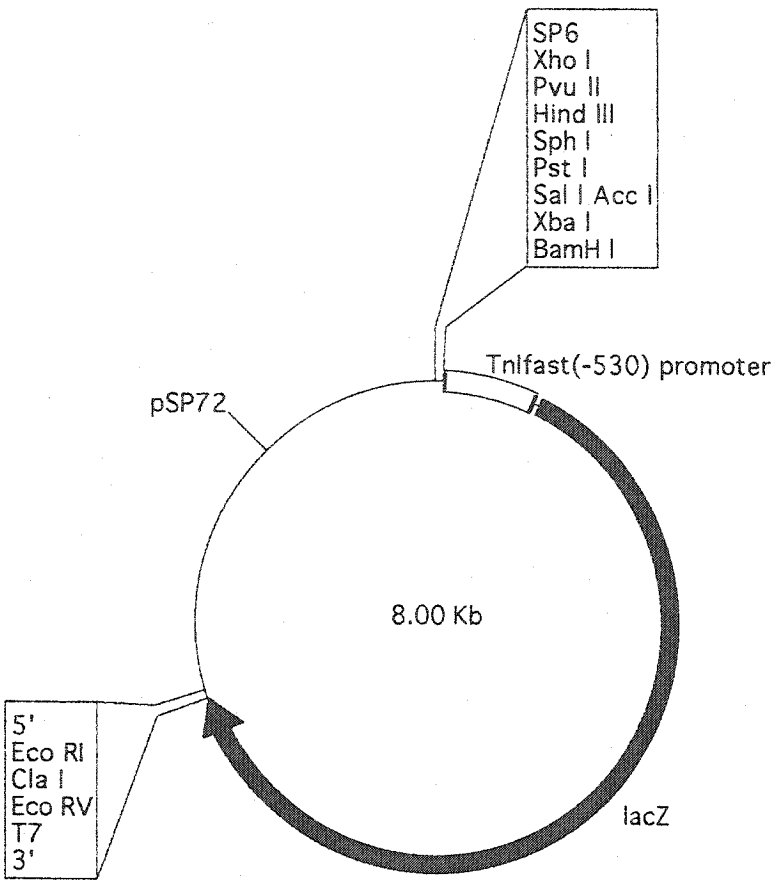


Figure 9

(-198)L3/4

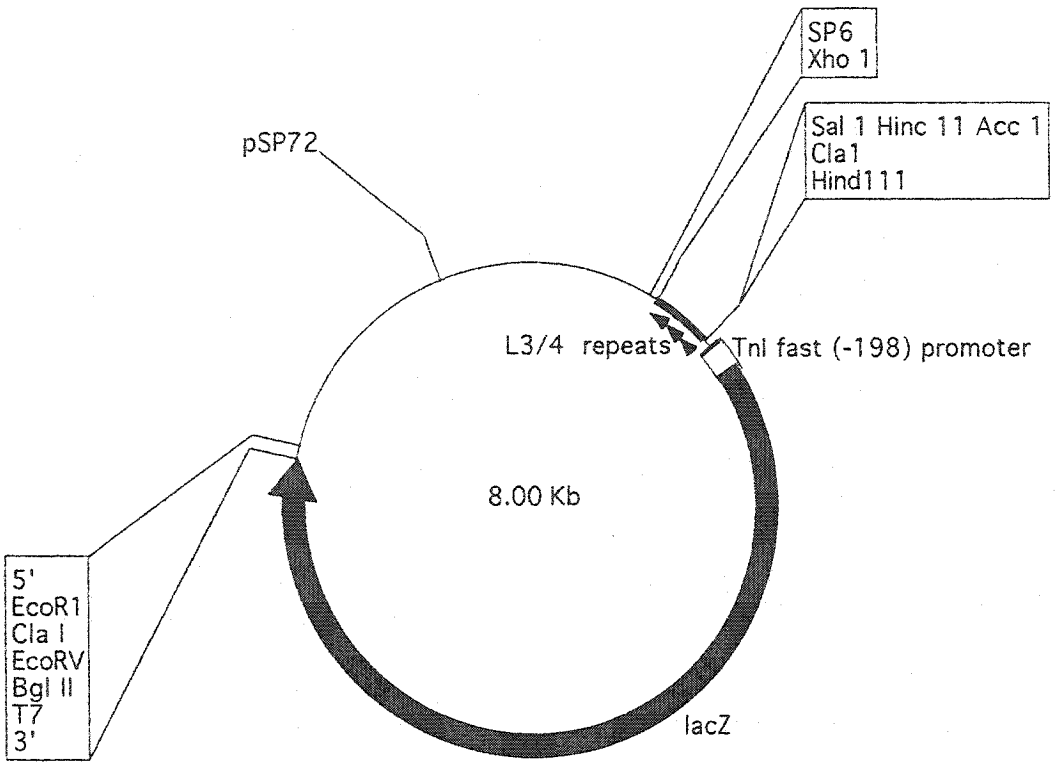


Figure 10

(-530)L3/4

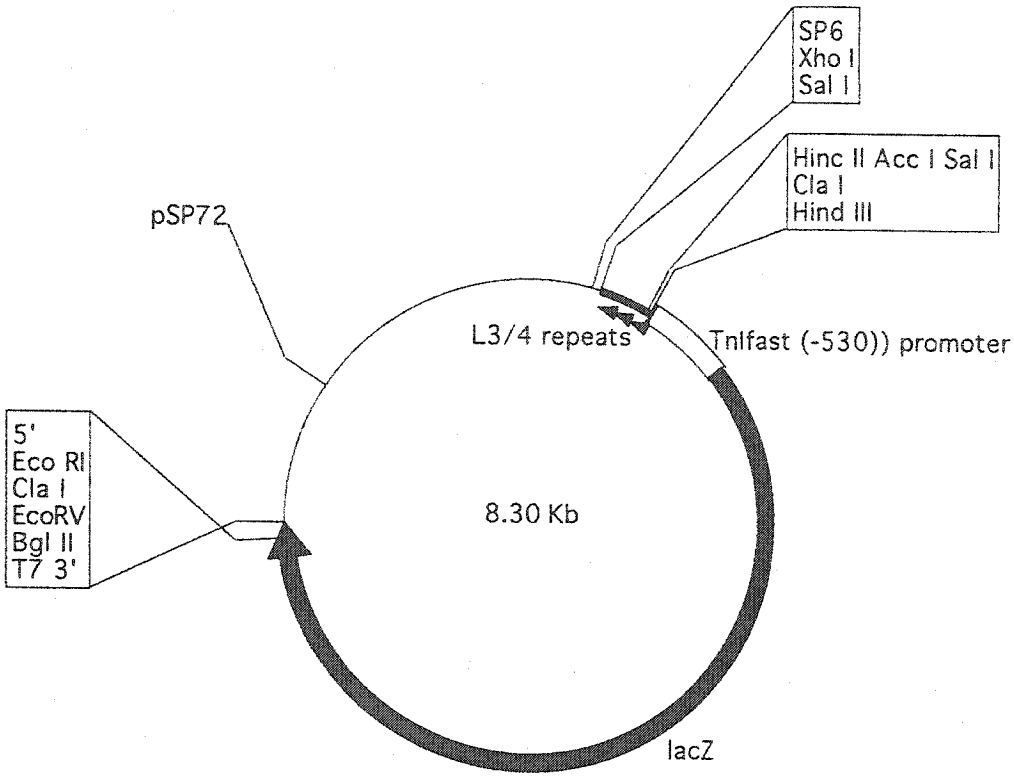


Figure 11

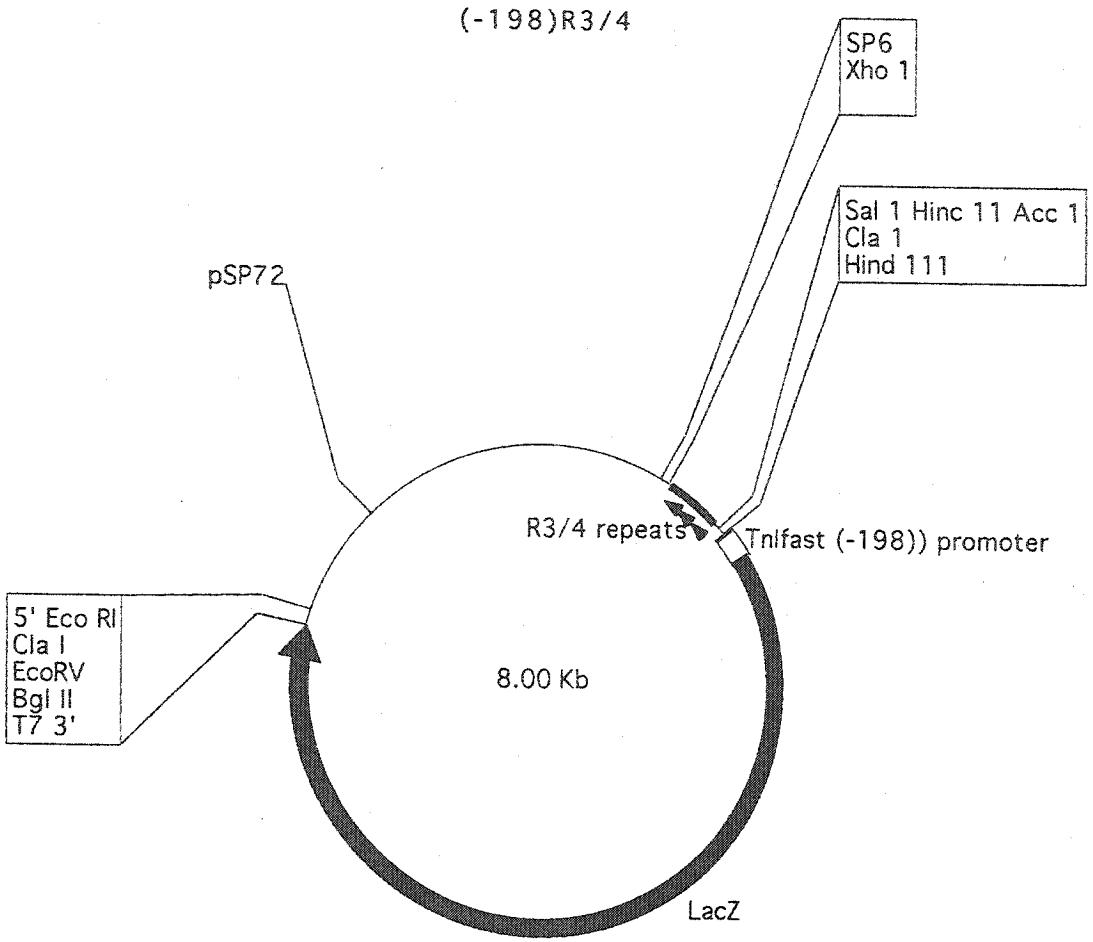

(-198)R3/4
SP6
Xho 1
Sal 1 Hinc 11 Acc 1
Cla 1
Hind 111
pSP72
R3/4 repeats
Tnlfast (-198)) promoter
5' Eco RI
Cla I
EcoRV
Bgl II
T7 3'
8.00 Kb
LacZ

Figure 12

(-530)R3/4

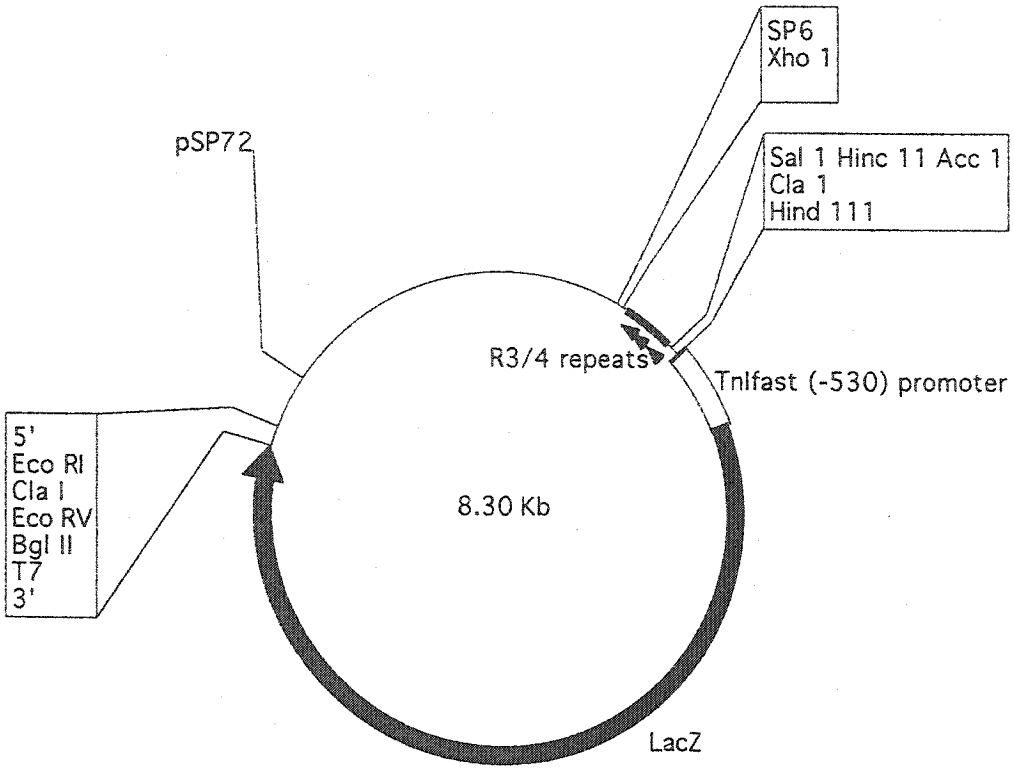


Figure 13

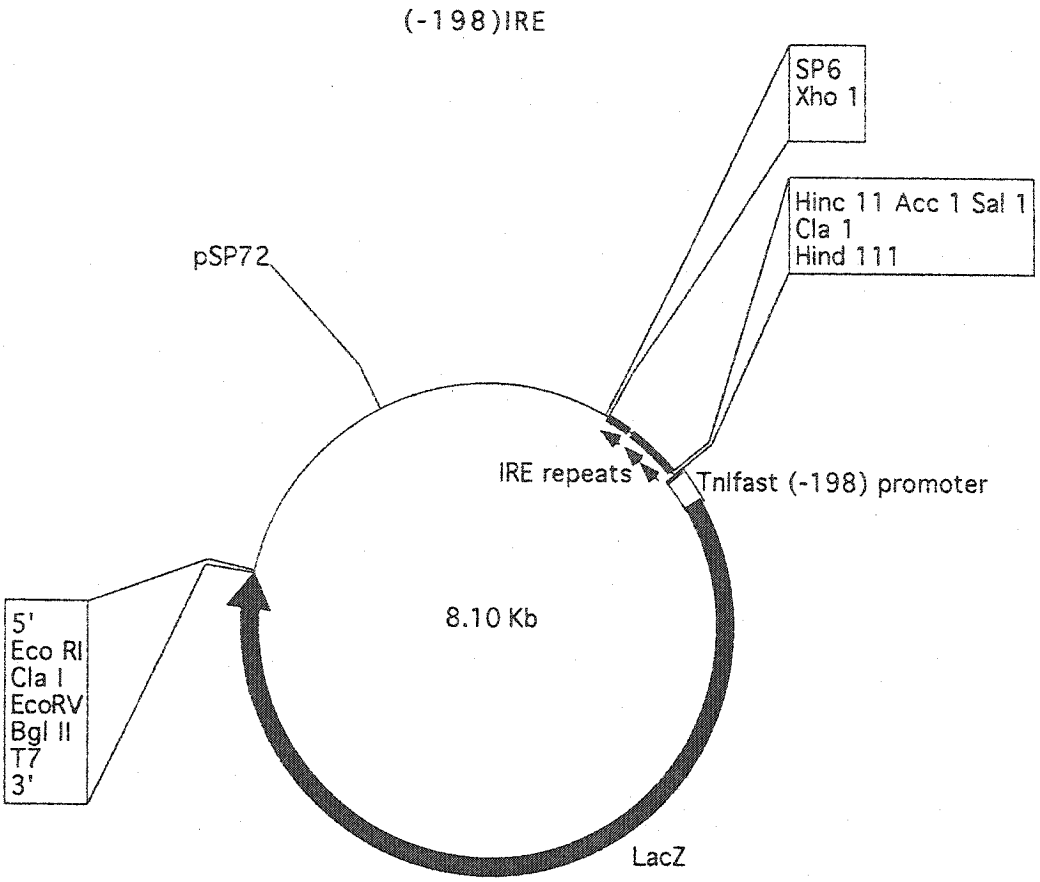

(-198)IRE
SP6
Xho 1
Hinc 11 Acc 1 Sal 1
Cla 1
Hind 111
pSP72
IRE repeats
Tnlfast (-198) promoter
8.10 Kb
5'
Eco RI
Cla I
EcoRV
Bgl II
T7
3'
LacZ

Figure 14

(-530)IRE

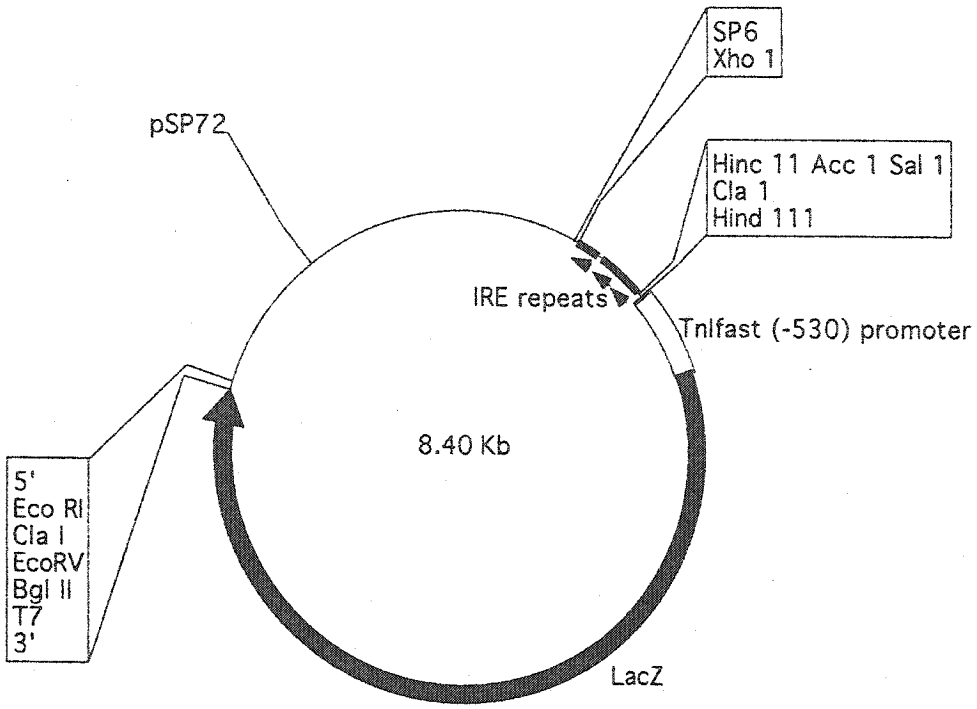


Figure 15

Preparation of large-scale plasmid DNA preps:

DNA preparations for transfection into mammalian cells were made using the Qiagen Midi Prep method. This procedure was started by streaking a (-70°C) glycerol stock of each clone onto an ampicillin agar plate, letting it grow overnight in a 37°C incubator, and then transferring a colony into 100ml of L-broth with ampicillin and letting the culture grow in a shaking incubator overnight at 37°C. Qiagen Endotoxin free Mega preps were made for direct gene transfer into mouse muscle. Glycerol stocks were streaked and cultured as described above except 2.5L of L-broth with ampicillin was used instead of 100ml, and this 2.5L volume of L-broth was inoculated with a 10ml starter culture with ampicillin grown at 37°C in a shaking incubator for 8h.

Mammalian Cell culture

Subculture of L6 rat myoblasts and 10T1/2 fibroblasts. Frozen samples (from liquid nitrogen stocks)were obtained courtesy the Muscle Biochemistry Lab, MNI. They were grown in 10% fetal bovine serum in DMEM (Dulbecco's Modified Eagle Medium,Gibco 11965-092) with antibiotic/ antimycotic (Gibco 15240-096) at 37° C and 5% CO_2 for 3 days in a 100mm tissue culture dish and subcultured as follows (all solutions prewarmed to 37°C): Growth medium was aspirated and cells rinsed with 5 ml Ham's HEPES buffered saline (HBS). Trypsin-EDTA (0.05%, Gibco 15400-054) with 0.02% NaEDTA in HBS (4ml) was added and the plate was left in the CO_2 incubator for 1-5 mins then shaken so the cells would detach from the plate. Growth medium (2ml) was added to inhibit the trypsin. Released cells were centrifuged in a 15ml conical tube at setting 4 on the IEC clinical centrifuge, and resuspended in 4 ml growth medium. Cells were counted on a haemocytometer, and were subcultured by seeding 3 x 10^5 cells per 100mm tissue culture dish. These cells were left to

grow another 3 days and then treated as described above or used to seed 35mm wells (2 x 10^5 cells in 2 ml growth medium) for a transfection experiment the following day.

Freezing and thawing cells. Instead of subculturing, excess cells were frozen in cryotubes at -70°C at a density of 5 to 10 x 10^5 cells / ml growth medium, with 10% dimethyl sulfoxide. Cryotubes were thawed in a 37°C waterbath for 1-3 mins. The contents were transferred to 13 ml prewarmed growth medium in a 50ml conical tube and cells were pelleted by centrifugation at setting 4 on the clinical centrifuge for 5 mins. Cells were resuspended in 10ml growth medium and plated in a 100mm tissue culture dish. The dish was placed in the 37°C/5%CO_2 incubator, left for 3 days and subcultured as described above.

DNA transfection experiments

L6 myofibers and 10T1/2 fibroblasts. Cells were plated in 35mm wells as described above, so that on the day of the transfection the cells were at 50-80% confluence. On occasion, if cell densities were too low, cultures were incubated an additional day. Solutions were kept at room temperature and sterile, except for DNA preps in TE (10mM Tris-HCl buffer, pH 7.5, 1mM EDTA). Plasmid DNA (2µg from ~1mg/ml stock solutions) including the test construct plus the internal pGL3 control (present in a 9:1 ratio respectively), were added to 100µl OptiMem Medium (Gibco 31985-070). Lipofectamine reagent (Gibco 18324-012) diluted to 6% in OptiMem (100µl) was added. This was mixed by pipetting and left to set for 30 mins. The DNA/lipofectamine mixture was diluted by adding 0.8ml OptiMem. This (total of 1ml) was mixed and added to 1 layer of cells that had just previously been rinsed with 2ml OptiMem. The cells were incubated in the 37°C/ 5%CO_2 incubator for 5h and the transfection was stopped by adding 1ml 20% fetal bovine serum in DMEM with 2 x antibiotic/antimycotic. The cells were placed back in the incubator and the next day the medium was changed to 2ml prewarmed fusion medium (2% horse serum in DMEM with

antibiotic/antimycotic) per well and cultures were returned to the incubator for 4 days. During this exposure to fusion medium, L6 cells fused extensively to form multinucleate myofibers, whereas 10T1/2 fibroblast cells did not fuse at all. The cells were harvested by washing each well twice with 2 ml PBS, and scraping in 0.25ml Reporter Lysis Buffer (Promega E397A). The cells were pipetted up and down several times and vortexed. They were then placed in eppendorf tubes and frozen at -20°C for at least 30 mins. The tubes were thawed on ice and centrifuged at 13,200 rpm for 2 min. The supernatant was collected and stored at -20°C until analysis by biochemical assay.

Undifferentiated L6 myoblasts. Cells were plated and transfected exactly as described above, except that on the day following the transfection, instead of adding 2 ml fusion medium, 2 ml prewarmed growth medium with antibiotic/antimycotic was added, and the cells were incubated for a further 2 days instead of 4 days. Microscopic inspection confirmed that, as expected, such myoblast cultures had not fused into muscle fibers. Harvest was as described above.

Biochemical assays

ß-galactosidase assay. Cell lysate duplicate aliquots (50µl) were assayed in 96-well microtiter plates. A β-gal standard curve of 0 to 3mU β-gal was made in the same plate, using β-galactosidase stock (Boehringer Mannheim 105031) diluted in Reporter Lysis Buffer (Promega E397A). The plate was then incubated at 48°C for 50 mins, in order to reduce background endogenous mammalian β-gal (42). One hundred fifty µl volumes of (+) ONPG buffer: [10mls 1M Na_2HPO_4 pH 7.2, 0.1ml 1M $MgCl_2$, 0.35ml β- mercaptoethanol (Fisher Biotech BP176-100), 67mg ONPG (Sigma N1127) and water to 50 mls] or (-) ONPG buffer: (same as (+) buffer but with no ONPG), were added to duplicate lysate samples and also to duplicate standard curve samples. The dish was placed in a 37°C incubator for 30 mins to 3

hrs. Optical densities were read in a microplate reader (BioRad model 450) at 405nm. In order to determine the optical density solely attributable to β-gal activity, (-)ONPG OD values for individual samples were subtracted from their corresponding (+)ONPG values. This eliminated any optical density due simply to the presence of cell lysates. A small additional correction was made to eliminate the optical density contributed by the ONPG per se (and its spontaneous hydrolysis products). The ONPG contribution to optical density was established by comparing +ONPG and –ONPG reactions that contained no cell lysate or added standard β-gal.

Luciferase assay. Cell lysate aliquots (20µl), or 20µl standard curve samples containing 0-500pg recombinant luciferase (Promega E170) diluted in Reporter Lysis Buffer were placed in luminometer tubes (Sarstedt 55.484). To each of the 20µl samples was added 0.2ml of luciferase assay substrate (Promega LuciferaseAssay System E4030) and light emission was quantified in a luminometer (BioOrbit 1250) which had been pre-calibrated to read zero with the "0 pg" standard curve sample, and set to take the light reading within a time of 10 sec.

Direct gene transfer in mice

CD1 mice aged 7-10 weeks were anaesthetized with 0.1ml/10g body weight of 5% chloral hydrate solution injected intraperitoneally. Soleus muscles were exposed by skin incision and pretreated with an injection of (~5µl) bovine hyaluronidase (Sigma H-4272) at a concentration of 0.4U/µl sterile 0.9% saline. After 2h the mice were given a top-off injection of 5% chloral hydrate (30-50% of the initial amount). The pretreated muscles were then injected with ~5µl plasmid DNA at 1mg/ml in sterile PBS. The skin was sutured, and conductive jelly (MTM Polygel Plus) was applied to the skin of the lower leg. Tweezertrodes (7mm paddle diameter) connected to an electroporator (BTX Electro Square Porator ECM 830) were then applied on either side of the leg at an angle which would allow the current to

pass straight to the injected muscles without having to cross bone. The electroporator settings were as follows: 175V/cm of space between paddles , 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 7 days before sacrificing them by cervical dislocation and harvesting the muscles by tendon-to-tendon dissection.

Processing of muscle tissue

Muscles were individually frozen by immersion (for 20s) in isopentane brought to the temperature of liquid nitrogen. Muscles were stored in eppendorf tubes with isopentane in a -70°C freezer.

Muscle cross-sections, 10μm thick, were cut on a cryostat. Serial sections were collected on cover slips at various points along the length of the muscle. Transgene β-gal reporter expression was visualized by X-gal staining as follows: sections were fixed in 0.25% glutaraldehyde for 3 mins and were washed several times in water. Excess water was lightly removed from the cover slip with a Kimwipe and the sections were laid face up in a weigh boat (in turn inside a petri dish whose bottom was layered with moist paper towel). The sections were then overlaid with a solution made up of 1.6mg/ml X-gal , 5mM ferrocyanide, 5 mM ferricyanide and 2 mM $MgCl_2$ 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 slide with Immu-Mount solution (Shandon 9990402).

Muscle fiber-types were determined by immunostaining unfixed sections with monoclonal antibodies specific for particular myosin heavy chain isoforms as follows: Primary antibody A4.840 or SC-71 (to identify types I and IIA myosin heavy chains respectively; type IIX fibers do not react with either of these antibodies, hence β-gal expressing fibers not stained for type I or IIA were classified as IIX) was overlaid on the sections for 1 h at room

temperature. After three PBS washes biotinylated secondary antibodies IgM (for primary A4.840) or IgG (for primary SC-71) were overlaid for 0.5 h. Then 3 PBS washes were followed by the overlaying the sections with avidin-coupled horseradish peroxidase (Dimension Labs Inc SA-5004) for 1/2 hr. This was followed by three 2 min PBS washes and a 5 min soak in diamino benzidine solution (2 drops/ml suspension buffer, LabVision Corp TA-060-HD). The sections were then washed several times with water and mounted as with X-gal stains.

III RESULTS

Our overall goal was to begin to localize functionally important cis-elements concerned with fiber-type-specific gene expression within the TnIfast IRE enhancer. The approach used was end-block-deletion. Two truncation mutants of the IRE were prepared each lacking approximately ¼ of IRE at either the left end or the right end (left and right defined by the left-to-right 5'-to-3' convention applied to the TnIfast gene sense strand). The construct lacking the IRE left end was termed R3/4 and the construct lacking the right IRE end was termed L3/4.

Because the L3/4 and R3/4 constructs would each lack an element known to contribute to expression levels in transfected muscle cell cultures (14, 27) it was anticipated that they would have weaker enhancer activity than the intact IRE. Because the "strength" of DNA regulatory elements can often be augmented by the production of serial multimers (40), it was decided to produce head to tail triplets of the L3/4 and R3/4 constructs in the hope of generating detectable enhancer activity despite the absence of quantitatively important cis-elements. As described in Materials and Methods, triplicated L3/4 and R3/4 were produced by PCR amplification from the native IRE DNA sequence using primers containing

restriction enzyme sites that permit the production of restriction enzyme resistant head-to-tail multimers by DNA ligase. Following electrophoresis of multimers in agarose gels (see Fig 6), the band corresponding to trimers was recovered and cloned into a plasmid vector. It was expected that both orientations would be recovered , and this was the case for R3/4. However, in the case of L3/4 although one orientation was obtained repeatedly, the other orientation was not found in several repeated attempts. This insert 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 so these recombinants would have given blue rather than white colour and have been counted as pBluesript II SK+ vector background (which appears as blue colonies on agar plates and was present in about a 20:1 ratio with the white recombinant colonies). It was decided for experimental uniformity to use all enhancer multimers in the same orientation as that for L3/4, namely the "reverse" orientation. In this orientation the enhancer is in the opposite orientation with respect to the transcriptional direction by comparison to the native gene. The IRE, like many enhancers, has been shown to activate transcription when present in either orientation (29). A triplicate of the full-length IRE was made by PCR as well and cloned into the pBluesript II SK+ vector in a similar fashion in order to assess the activity of the fully active IRE enhancer in this experimental setting.

In preparing the TnIfast minimal promoter construct to act as our enhancer-dependent test gene, we decided to use two slightly different promoter constructs, one extending farther in the 5' (upstream) direction. The restriction enzyme sites Bam HI and Pst I located at (-530) and (-198) of the TnIfast gene respectively were used to cleave the promoter to give the two test promoters. Both test promoters shared the same 3' endpoint located at +22 of TnIfast,

where a Bal I site was cleaved. The –530 site corresponds to the end of the TnILacZ1B construct, which has been shown to be effectively expressed in transgenic mouse muscle (16). Use of the –198 site reduces the amount of 5'- flanking DNA by ~330bp which, if the shorter promoter still effectively responds to the IRE enhancer, would minimize the amount of non-IRE DNA in the test constructs, and more clearly indicate the degree of functional autonomy of the IRE.

The β-gal (LacZ) gene was selected as the reporter gene for all test constructs. The advantages of this reporter were 1) that it would give blue colour selection in the cloning stage when inserted into the enhancer-containing pBluescript II SK+ vector (which gave white colonies.) 2) it would allow for an internal luciferase control to be used in addition, in cell transfection experiments; and 3) it provides a way of visualizing trangene expression by X-gal staining of muscle tissue cross-sections. Cassettes containing either the (-198) or (-530) TnIfast promoter plus the LacZ gene were cloned into the enhancer multimer-containing pBlueScript II SK+ vectors. With this step completed, an unexpected difficulty emerged. None of the clones could be grown up (by Qiagen plasmid preps) to sufficient yields at which to begin the experiments. This technical problem was eventually solved by subcloning all (enhancer+promoter+LacZ) cassettes into the pSP72 vector.

To complete the suite of test plasmids, we also made enhancerless control constructs otherwise identical to the enhancer-containing constructs: (-198)control and (-530)control, containing the (-198) or (-530) TnIfast promoters respectively. We also made Z72, an enhancerless and promoterless control, with only the LacZ gene in the pSP72 vector. By comparing (-198)control and (-530)control expression levels to that of the promoterless construct Z72, we could directly assess how much expression was attributable to the TnIfast promoters alone. In addition, Z72 expression levels could be subtracted from those of test

constructs in order to eliminate any background, promoter-independent "leaky" transcription. This is described in further detail below. Finally, comparison of (-198)control and (-530)control expression levels to those of enhancer-containing constructs having the same promoter, could directly reveal how much expression was attributable to the enhancers.

Cell culture transfection studies

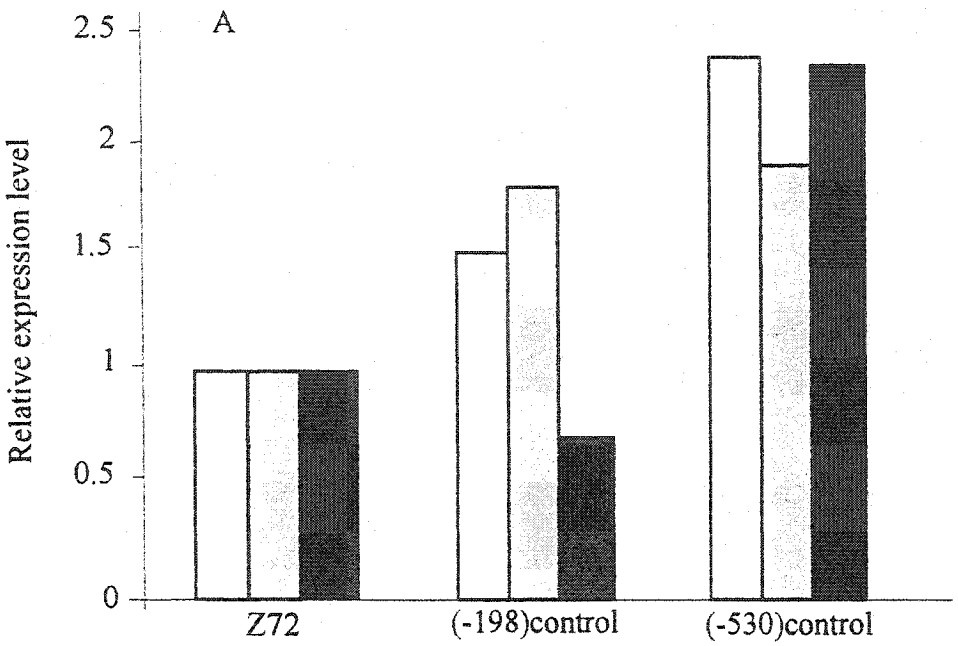
In order to elucidate the gene regulatory capabilities of the IRE derivatives and of the (-198) and (-530) TnIfast promoters, constructs were introduced by transfection into muscle and non-muscle cell lines. To correct for intrinsic differences in transfection efficiencies of the various plasmid preparations, and well-to-well variation in transfection efficiency, an internal transfection control plasmid was used in each transfection cell culture well, namely pGL3 control (Promega), expressing luciferase from a SV40 promoter and enhancer. (The SV40 promoter and enhancer give strong expression of the luciferase gene in many mammalian cell lines). Also, so that experiments done in different cell types or in different culture conditions could be directly compared, a "standard" β-gal -expressing plasmid pRSVZ based on the LTR promoter of the Rous sarcoma virus was also assayed in each experiment. Normalizing experimental β-gal/ luciferase ratios to those obtained in parallel with the standard pRSVZ/ pGL3 pair would be expected to correct for any experiment-to-experiment differences in either β-gal or luciferase assay conditions, and also for any β-gal or luciferase differential protein stability that might exist in different cell types or under different conditions. The underlying assumption is that the relative activities of the RSV and SV40 promoters would be similar in all conditions. In practice, such normalization involved relatively small factors (<1.5).

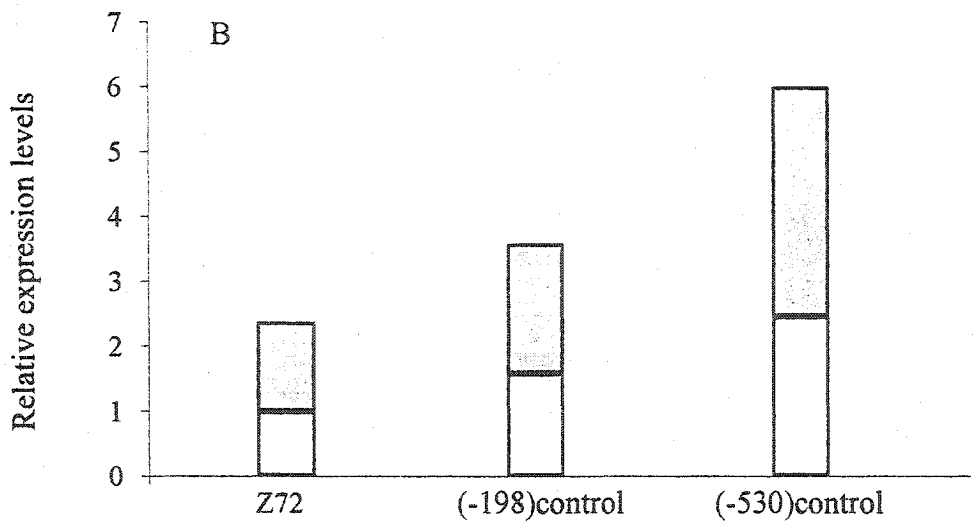
Transfection studies of L6 myofibers

Initial transfection experiments were carried out in L6 myofibers. L6 cells are a rat-derived line of myoblast (muscle precursor) cells which, when induced to stop proliferation in culture, fuse with each other to form multinucleated muscle fibers, also termed myofibers or myotubes, and activate expression of many muscle-specific genes (46, 49). After transfecting the plasmid DNA into L6 myoblasts, growth medium containing high levels of serum-borne growth factors was replaced by fusion medium containing low levels of serum. The cells were subsequently allowed to fuse for 4 days and then cell extracts were harvested from individual wells and biochemical assays were used to determine β-gal and luciferase control reporter gene expression levels. In each experiment, 3 wells of cells were transfected with each test construct and luciferase control, and β-gal and luciferase levels were separately measured in each well. It was usually the case that the replicate samples were similar. In several cases one of the β-gal or luciferase values departed markedly from the other two (> 3 fold difference). Such outlying points were discarded. Figs 16 and 17 show results of L6 myofiber transfection studies. Figure 16 shows experiments done to assess the transcriptional activity of the long (-530) and short (-198) TnIfast promoters in the absence of enhancer elements. We compared the expression of the enhancerless constructs (-198)control and (-530)control with the enhancerless and promoterless control plasmid Z72. Both promoters generally increased expression over that seen with Z72 with the (-530) promoter showing greater activity than the (-198) promoter (Fig 16), although differences did not achieve statistical significance ($p=0.47$). Previous studies had characterized the TnI promoter as being highly responsive to enhancers (29) Nonetheless our results suggested that the –530 promoter and, to a lesser extent, the –198 promoter, may have some transcription-promoting activity even in the absence of enhancers.

We also observed low but non-zero levels of β-gal activity from the enhancerless and promoterless control plasmid Z72. This presumably reflects a low level of "leaky" transcription not occuring through the normal transcriptional initiation mechanisms. In all subsequent data presented in this thesis, the Z72 expression level value (average of three transfection wells) was subtracted from the average expression level values of each of the other constructs in each cell culture experiment, in order to eliminate this leaky transcription contribution.

Figure 16. Expression levels in transfected L6 myofiber cultures of promoterless Z72 β-gal reporter gene plasmid compared to (-198)TnIfast-promoter-containing (-198)control and (-530)TnIfast-promoter-containing (-530)control constructs. A: Each bar represents the average β-gal activity in triplicate culture dish wells in one experiment divided by the average cotransfected pGL3 luciferase activity in the same three wells and normalized to Z72 levels. Each set of 3 similarly marked bars represents a separate experiment comparing the three constructs. Inclusion of either the long (-530) or short (-198) TnIfast promoters generally increased expression levels over the promoterless Z72 background levels, and the -530 promoter generally showed higher expression levels than the -198 promoter. B: Data from the three experiments shown in A were pooled on the basis of β-gal and luciferase activities in each culture well (i.e each bar represents 9 culture wells). Histogram bars show mean and standard deviation. The (-530) control > (-198) control > Z72 trend is shown but differences did not achieve statistical significance ($p=0.47$, ANOVA).


A
2.5
2
1.5
1
0.5
0
Relative expression level
Z72
(-198)control
(-530)control


B
7
6
5
4
3
2
1
0
Relative expression levels
Z72
(-198)control
(-530)control

In order to confirm that transcription from TnI promoter constructs could be stimulated by the IRE, we compared (-198)control and (-530)control plasmids to constructs in which the IRE had also been inserted, in 3 copies: (-198)IRE and (-530)IRE. As expected, presence of the IRE enhancer stimulated expression several fold (Fig 17).

The construct TnILacZ1B was used as a benchmark to which the expression levels of test constructs could be compared. This is a β-gal construct containing 5' upstream sequence of the TnIfast gene followed by the first two exons plus the IRE- containing intron 1. This construct has been shown to give effective muscle-specific and fiber-type-specific expression in transgenic mice (16). TnILacZ1B expression levels were 38% to 173% of those seen for IRE constructs. Thus the IRE constructs are expressed at levels comparable to that of the closer-to-native gene construct TnILacZ1B.

The transcription promoting activities of the IRE end-deletion constructs L3/4 and R3/4 were also assessed. The L3/4 element consistently stimulated expression. The R3/4 also stimulated expression, although this was consistently weaker than that observed for L3/4 (Fig 17). In some experiments stimulation by R3/4 was very small, but with few exceptions, R3/4 expression was higher than observed for enhancerless controls (-198)control and (-530)control.

The different enhancer constructs conferred a consistent hierarchy of expression levels. Complete-IRE constructs gave the highest levels of expression, followed by the L3/4 constructs. This in turn was followed by the R3/4s (Fig 17). This pattern was seen with both (-530) and (–198) TnIfast promoters. The (-198) promoter showed greater stimulation by the IRE enhancer (6.46, 10.04 and 28.5- fold in three experiments) than did the (-530) promoter

(2.15, 4.33 and 7.8-fold). This is a consequence of the relatively high transcriptional background of the (-530) promoter, which reduced the apparent enhancement of expression. These results show that, despite the absence of elements previously shown to contribute to enhancer activity, the L3/4 and R3/4 IRE derivatives are nonetheless functional transcriptional enhancers in differentiated L6 muscle cultures when present in triplicate.

Transfection studies of undifferentiated L6 myoblasts

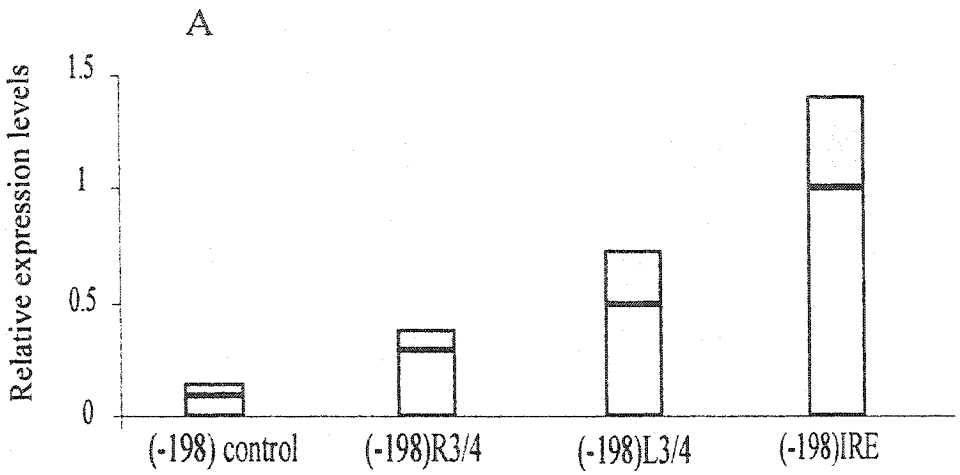
Transfection experiments were also carried out in undifferentiated L6 myoblast cells. Previous studies of the IRE showed that its enhancer activity was markedly upregulated during myoblast differentiation (29). I found that in undifferentiated L6 myoblasts all TnIfast constructs had little activity, although pRSVZ was actively expressed in both L6 myoblast and L6 myofiber cultures (Fig 18). Enhancerless (-198)control and (-530)control plasmids showed little difference in expression in myofibers versus myoblasts. In contrast, L3/4 and R3/4 constructs showed markedly stronger expression in differentiated myofibers - 5 to 10 fold higher than in myoblasts (Fig 18). These results indicate that the enhancer activity of not only the IRE as a whole, but also its L3/4 and R3/4 fragments are markedly upregulated during myoblast differentiation.

Transfection studies of 10T1/2 embryonic fibroblasts

In order to assess cell-type specificity of expression, transfection studies of the short promoter construct series were also carried out in a non-muscle cell line - the 10T1/2 mouse embryonic fibroblast cell line. Most of the TnIfast constructs showed relatively little activity in 10T1/2 cells, although pRSVZ was active in both 10T1/2 cells and L6 myofibers (Fig 19). This is consistent with the known muscle cell type specificity of the IRE enhancer established by other investigators (29). However we found, surprisingly, that the R3/4 is an active enhancer

in 10T1/2 cells. Our data show that the (-198) promoter is extremely specific for L6 myofibers as opposed to 10T1/2 cells, whether by itself, or when activated by IRE or L3/4 enhancers. However, in the presence of the R3/4 enhancer, expression in 10T1/2 cells was strongly stimulated (30-fold stimulation in 10T1/2 cells (Fig 19) compared to 2-fold stimulation in L6 myofibers (Fig 17)). These results indicate that the R3/4 IRE segment contains elements that can effectively drive transcription in 10T1/2 cells, but this activity is masked in the setting of the complete IRE, presumably because of interference from the leftmost 30 bases, which are deleted in the R3/4 enhancer.

Figure 17. Effect of IRE-derived enhancer segments on TnIfast promoter/β-gal reporter gene expression in differentiated transfected L6 myofiber cultures. A: (-198)control and the enhancer-containing (-198) constructs. B: (-530)control and enhancer-containing (-530) constructs. Each panel shows pooled data from 3 independent experiments involving triplicate culture wells; each bar represents the average and standard deviation over 9 culture wells (as in Fig 16B) and normalized to the relevant IRE-containing construct. The intact IRE enhancer showed evident stimulation of both -198 and -530 TnIfast promoter constructs. The L3/4 and R3/4 segments showed less enhancer activity than the intact IRE, but in most experiments expression was higher than the enhancerless control plasmids. L3/4 constructs generally showed higher expression levels than R3/4 constructs. Differences in each panel were statistically significant (ANOVA, $p=1.5x10^{-6}$ for A, $p=1.4x10^{-4}$ for B).


A
Relative expression levels
1.5
1
0.5
0
(-198) control
(-198)R3/4
(-198)L3/4
(-198)IRE

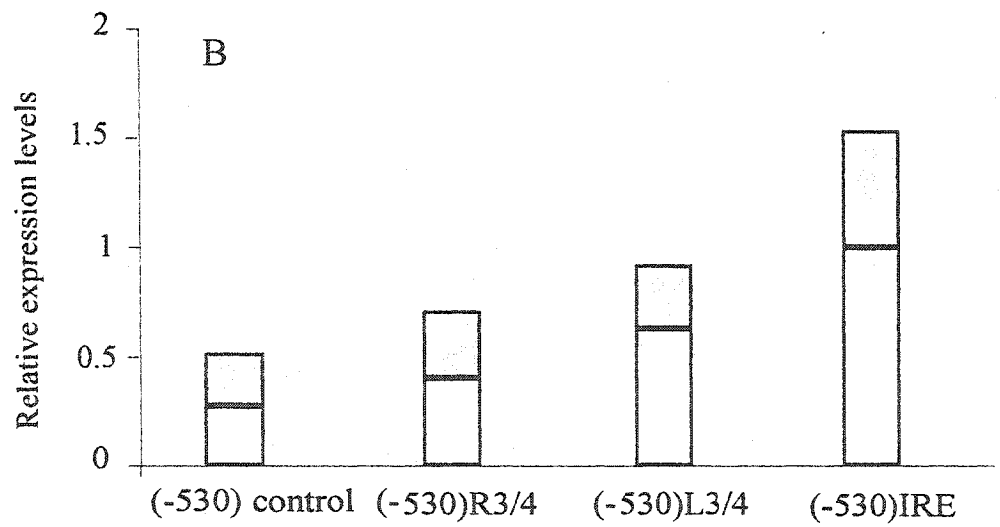
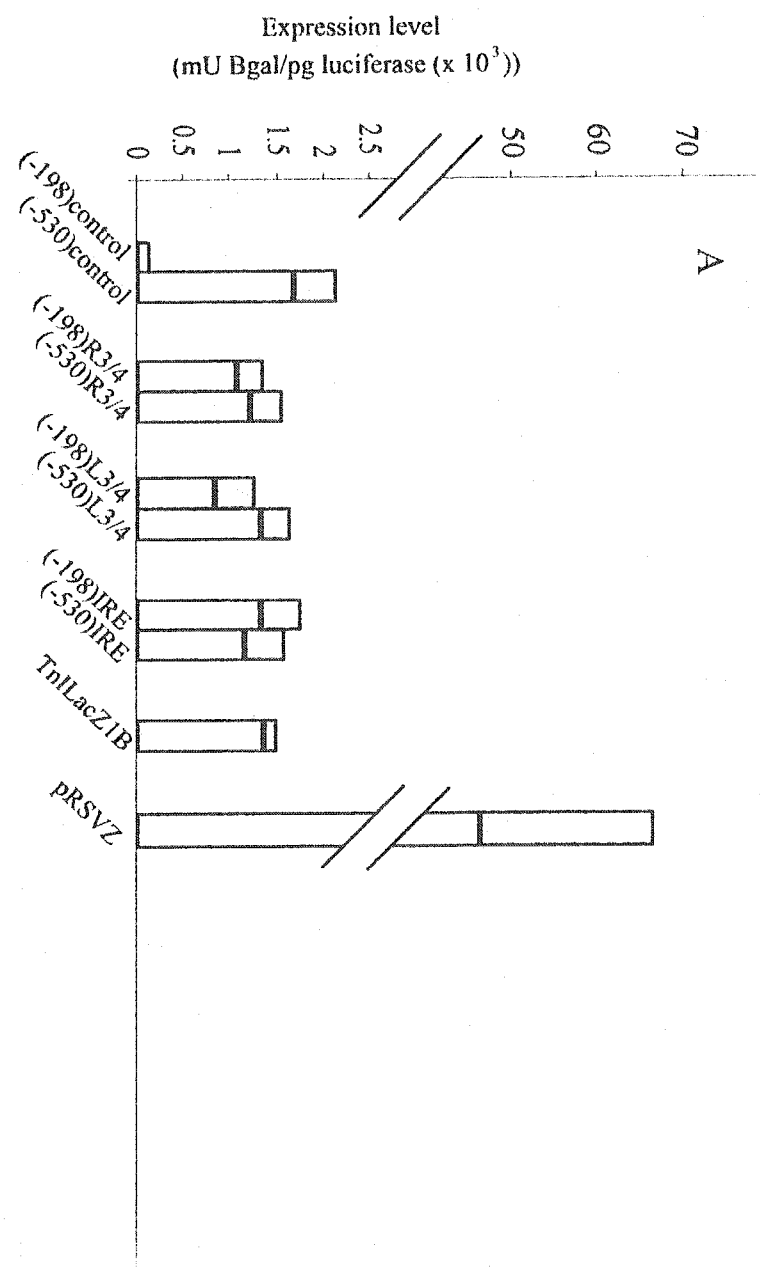

B
Relative expression levels
2
1.5
1
0.5
0
(-530) control
(-530)R3/4
(-530)L3/4
(-530)IRE

Figure 18. Expression of test constructs in undifferentiated transfected L6 myoblasts. A: Expression levels in myoblasts. Each bar represents the average and standard deviation of β-gal values obtained from triplicate culture dish wells, corrected for differences in transfection efficiencies with pGL3 luciferase control. pRSVZ was used as a non-muscle-specific control construct. B: Myofiber/myoblast expression ratio. To compare myofiber and myoblast transfections, the ratio of luciferase-corrected pRSVZ expression values in myofiber and myoblast transfection experiments was set at 1.00 (the myofiber/ myoblast ratio was 0.7 before this correction) and other expression values were adjusted by this factor. This normalization was done to correct for any possible differential effects of the two different cell culture conditions on stability or assay sensitivities for β-gal and transfection control luciferase enzymes. TnILacZ1B was used as a known muscle gene positive control construct. The enhancer-containing constructs showed myofiber/ myoblast expression ratios>1, indicating that these constructs are upregulated during myoblast differentiation. Note also that the (-198)control construct showed marked upregulation during myoblast differentiation, whereas the (-530)control construct did not.


Expression level
(mU Bgal/pg luciferase (x 10^3))
0
0.5
1
1.5
2
2.5
50
60
70
A
(-198)control
(-530)control
(-198)R3/4
(-530)R3/4
(-198)L3/4
(-530)L3/4
(-198)IRE
(-530)IRE
TnILacZ1B
pRSVZ

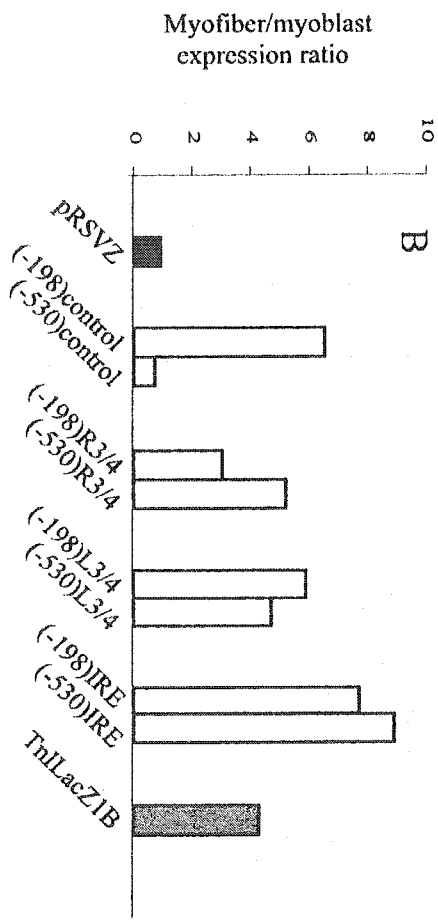
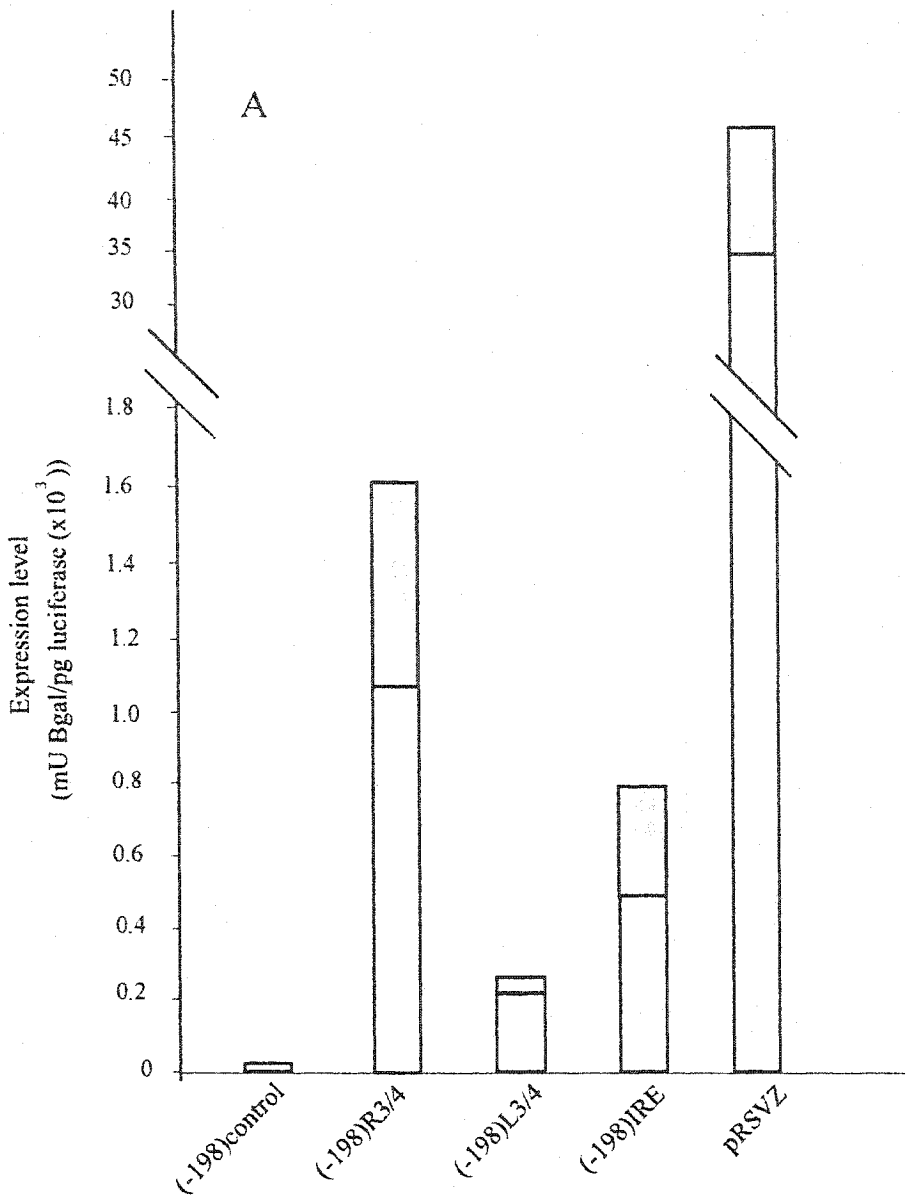
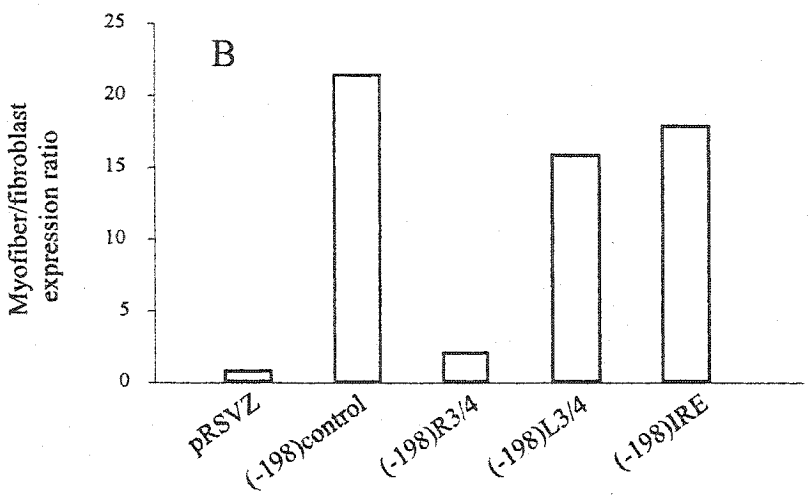

Myofiber/myoblast
expression ratio
0
2
4
6
8
10
B
pRSVZ
(-198)control
(-530)control
(-198)R3/4
(-530)R3/4
(-198)L3/4
(-530)L3/4
(-198)IRE
(-530)IRE
TnILacZ1B

Figure 19. Expression of test constructs in transfected 10T1/2 fibroblasts. A: Expression levels in 10T1/2 fibroblasts. Each bar represents the average and standard deviation of β-gal values from triplicate culture dish wells, corrected for variation in transfection efficiencies with the pGL3 luciferase control. pRSVZ was used as a non-muscle-specific control. Note the higher expression levels of (-198)R3/4 compared against (-198)L3/4 and (-198)IRE. B: Myofiber/fibroblast expression ratio. To compare myofiber and fibroblast transfections, the ratio of luciferase-corrected pRSVZ expression values in myofiber and fibroblast transfection experiments was set at 1.00 (the myofiber/ fibroblast ratio was 0.9 before this correction) and other expression values were adjusted by this factor. This normalization was done to correct for any possible differential effects of the two different cell culture conditions on stability or assay sensitivities for β-gal and transfection control luciferase enzymes. Panel B illustrates that whereas the

(-198)control, (-198)L3/4 and (-198)IRE constructs showed marked preferential expression in myofibers as opposed to 10T1/2 fibroblasts, the (-198)R3/4 construct was expressed at similar levels in myofibers and 10T1/2 fibroblasts.


A
Expression level
(mU Bgal/pg luciferase (x10^3))
50
45
40
35
30
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
(-198)control
(-198)R3/4
(-198)L3/4
(-198)IRE
pRSVZ


B
Myofiber/fibroblast
expression ratio
25
20
15
10
5
0
pRSVZ
(-198)control
(-198)R3/4
(-198)L3/4
(-198)IRE

***In vivo* direct gene transfer studies**

Having established that the L3/4 and R3/4 IRE segments had detectable enhancer activity in muscle cells in culture, it was then decided to test them *in vivo*, for the purpose of assessing possible fiber-type-specificity. The (-198) promoter series of constructs was selected for this purpose, based on their higher degree of enhancer-dependence. The soleus muscle was chosen because it consists of fast and slow fibers in an approximately 50:50 ratio. Such a muscle would allow us to better be able to determine whether the TnI constructs showed fast versus slow fiber-type-specificity.

Incorporating a novel method using limb electroporation to enhance DNA transduction (41), DNA was injected into soleus muscles of CD1 mice. The muscles were harvested after 7 days and sectioned for histological/ immunological analysis. X-gal staining showed fibers expressing the β-gal reporter gene as blue; and immunostaining of serial sections with anti-myosin heavy chain isoform-antibodies A4.840 and SC-71 identified slow fibers (type 1) and fast fibers (type IIA) respectively (see Fig 20). Fibers not reacting with either of these two antibodies were classified as type IIX (Figs 21, 22). The actual proportions of fast and slow fibers in each muscle were established by fiber typing a contiguous patch of about 100 fibers. The total number of blue (transgene expressing) fibers in the same muscle sections was also determined, and these fibers were also categorized as to fiber type(Fig 21). By comparing the fiber type composition of the transgene-expressing fibers with the fiber type composition of the muscle overall, it could be assessed whether constructs showed fiber-type preferential expression.

When we injected the plasmid pRSVZ we observed expression in both fast and slow fibers (Fig 21). No fiber type specificity was expected for the viral transcriptional elements driving

β-gal expresion in this plasmid. These results establish that both fast and slow fiber types are efficiently transduced by the injection/ electroporation method.

When we injected the enhancerless (-198)control there was no detectable expression, ie, no blue fibers were seen (data not shown). When we injected (-198)IRE numerous transduced fibers were observed (Fig 21). However, unlike pRSVZ , (-198)IRE was expressed preferentially in fast fibers (97% of transduced fibers were fast, Fig 21; $p<0.001$, chi-square analysis). Thus the plasmid injection/ electroporation method unlike the earlier, less efficient approach of injection without electroporation (45) does not de-regulate TnIfast fiber type specificity. When we injected (-198)L3/4 and (-198)R3/4 constructs, numerous blue fibers were observed. Thus, the L3/4 and R3/4 enhancer segments have detectable activity *in vivo* as well as *in vitro.*

Like (-198)IRE, the (-198) L3/4 construct also showed preferential expression in fast fibers (91% of transduced fibers were fast, Fig 21; $p= 2x10^{-4}$, chi-square analysis). (-198) R3/4 , on the other hand showed a loss of fast fiber specificity (Fig 21; $p>0.6$). For this construct, transgene expression was uniform across fiber types, as was the case with the control plasmid pRSVZ (Fig 21; $p>0.6$). (-198)R3/4 and pRSVZ were expressed in fast and slow fibers in the same proportions as their relative abundance in the muscle overall (Fig 21).

Quantifying the optical densities of blue stain in the transgene expressing fibers (Fig 22) showed that not only were similar numbers of fast and slow fibers transduced by the (-198)R3/4 construct, but β-gal expression levels were at similar levels in both major fiber types ($p=0.25$, t-test). In contrast, the small numbers of slow fibers transduced by the (-198)IRE and (-198)L3/4 constructs expressed β-gal at much lower levels ($p= 9x10^{-18}$ and $p= 3x10^{-8}$, respectively) than did the larger numbers of fast fibers transduced by the constructs. These results show that the R3/4 IRE fragment has very different fiber type regulatory

properties from the IRE itself, or the L3/4 fragment. Whereas the latter two enhancers show a marked fast fiber specificity, the R3/4 element does not, but drives expression at similar levels in fast and slow fibers. This result indicates that somewhere in the 30 bases of the left 1/4 of the IRE lies a cis-element which confers fast-fiber-specificity to the TnIfast gene.

Figure 20. Microphotographs showing expression of test constructs following gene transfer to soleus muscle *in vivo*. Muscle tissue sections were subjected to X-gal histochemistry to reveal β-gal reporter gene expression (Panels A,C,E,G). Serial sections were subjected to immunohistochemistry with an antibody specific for the type IIA (fast) myosin heavy chain isoform, to identify type IIA (fast) fibers (Panels B, D, F, H). The majority of unstained fibers in panels B, D, F, H are type I (slow) fibers. The same set of muscle fibers is shown in each left/right pair of panels; some corresponding fibers are marked with an asterisk. Expression of (-198)L3/4 and (-198)IRE constructs was predominantly in type IIA (fast) fibers, whereas the (-198)R3/4 construct (and the non-fiber-type-specific control pRSVZ) were expressed both in fast and slow fibers.

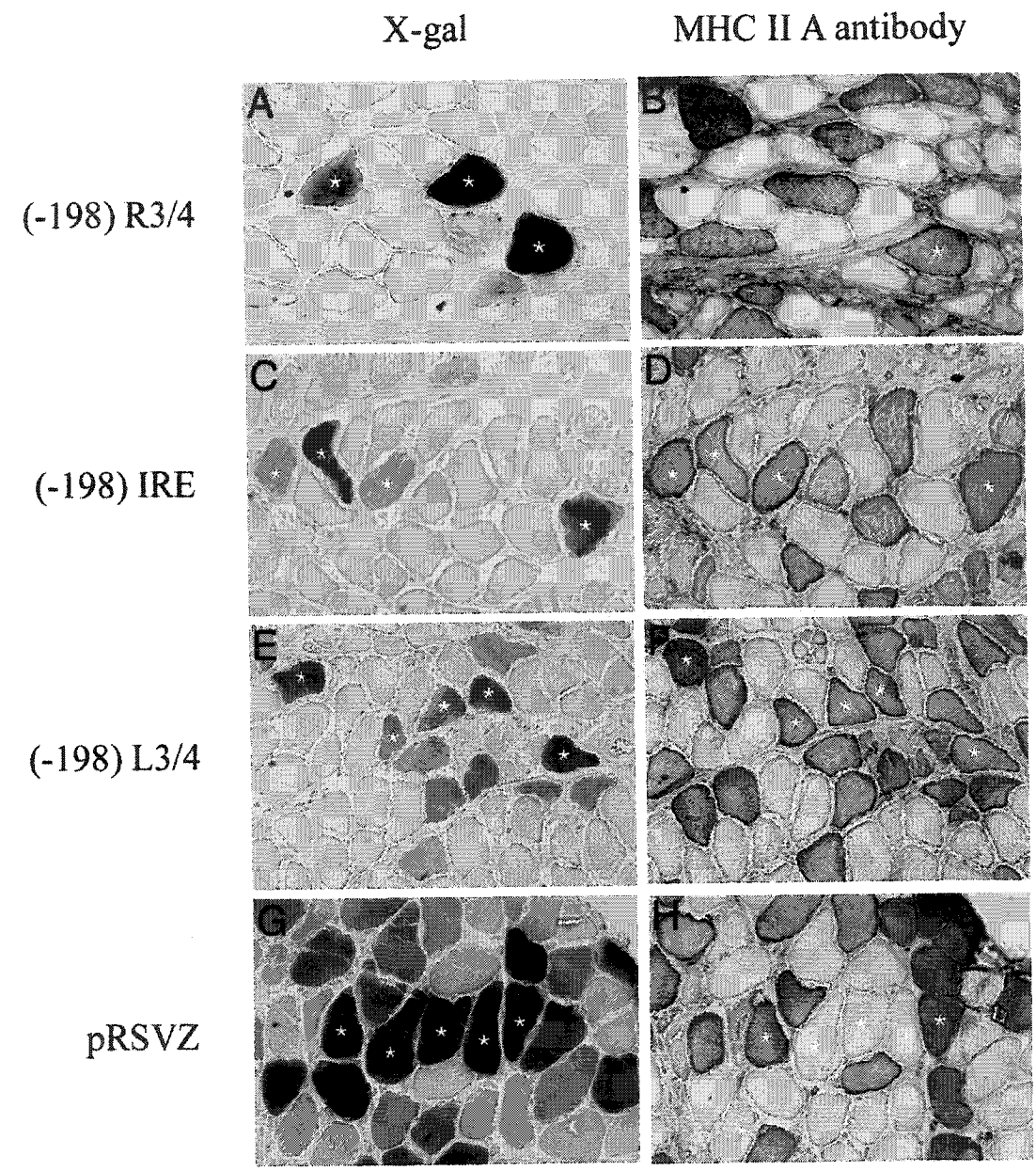
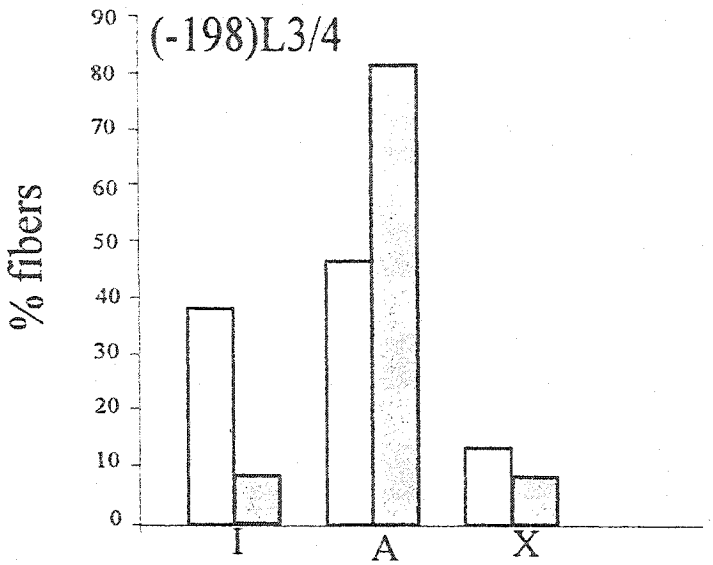
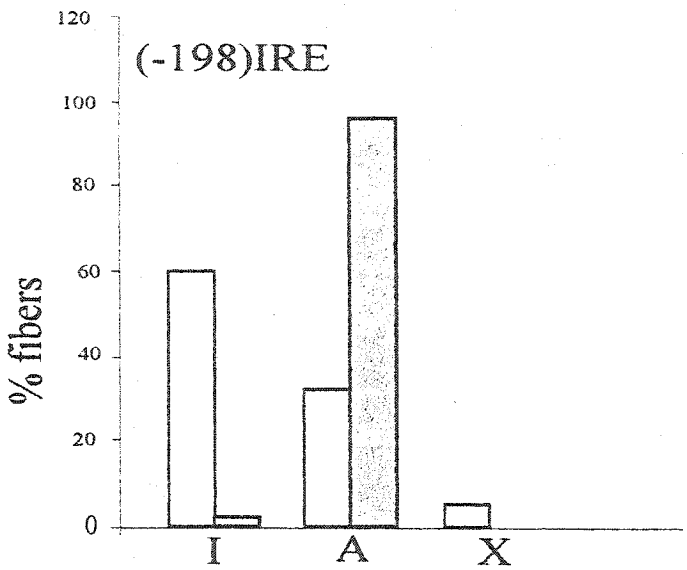
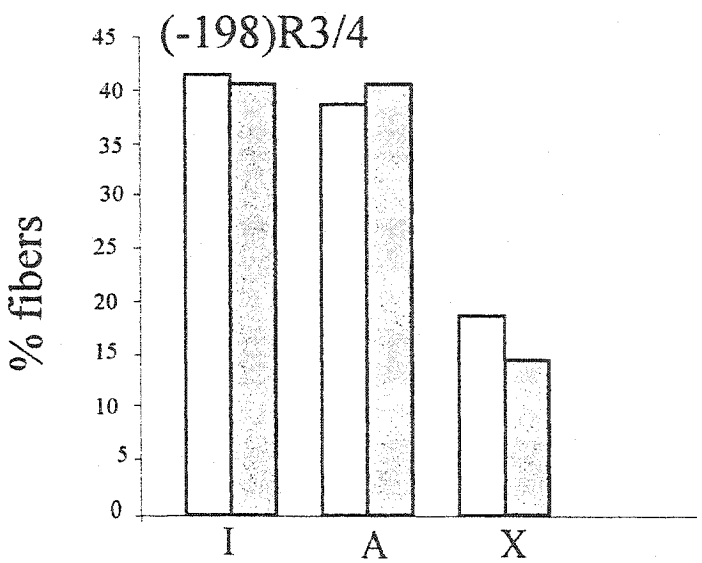

X-gal
MHC II A antibody
(-198) R3/4
(-198) IRE
(-198) L3/4
pRSVZ
A
B
C
D
E
G
H

Figure 21. Fiber type expression patterns of test constructs following gene transfer to skeletal muscle *in vivo*. Plasmid constructs were introduced into the soleus muscle and expression was analyzed at the level of individual muscle fibers by histochemical display of β-gal reporter gene expression by X-gal staining of muscle tissue cross sections. Muscle fiber types were determined by myosin isoform immunohistochemical analysis of serial sections. White bars show the distribution of fiber types in the muscle: I= type I (slow), A= type IIA (fast), X=type IIX (fast), based on a sample of 60 to 100 fibers in a contiguous patch. Grey bars show the distribution of (X-gal-stained) blue fibers in the different fiber types, based on a sample size of 30-100 fibers. (-198)L3/4 and (-198)IRE were preferentially expressed in the fast fibers ($p<2x10^{-4}$, $p<0.001$, respectively, by chi-square analysis pooling IIA and IIX fiber types) whereas (-198)R3/4, like the control plasmid pRSVZ, did not show differential expression in fast and slow fibers ($p>0.6$ by chi-square analysis in both cases).


(-198)L3/4
% fibers
90
80
70
60
50
40
30
20
10
0
I
A
X


(-198)IRE
% fibers
120
100
80
60
40
20
0
I
A
X


(-198)R3/4
% fibers
45
40
35
30
25
20
15
10
5
0
I
A
X

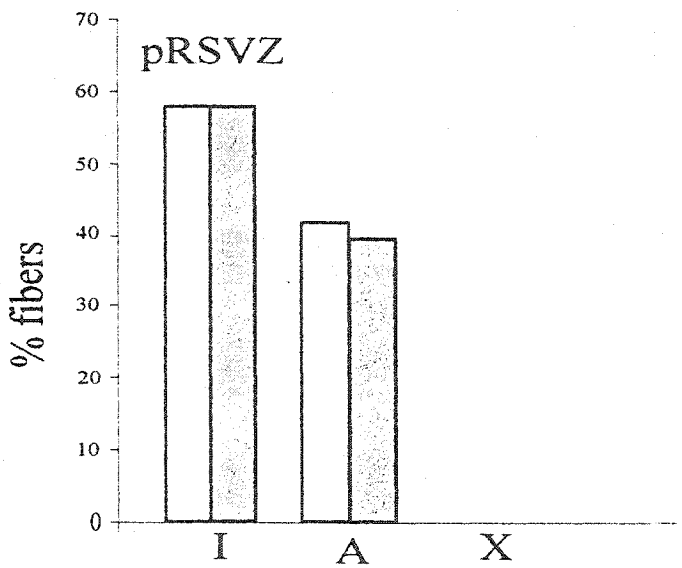
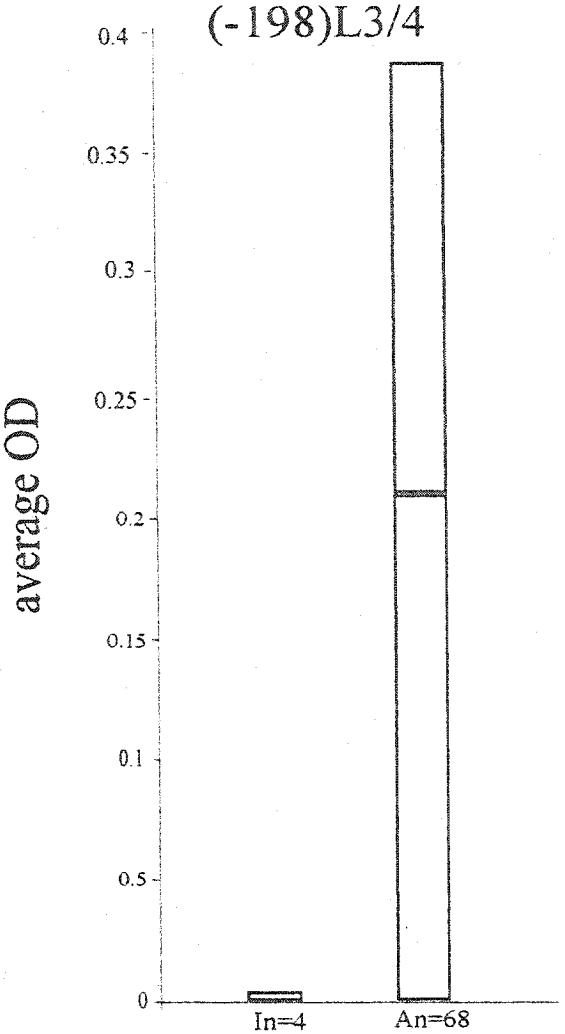
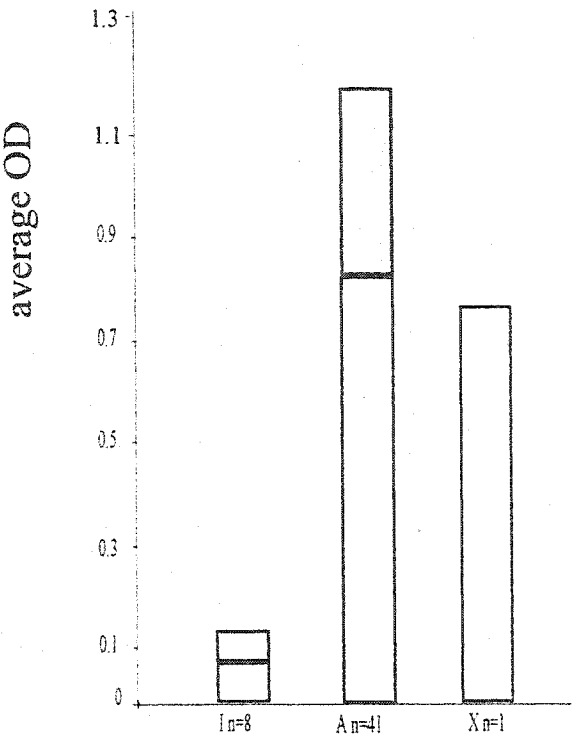

pRSVZ
% fibers
70
60
50
40
30
20
10
0
I
A
X

Figure 22. Quantitation of expression levels of test constructs in individual muscle fibers following gene transfer to the soleus muscle. Following X-gal histochemistry of muscle sections the optical densities (OD) of individual stained muscle fibers were determined by microdensitometry. Muscle fiber types were determined by myosin isoform immunohistochemical analysis of serial sections: I= type I (slow), A= type IIA (fast), X= type IIX (fast). The numbers of fibers analysed for each fiber type (n) are indicated below the histogram bars. The small numbers of type I (slow) fibers with detectable expression of the (-198)L3/4 and (-198)IRE constructs showed levels of expression lower than was observed in the fast fibers (types IIA and IIX), (t-test, $p=3x10^{-8}$ and $9x10^{-18}$ respectively). The larger numbers of type I (slow) fibers with detectable expression of the (-198)R3/4 construct showed levels of expression comparable to that observed in the fast fibers (types IIA and IIX), ($p=0.25$, t-test). pRSVZ showed similar expression in fast and slow fibers, with slightly higher levels in slow fibers ($p=0.02$, t-test).

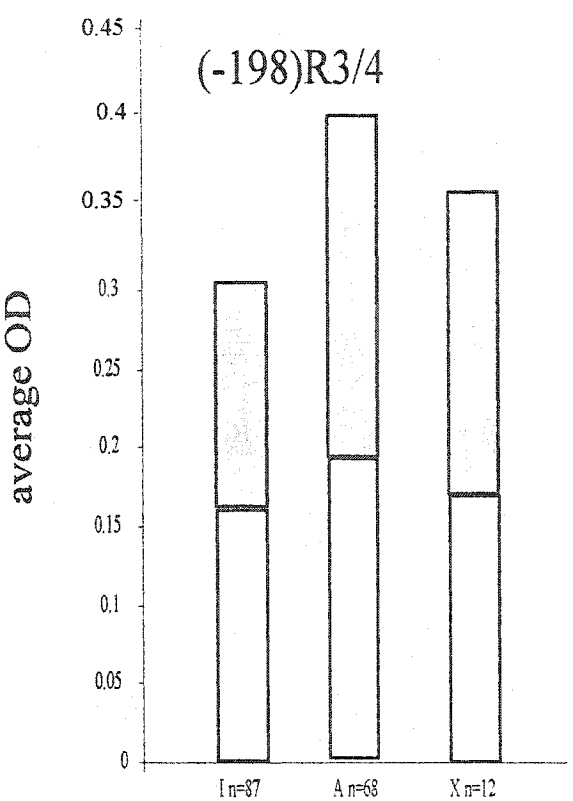
(-198)L3/4



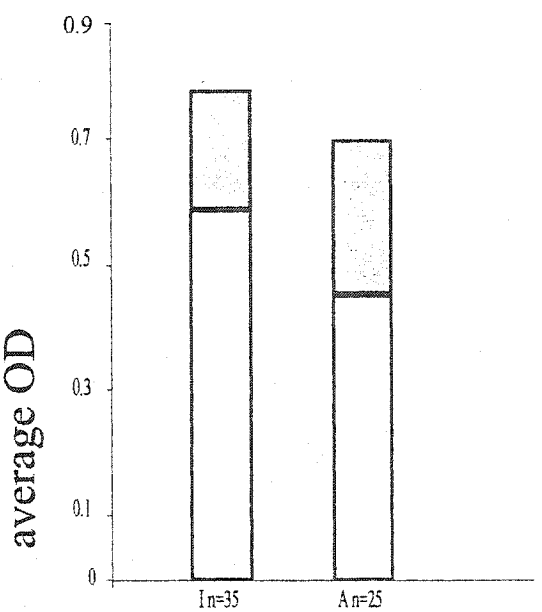
(-198)IRE



(-198)R3/4



pRSVZ



IV DISCUSSION

Validity of the TnIfast -198 promoter construct as an IRE enhancer test system

The ideal enhancer test construct would consist of a convenient reporter gene driven by a promoter that is highly responsive to the enhancer in question, but that has no intrinsic transcriptional activity of its own. Previous studies by Konieczny and coworkers (29, 44) indicated that the TnIfast promoter was highly enhancer-dependent, although Nikovits et al (51) reported detectable expression of TnIfast promoter constructs in the absence of added enhancers. We found that, while it is indeed responsive to the IRE enhancer, the TnIfast promoter has intrinsic transcriptional activity and drove readily detectable expression in transfected cell cultures in the absence of the IRE.

Our data showed that the -198 promoter construct was more active in L6 myofiber cultures than was the promoterless control construct Z72 (Fig 16). Moreover this expression was highly specific for differentiated muscle cells; activity of the -198 promoter in undifferentiated L6 myoblasts , as well as in 10T1/2 fibroblasts, was far lower (5 - 25-fold) than was observed for differentiated L6 myofiber cultures (Figs 18B and 19B) . These results suggest the presence of an element within the -198 to +22 region of the quail TnIfast promoter that is capable of driving muscle-specific expression in cultured cells. This is consistent with the findings of Nikovits et al (51) who reported that a -160 to +61 promoter segment of the chicken TnIfast gene (very similar to the quail gene) drove much higher expression in chicken primary muscle cultures than in fibroblast cultures.

Nikovits et al (51) also tested longer TnIfast promoter fragments extending as far upstream as -1000, but none of the longer constructs showed greater activity in muscle cultures than did the -160 construct. However, other studies have suggested the presence of additional functional elements upstream of -160. In experiments based on transfection of 5'-deletion constructs of the intact quail TnIfast gene into the 23A2 mouse muscle cell line, Konieczny and Emerson (44) obtained evidence for an upstream element between -484 and -436 that had a modest (3-fold) positive effect on expression. Our results are consistent with the presence of such an element, because we found that the enhancerless -530 promoter construct was more active than the -198 promoter construct (Fig 16). Our results further indicate that this upstream element is not muscle-differentiation-specific, because the activity of the enhancerless -530 promoter was similar in both differentiated L6 myofibers and in undifferentiated L6 myoblasts (Fig 18B).

The reports of Konieczny and coworkers (29, 44) seemed to indicate a higher degree of enhancer dependence of the TnIfast promoter than we observed, at least in some of our experiments. Konieczny and Emerson (44) carried out a series of 23A2 mouse myofiber transfection experiments based on internal deletions of the intact quail TnIfast gene starting from position -530 (i.e the same as in our -530 promoter construct). They found that deletion constructs lacking the IRE but having intact promoter retained ~ 5-10% of the initial activity. This suggests the TnIfast promoter is stimulated 10 - 20 - fold by the presence of the IRE in the wild type gene, although adding back IRE-containing fragments in other locations/orientations resulted in at most a 6-fold stimulation. In a different approach based on CAT reporter gene transfection studies of 3' deletions having a 5'-end at -530 Yutzey et al (29) found that a construct containing the IRE (TnICAT1) was 100- fold more active in 23A2

myofiber cultures than a construct (TnICAT2) that did not, but did contain the entire TnIfast promoter region. However, here again, "adding-back" experiments gave less striking results; addition of IRE-containing DNA fragments upstream of TnICAT2 increased expression in myofiber cultures by ~7 - 17-fold.

In our experiments we found that addition of the triplicated IRE enhancer to TnIfast promoter constructs consistently increased expression in L6 myofiber cultures, although the enhancement varied from experiment to experiment and differed between the different promoter constructs employed (see Fig 17). The high end of the range of enhancement effects observed (~2-25 fold) was within the range of IRE enhancer effects (~6 - 17 fold) on the TnIfast promoter in the "adding-back" experiments of Konieczny and coworkers (29,44). The lower values observed in some of our experiments e.g. only several-fold in experiment I in Fig 17, could reflect variations in the extent of myogenic differentiation of the L6 cultures. Because the IRE is a differentiation-specific enhancer, any variations in the extent of myogenic differentiation from one experiment to the next will be directly reflected in variations in the expression levels of IRE-driven constructs. In all our experiments microscopic inspection showed extensive myoblast fusion in L6 myofiber cultures, however, there may still have been significant quantitative differences not apparent to the eye, or there may have been different extents of myofiber biochemical maturity rather than major differences in the extent of myoblast fusion per se.

The -530 promoter construct consistently gave lower fold-enhancement responses to the IRE than did the -198 promoter, due largely to its higher intrinsic activity (Fig 17). Because of its lower intrinsic "background" activity, and strong response to enhancement by the IRE, the -198 promoter construct was the more suitable IRE-enhancer-test construct of the two

promoters tested, and it was selected as the test system for our *in vivo* gene transfer studies. Our *in vivo* gene transfer observations paralleled those of cell culture transfection studies. Addition of the triplicated IRE enhancer caused a marked increase in gene expression from the -198 promoter in skeletal muscle. However, whereas we could readily detect intrinsic activity of the -198 promoter construct in cultured L6 myofibers, there was no detectable expression by the method used to assess expression in skeletal muscle *in vivo*, namely X-Gal staining of muscle cross-sections. It is possible that the -198 promoter has less intrinsic activity in adult skeletal muscle fibers than in myofibers in cell culture, or is more responsive to stimulation by the IRE. However, it is also possible that quantitative analysis of beta-gal expression by enzyme biochemical assay of muscle homogenates might reveal a low but measurable expression from the -198 promoter. However, using X-Gal staining as the assay, the -198 promoter construct has the properties of the ideal enhancer test construct for *in vivo* functional studies of the IRE enhancer, i.e. undetectable intrinsic activity and strong enhancer response. In cell culture experiments the detectable intrinsic activity in L6 myofibers makes the -198 construct less than ideal. However it is serviceable nonetheless even for cell culture experiments because although the intrinsic background activity is significant, the response to the IRE is still very clear. The -530 construct also responds to the IRE but its higher intrinsic activity renders it less useful as an IRE enhancer test construct.

Functional analysis of IRE end-deletion constructs

The goal of this work was to begin to associate specific IRE cis-elements with expression patterns. Deletion of the left-most IRE segment including the E box markedly reduced expression levels in L6 muscle cells, as seen with the R3/4 constructs. To find a reduction was not surprising. Mutation of the E box was previously shown to reduce the gene activation

capability of the IRE in cell culture by 95% (27) presumably because the muscle-specific MyoD factors in this case have no DNA binding site, and therefore cannot transactivate the gene. Our results suggest a less dramatic loss of function in the R3/4 constructs. The triplication in our constructs may efficiently augment transcriptional activity. Alternatively, other elements than the E box may be present in the 30bp segment lacking in the R3/4 construct; elements that may repress expression in the absence of a functional E box.

L3/4 constructs, in which the right-most IRE segment including the CAGG conserved sequence was missing, showed expression levels intermediate between R3/4 and IRE, indicating that the CAGG element may contribute to IRE activity but is quantitatively less important than is the E box. This element seems to be more crucial to the functioning of the TnIslow SURE enhancer; mutation of this element completely abolished SURE-driven transcription in cell culture as well as in transgenic mice (14). The CAGG element may play a less important role in the TnIfast enhancer. Alternatively, the triplication of the element in our constructs may efficiently augment expression, or additional elements, which repress transcription in the absence of a functional CAGG element, may be present in the 43 bp IRE segment lacking in the L3/4 constructs.

All enhancer-containing constructs were expressed at higher levels in L6 myofibers than in undifferentiated myoblast cultures. This suggests that sequences common to L3/4, R3/4 and IRE, namely the central portion of the IRE including MEF2 and CCAC elements, may be sufficient to activate transcription during myoblast differentiation. However the surprising expression of the R3/4 construct in 10T1/2 cells (Fig 19) shows that this enhancer, unlike the L3/4 and IRE enhancers, is not muscle-specific. Thus the higher expression of R3/4

constructs in myofiber cultures than in myoblast cultures may reflect not the process of myogenic differentiation per se, but some other difference between differentiated and undifferentiated cultures, e.g., the medium conditions or proliferative status of the cells. In our experiments with 10T1/2 fibroblasts the medium conditions matched those of differentiated (but not undifferentiated) L6 cultures, so that like the differentiated muscle cells the 10T1/2 cultures were quiescent nonproliferating cells. This suggests the hypothesis that the R3/4 enhancer may be more active in quiescent than in proliferating cells in general, without regard to developmental cell lineage. This hypothesis could be tested in future studies by assessing R3/4 enhancer activity in 10T1/2 cells (and in other nonmuscle cell lines) under both proliferating and quiescent culture conditions.

The (-198)IRE construct was not expressed in 10T1/2 cultures (as expected from previous studies showing that the IRE is a muscle-specific enhancer (29)) despite the fact that it contains all of the cis-elements present in the R3/4 construct, which is expressed in 10T1/2 cultures. This suggests that the extra DNA sequence present in the IRE, namely the leftmost 30 bases including the E box, inhibits the expression that the R3/4 enhancer would otherwise drive in 10T1/2 cells. This suggests a model of muscle-specificity in which some element in the left-most 30 bases of the IRE acts to inhibit expression in nonmuscle cells. It is conceivable that this element could be the E box itself, as several negatively-acting E box binding factors have been identified including ZEB (42) and the Hes factors (43). However this would depart from the conventional view that the E box confers muscle-specific expression through a positive (not a negative) mechanism in which muscle-specific E box-binding factors of the myoD family bind to the E box and activate expression only in muscle cells (20). The possibility that the E box might be the non-muscle silencer element in the left-most 30 bases could be addressed in future studies by site-directed mutagenesis, and

transfection studies in muscle and nonmuscle cells.

We found that upon introduction into adult soleus muscle by intramuscular injection/electroporation the IRE construct showed marked preferential expression in fast as opposed to slow fibers. This pattern corresponds to the fast-fiber-specificity of the TnIfast gene (44) and of IRE-driven transgenes in transgenic mice (15, 18). However it was a departure from previous observations made using intramuscular injection without electroporation. In these previous studies, transferred TnIfast plasmid constructs showed expression in only a small number of muscle fibers in each muscle and, more important, this expression was similar in fast and in slow fibers, i.e., TnIfast fiber-type-specificity was deregulated in that experimental setting (45). This deregulation was attributed to a low level of muscle fiber segmental necrosis/regeneration which is associated with the intramuscular injection procedure. Although regenerated fiber segments were a minority of the muscle fiber population, they appeared to be especially targetted for gene transfer. Moreover it was already known that TnIfast fiber-type-specificity is perturbed by regeneration in that regenerated slow muscle fibers were known to activate TnIfast expression (50).

From these previous findings it appeared that gene transfer to adult muscle would not be applicable to the study of the molecular genetic mechanisms of TnIfast fiber-type-specific expression. Thus, our finding of proper fast-fiber-enriched expression of TnIfast IRE-based constructs in the combined intramuscular injection/electroporation protocol represents an important development in experimental studies of fiber type specificity. Presumably when electroporation is used there is direct transfer of injected plasmids into adult muscle fibers, without passing through a necrosis/regeneration stage.

We found that not only the IRE construct but also the L3/4 and R3/4 constructs were also

effectively expressed when transferred into soleus muscle. Thus, despite the absence of elements thought to be of quantitative importance, these partial IRE derivatives are effectively expressed in muscle fibers *in vivo* in addition to being detectably expressed in transfected cell cultures. Our results clearly showed that, like the IRE construct, the L3/4 construct gave preferential expression in fast muscle fibers. Thus the right-most 40 bases of the IRE including the CAGG element, which are missing in the L3/4 construct do not play any essential role in fiber type specificity. On the other hand we found that fast fiber type specificity was severely perturbed in the R3/4 construct. This construct showed similar expression in fast and slow muscle fibers. This result clearly implicates the left-most 30 bases of the IRE, which include the E box, in directing fast fiber type specific expression of the IRE. Moreover it is evident that a negative mechanism is involved, i.e., the key element in the left-most 30 bases functions by repressing expression in slow fibers.

Previous studies have implicated negatively-acting mechanisms in fiber type specific expression of TnIfast genes. Hallauer and Hastings showed that TnIfast transgenes are activated during primary muscle fiber formation (18), even though most primary fibers are destined to mature as slow fibers which will no longer express TnIfast transgenes. Moreover Dhoot and Perry showed evidence that the lack of expression of TnIfast in adult soleus muscle slow fibers was based at least in part on repression that occurred during early postnatal life (47, 48). Our results indicating that negative mechanisms contribute to the fast fiber specific expression of TnIfast IRE-driven constructs is consistent with these findings. The nature of the element(s) responsible for repressing IRE-driven expression in slow fibers is not known. Site-directed mutagenesis of the left-most 30 bases coupled with gene expression assay by transfer into adult soleus muscle provides an approach for future studies to map and characterize such elements. A reasonable initial focus for such studies would be

the E box. Indeed there are reasons to believe that E box-binding members of the MyoD family of transcription factors could play a role in differential expression among muscle fibers. Hughes et al have shown that MyoD and myogenin are differentially expressed in adult muscles, with MyoD being preferentially expressed in fast glycolytic fibers and myogenin in slow (and fast oxidative) fibers (21, 22). Thus it is possible to envisage a mechanism in which myogenin (but not MyoD) could negatively regulate IRE-driven expression in slow fibers. Studies for the future might therefore include co-transfer into adult muscle of TnIfast reporter plasmids along with plasmids encoding myogenin or MyoD to document whether these factors have different effects on IRE-driven gene expression.

REFERENCES

1. Pette D and Staron R S. 1990. Cellular and molecular diversities of mammalian skeletal muscle fibers. Rev Physiol Biochem Pharmacol 116.
2. Hennig R and Lomo T. 1985. Firing patterns of motor units in normal rats. Nature 314: 164-66.
3. Pette D and Staron R S. 1997. Mammalian skeletal muscle fiber type transitions. Int Rev of Cytology 170: 143-223.
4. Schiaffino S and Reggiani C. 1996. Molecular diversity of myofibrillar proteins: gene regulation and functional significance. Physiological reviews 76 (2): 371-423.
5. Burke RE. 1994. Physiology of motor units. In *Myology* (A.G., E., and C., F.-A., eds) Vol 1 pp 464-483, McGraw-Hill, Montreal.
6. Miller JB. 1991. Myoblasts, myosins, MyoDs, and the diversification of muscle fibers. Neuromuscular Disorders 1 (1): 7-17.

6a. Wolf JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, and Felgner PL. 1990. Direct gene transfer into mouse muscle *in vivo*. Science 247:1465-68.

7. Kelly R, Alonso S, Tajbakhsh S, Cossu G and Buckingham . 1995. Myosin light chain 3F regulatory sequences confer regionalized cardiac and skeletal muscle expression in transgenic mice. J Cell Biol 129: 383-96.
8. Donoghue MJ, Alvarez JD, Merlie JP and Sanes JR. 1991. Fiber type- and position-dependent expression of a myosin light chain -CAT transgene detected with a novel histochemical stain for CAT. J Cell Biol 115: 423-34.

9. Spitz F, Salminen M, Demignon J, Kahn A, Daegelen D and Maire P. 1997. A combination of MEF3 and NF1 proteins activates transcription in a subset of fast-twitch muscles. Mol Cell Biol. 17: 656-66.

10. Spitz F, Demignon J, Demeurie J, Sabourin JC, Kahn A Daegelen D and Maire P. 1998. A binding site for nuclear receptors is required for the differential expresssion of the aldolase A fast-twitch muscle promoter in body and head muscles. J Biol Chem 273: 561-67.

11. Spitz F, De Vasconcelos ZA, Chatelet F, Demignon J Kahn A, Mira JC, Maire P and 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.

12. Spitz F, Demignon J, Kahn A, Daegelen D and Maire P. 1999. Developmental regulation of the aldolase A muscle-specific promoter during in vivo muscle maturation is controlled by a nuclear receptor binding element. J Mol Biol 289: 893-903.

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

14. Calvo S, Venepally P, Cheng J and A Buonanno. 1999. Fiber-type specific expression of the troponin I slow gene is regulated by multiple elements. Mol Cell Biol. Jan; 19 (1): 515-25.

15. Nakayama M, Stauffer J, Cheng J, Banerjee-Basu S, Wawrousek E and A Buonanno. 1996. Common core sequences are found in skeletal muscle slow- and fast-fiber-type specific regulatory elements. Mol Cell Biol 16: 2408-17.

16. Hallauer PL, Bradshaw HL and KEM Hastings. 1993. Complex fiber-type-specific expression of fast skeletal muscle troponin I gene constructs I transgenic mice. Development 119: 691-701.

17. Hallauer PL, Hastings KEM and AC Peterson. 1988. Fast skeletal muscle-specific expression of quail troponin I gene in transgenic mice. Mol Cell Biol 8:5072-5079.

18. Hallauer PL and Hastings KEM. 2002. The TnIfast IRE enhancer: Multistep developmental regulation during skeletal muscle fiber type differentiation. Devel Dynam 224: 422-31.

19. Hallauer PL and Hastings KEM. 2002. Co-regulation of fast contractile protein transgene and glycolytic enzyme expression in mouse skeletal muscle. American Journal of Physiology: Cell Physiology 282: C113-24.

20. Firulli A B and Olson E N. 1997. Modular regulation of muscle gene transcription: a mechanism for muscle cell diversity. TIG 13 (9): 364-69.

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

22. Hughes S M, Koishi K, Rudnicki M and 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.

23. Rudnicki MA, Braun T, Hinuma S and Jaenisch R. 1992. Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. Cell 71: 383-90.

24. Farah CS and Reinach FC. 1995. The troponin complex and regulation of muscle contraction.. FASEB J 9: 755-767.

25. Dhoot GK, and Perry SV. 1979. Distribution of polymorphic forms of troponin components and tropomyosin in skeletal muscle. Nature 278: 714-18.

25a. Hastings K E M.1997. Molecular evolution of the vertebrate troponin I gene family. Cell Struct Funct 22: 205-11.

26. Corin SJ, Levitt LK, O'Mahoney JV, Joya JE, Hardeman EC and R Wade. 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.

27. Lin H, Yutzey KE and Stephen E Konieczny. 1991. Muscle-specific expression of the troponin I gene requires interactions between helix-loop-helix muscle regulatory factors and ubiquitous transcription factors. Mol Cell Biol 11: 267-280.

28. O'Mahoney JV, Guven KL, Lin J, Joya JE, Robinson CS, Wade RP and Hardeman EC. 1998. Identification of a novel slow-muscle-fiber enhancer binding protein, MusTRD1. Mol Cell Biol 18: 6641-52.

28a. Wheeler MT, Snyder EC, Patterson MN and Swoap SJ. 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 276: C1069-78.

29. Yutzey KE, Kline RL and Koniezny SE. 1989. An internal regulatory element controls troponin I gene expression. Mol. Cell. Biol. 9: 1397-1405.

30. Banerjee-Basu S and 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: 7019-7028.

31. Breitbart RE, Liang CS, Smoot LB, Laheru DA, Mahdavi V and B Nadal-Ginard.1993. A fourth human MEF2 transcription factor, hMEF2D, is an early marker of the myogenic lineage. Development 118: 1095-1106.

32. Pollock R and R Treisman. 1991. Human SRF-related proteins: DNA-binding properties and potential regulatory targets. Genes Dev 5: 2327-2341.

33. Dodou E, Sparrow DB, Mohun T and R Treisman. 1995. MEF2 proteins, including MEF2A, are expressed in both muscle and non-muscle cells. Nucleic Acids Res. 23:4267-4274.

34. Molkentin JD and EN Olson. 1996. Combinatorial control of muscle development by basic helix-loop-helix and MADS-box transcription factors. Proc Natl Acad Sci USA 93: 9366-9373.

35. Parmacek MS, Ip HS, Jung F, Shen T, Martin JF, Vora AJ, Olson EN and JM Leiden. 1994. A novel myogenic regulatory circuit controls slow/cardiac troponin C gene transcription in skeletal muscle. Mol Cell Biol. 14: 1870-1885.

36. Schreier T, Kedes L and R Gahlman. 1990. Cloning, structural analysis and expression of the human slow-twitch skeletal muscle cardiac troponin C gene. J Biol Chem265: 21247-21253.

37. Edmondson DG and EN Olson. 1989. A gene with homology to the myc similarity region of Myo D1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. Genes Dev 3: 628-640.

38. Horlick RA, and Benfield PA. 1989. The upstream muscle-specific enhancer of the rat muscle creatine kinase gene is composed of multiple elements. Mol Cell Biol. 9: 2396-2413.

39. Hidaka K, Yamamoto I, Arai Y and 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.

40. Wu H, Naya FJ, McKinsey TA, Mercer B, Shelton JM, Chin ER, Simard AR, Michel RN, Bassel-Duby R, Olson EN and Williams RS. 2000. MEF2 responds to multiple calcium-regulated signals in the control of skeletal muscle fiber type. EMBO J 19: 1963-73.

41. McMahon JM, Signori E, Wells KE, Fazio VM and Wells DJ. 2001. Optimization of electrotransfer of plasmid into skeletal muscle by pretreatment with hyaluronidase - increased expression with reduced muscle damage. Gene Therapy 8: 1264-1270.

42. Brabletz T, Jung A, Hlubek F, Löhberg C, Meiler J, Suchy U and Kirchner T. 1999. Negative regulation of CD4 expression in T cells by the transcriptional repressor ZEB. Int Immunol 11:1701-8.

43. Kageyama R, Nakanishi S. 1997. Helix-loop-helix factors in growth and differentiation of the vertebrate nervous system. Curr Opin Genet Dev 7: 659-65.

44. Konieczny SF and Emerson CP. 1987. Complex regulation of the muscle-specific contractile protein (troponin I) gene. Mol Cell Biol 7: 3065-75.

45. Hallauer PL, Karpati G and Hastings KEM. 2000. Skeletal muscle gene transfer: regeneration-associated deregulation of fast troponin I fiber type specificity. Am J Physiol Cell Physiol 278: C1266-74.

46. Yaffe D. 1968. Retention of differentiation potentialities during prolonged cultivation of myogenic cells. Proc Natl Acad Sci USA 61: 477-483.

47. Dhoot GK, Perry SV The components of the troponin complex and development in skeletal muscle. Exp Cell Res. 1980 127, 75-87.

48. Dhoot GK, Perry SV. The effect of denervation on the distribution of the polymorphic forms of troponin components in fast and slow muscles of the adult rat. Cell Tissue Res. 1982 225, 201-215.

49. Zevin-Sonkin D, and Yaffe D. 1980. Accumulation of muscle-specific RNA sequences during myogenesis. Dev Biol 74: 326-34.

50. Sutherland CJ, Esser KA, Elsom VL, Gordon ML and Hardeman EC. 1993. Identification of a program of contractile protein gene expression initiated upon skeletal muscle differentiation. Devel Dynam 196: 25-36.

51. Nikovits W, Mar JH, and Ordahl CP. 1990. Muscle-specific activity of the skeletal troponin I promoter requires interaction between upstream regulatory sequences and elements within the first transcribed exon. Mol. Cell. Biol. 10: 3468-3482.