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Secondary enhancers synergise with primary enhancers to guarantee fine-tuned muscle gene expression

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ABSTRACT

Although tight quantitative control of gene expression is required to ensure that organs and tissues function correctly, the transcriptional mechanisms underlying this process still remain poorly understood. Here, we describe novel and evolutionary conserved secondary enhancers that are needed for the regulation of the expression of Troponin I genes. Secondary enhancers are silent when tested individually in electroporated muscles but interact with the primary enhancers and are required to precisely control the appropriate timing, the tissue and fibre specificity, and the quantitative expression of these genes during muscle differentiation. Synergism is completely dependent of the fully conserved MEF2 site present on the primary enhancers core of skeletal muscle Troponin I genes. Thus, while each of these paired enhancers has a different function, the concerted action of both is crucial to recapitulate endogenous gene expression. Through comparative genomics, we predict that this mechanism has also arisen in other mammalian muscle genes. Our results reveal the existence of a novel mechanism, conserved from flies to mammals, to fine-tune gene expression in each muscle and probably other tissues.

Introduction

Enhancers are the most prevalent class of *cis*-regulatory DNA elements that determine when and where a given gene is expressed during development and, in recent years, many *cis*-acting elements that regulate spatio-temporal gene expression have been described (Arbeitman et al., 2002; Arnone et al., 2004; Brown et al., 2007; Carvajal et al., 2008; Hong et al., 2008; Philippakis et al., 2006). Enhancers interact with basal promoters to precisely modulate the rates of transcription, the quantitative levels of gene expression, and to confer spatial and temporal specificity. Hence, they guarantee the correct functioning of tissues and organs by ensuring that thresholds of a specific regulator are reached appropriately, and that the correct stoichiometry is established for the assembly of multi-protein complexes. Thus, a better understanding of the complex mechanisms underlying their activity is crucial to be able to define how gene expression is controlled.

Muscle differentiation is an exceptional model system to investigate the precise developmental regulation of tissue-specific genes. In *Drosophila*, two separate enhancers control highly dynamic and exquisitely coordinated patterns of sarcomeric gene expression (Garcia-Zaragoza et al., 2008; Marco-Ferreres et al., 2005; Mas et al., 2004). When tested in transgenic animals, each enhancer of the pair is transcriptionally active and it can independently drive similar expression patterns to the paired enhancers in all muscle-types. Interestingly, although each enhancer can determine muscle specification, it cannot recapitulate the levels of expression of the endogenous gene in each muscle subtype. Thus, the concerted action of both enhancers in a differentiated and synergistic manner is required to control global regulation *in vivo* (Garcia-Zaragoza et al., 2008; Marco-Ferreres et al., 2005; Marin et al., 2004; Mas et al., 2004). Generally, one of the enhancers of the pair is situated at the 5′ flanking region of the gene while the other is associated with the first intron. In general, enhancers are occupied by macromolecular complexes that interact among themselves, as well as with proximal promoters to trigger transcription. Enhancer occupancy and the composition of the binding complexes may vary depending on the muscle subtype and the developmental stage in order to ensure the appropriate spatio-temporal expression of the gene in question. On the basis of our previous studies, we suggested that paired functional enhancers might arise from duplication to guarantee fibre specificity and the fine-tuning of gene expression levels in each particular muscle (Garcia-Zaragoza et al., 2008; Marco-Ferreres et al., 2005; Mas et al., 2004).

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Recently, a similar enhancer organisation was described in several *Drosophila* genes, such as some neurogenic target genes of the Dorsal transcription factor, a protein related to the mammalian NF-κB, that controls the dorsal–ventral patterning of the early embryo (Hong et al., 2008). These genes contain two enhancers referred to as primary and secondary enhancers. The primary enhancer usually lies in the 5′ flanking region of the gene, while the secondary enhancer in some cases maps within 5 kb of the transcription initiation site, although in some other cases it may lie tens of kilobases away. Indeed, it was suggested that these remote secondary enhancers, which mediate activities overlapping the primary enhancer and that ensure precise and reproducible patterns of gene expression during embryogenesis, be known as "shadow enhancers" (Hong et al., 2008).

Within this scenario, it is relevant to determine whether the mechanisms used by *Drosophila* to fine-tune the expression of muscle and neurogenic genes are evolutionary conserved (Cervera et al., 2006; Hong et al., 2008; Marco-Ferreres et al., 2005; Marin et al., 2004; Mas et al., 2004). In other words, how do mammalian genes fine-tune spatio-temporal gene expression? The Troponin I gene family contains three related genes that encode slow (*s*TnI; TNNI1) and fast (*f*TnI; TNNI2) skeletal muscle, and cardiac (*c*TnI; TNNI3) muscle isoforms. These three genes have been widely used as model system to study muscle-specific gene transcription in mammals (Cullen et al., 2004; Dhoot and Perry, 1979; Hastings, 1997). The presence of a *cis*-regulatory element in each gene has been shown to be relevant in order to obtain fibre-type specific expression in transgenic mice. Thus, a 128 bp troponin I *S*low *U*pstream *R*egulatory *E*lement (SURE) and a 144 bp troponin I *F*ast *I*ntronic *R*egulatory *E*lement (FIRE) have been described that restrict *s*TnI and *f*TnI expression to slow and fast fibres, respectively, thereby conferring fibre-type specificity (Calvo et al., 1999; Calvo et al., 2001; Hallauer and Hastings, 2002; Levitt et al., 1995; Nakayama et al., 1996; Rana et al., 2008; Yutzey et al., 1989). In addition, it has been demonstrated that a 356 bp upstream regulatory element within *cardiac* Troponin I controls heart muscle specificity (Di Lisi et al., 1998; Di Lisi et al., 2007). However, FIRE-driven transgene expression does not exactly correlate with the endogenous *f*TnI gene (Hallauer et al., 1993; Hallauer and Hastings, 2002).

To investigate the mechanisms that mediate the precise developmental regulation of tissue-specific genes in mammals, we have focused our studies on the TnI gene family. Here, we describe the identification of new evolutionary conserved enhancers that act as secondary enhancers in all three mammalian TnI genes.

Materials and methods

Sequence analysis and comparative genomics

The evolutionary conserved regions in the genomic sequences containing the *s*TnI, *f*TnI and *c*TnI genes of several mammal species (mouse, rat, dog and human) were analysed using the ECR Browser (ecrbrowser.dcode.org: Ovcharenko et al., 2004). Conserved potential binding sites for transcription factors were analysed with the Mulan (mulan.dcode.org) and MultiTF (multitf.dcode.org) servers.

Reporter constructions

The SURE (−2508/+56), SIRE (+36/+3021), SIRE2 (+2999/+5468), SURE–SIRE (−2508/+3021) fragments from the *s*TnI gene, the FURE (−1243/+37), FIRE (+17/+672), FIRE2 (+651/+1359), FURE–FIRE (−1243/+672), FURE–FIRE–FIRE2 (−1243/+1359), FURE–FIRE2 (−1243/+37 and +651/+1359), FURE promoterless (pl) (−1243/−86), FURE–FIRE pl (−1243/+672 Δ−86/+17), core FIRE (+251/+522), FURE–core FIRE (−1243/−86 and +251/+522) fragments from the *f*TnI gene and the CURE (−230/+126), CIRE (+127/+1363), CURE–CIRE (−230/+1363) fragments from the *c*TnI gene were amplified by PCR with specifically designed primers (primer sequences are available upon request). FIRE-ΔMEF and FURE–FIRE-ΔMEF are exactly the same than FIRE and FURE–FIRE pl, respectively, in which the MEF2 binding site has been deleted by PCR following standard protocols. All fragments were cloned into a pBluescript-derived vector upstream of the β-globin minimal promoter and the LacZ gene (the plasmid was a gift from M. Manzanares).

Cell culture and transfections

C2C12 mouse myoblasts were maintained in DMEM (Gibco) supplemented with L-glutamine, gentamycin and 10% foetal bovine serum (FBS). Differentiation of confluent monolayers of myoblasts was induced by replacing FBS with 2% horse serum (differentiation medium). Cultured cells were maintained at 37 °C in 5% CO_2.

For transient transfection assays, 1.75×10^5 cells were plated in 35 mm culture dishes and after 24 h, proliferating myoblasts were transfected using Lipofectamine (Invitrogen) with 1.5 μg of each LacZ construct. Differentiation was induced 24 h after transfection and β-galactosidase activity was analysed after 0 and 5 days of differentiation. All transfection experiments were repeated at least five times unless otherwise indicated.

To normalise for transfection efficiency, the cells were co-transfected with 0.1 μg of either pEGFP when the cells were to be analysed in Western blots, or pCMVluc when the cells were used to measure β-galactosidase activity. Expression of EGFP and Luciferase is driven by a CMV promoter.

Primary cardiomyocyte culture and transfection

One day old rat cardiomyocytes were isolated and cultured using a commercially available neonatal rat/mouse cardiomyocyte isolation kit (Cellutron Life Technologies) according to the manufacturer's instructions. For transient transfection, 7×10^5 cells were plated in 35 mm culture precoated dishes and the cells were maintained in serum free medium at 37 °C in 5% CO_2. Cardiomyocyte cultures were transfected 48 h after plating using Lipofectamine (Invitrogen) with 1.5 μg of each LacZ construct and 0.1 μg of pCMVluc as a control for transfection efficiency. Differentiation was induced 24 h after transfection and β-galactosidase activity was analysed after 0 and 3 days of differentiation. Transfection experiments were repeated 4 times and each experiment contained triplicates for each construct.

X-Gal staining

Cells were rinsed in cold PBS and then fixed for 10 min in 2% formaldehyde and 0.2% glutaraldehyde in PBS. The cells were then washed twice for 10 min in PBS and stained for 4 h at 37 °C in a PBS solution containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$ and X-Gal 1 mg/ml. The cells were washed in PBS and then observed under a light microscope. The same staining procedure was used for muscle sections on slides to detect fibres expressing LacZ. Incubation times varied from 2 to 24 h to visualise the LacZ expressing fibres and the staining intensity was monitored under the microscope at regular time intervals.

Quantification of β-galactosidase activity

Cells were lysed in Glo Lysis Buffer (Promega) and β-galactosidase activity was determined with the Beta-Glo Assay System (Promega). Briefly, 40 μl of each cell extract was mixed with 40 μl of the Beta-Glo reagent and incubated for 30 min. Luminescence was then measured in an Optocom I luminometer and the β-galactosidase units were normalised to the luciferase activity of the pCMVLuc control plasmid. Luciferase activity was determined in the same extracts with the Luciferase Assay System (Promega), following the manufacturer's instructions. Relative β-galactosidase activity is given as the β-gal/Luc

ratio and all data were normalised to the protein concentration of each sample measured with the Bio-Rad Protein Assay (Bio-Rad) or Micro BCA Protein Assay (Pierce).

Western blotting

Rat muscles were homogenised in lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitors in PBS) and cleared at 10,000 rpm for 10 min at 4 °C. Each protein extract (20 μg) was loaded onto 10% SDS-polyacrylamide gels, and transferred to PVDF membranes (Inmobilon, Millipore). Western blotting was carried out as described previously (Marco-Ferreres et al., 2005) and the membranes were probed with antibodies against TnI (H-170, Sta. Cruz Biotech.) and GFP (molecular probes) diluted 1:1000, or with an antibody against Luciferase (251-550, Sta. Cruz Biotech.) or β-galactosidase (Cappel) at 1: 2000.

Muscle transfection by in vivo electroporation

The specificity of the different plasmid constructs for slow and fast muscles was studied in adult 200–250 g male Wistar rats. Soleus and extensor digitorum longus (EDL) muscles were transfected by intramuscular injection of plasmid DNA (25 μg), followed by electroporation to increase gene transfer efficiency as published previously (Serrano et al., 2001). Co-transfection of 2.5 μg of a GFP-expressing plasmid was used to identify the transfected fibres. The muscles were removed 7 days after electroporation, frozen in isopentane, cooled in liquid nitrogen and stored at −80 °C before they were analysed.

Histological procedures and data analysis

Serial cryostat cross-sections (10 μm thickness) from control and transfected muscles were obtained and examined by standard immunohistochemical procedures for the expression of LacZ (see above), GFP and myosin heavy chain (MHC) isoforms (Serrano et al., 2001). The primary monoclonal antibodies employed were A4.840 specific to rat slow MHC (Developmental Studies Hybridoma Bank); A4.74, which stains rat 2A strongly and 2X/2D MHC weakly (Developmental Studies Hybridoma Bank); and BF-F3 specific for rat 2B MHC (Schiaffino et al., 1989). Micrographs were obtained on a Leica DMR microscope equipped with a camera and serial digitalised images were taken from the same GFP positive areas on different cryosections to study the correspondence between the LacZ and MHC expression in individual muscle fibres. Five muscles of each type were analysed for each LacZ expressing plasmid construct transfected.

Results

Evolutionary conserved sequences and modules in the slow and fast TnI genes

Conserved non-coding regions are likely to define elements that are important for the regulation of gene transcription. Indeed, comparative genomic tools have helped to identify a significant number of functional regulatory sequences based on evolutionary constraints (Cheng et al., 2007; Konig et al., 2002; Li et al., 2007). To determine whether mammalian TnI genes follow the same mechanistic logic as *Drosophila* muscle genes, involving conserved regulatory modules that interact synergistically to fine-tune gene expression, we performed a detailed in silico and functional analysis of new regulatory elements in the two mouse skeletal Troponin I genes: *s*TnI and *f*TnI genes. We examined the sequences of the *s*TnI and *f*TnI loci using the ECR Browser bioinformatics tool (Ovcharenko et al., 2004), comparing both loci from the human genome with those from mouse, rat and dog. With a conservation cut-off of 70% identity over 100 base pairs, values that are reasonable to determine only significant alignments, several highly conserved non-coding regions were identified in both genes, which lay upstream of the transcription start site and in the first introns (Fig. 1 and Supplementary Figs. S1 and S2). In the mouse *s*TnI gene, our bioinformatics analysis revealed four highly conserved modules in the 5′ upstream region (at positions −2462/−2252, −2101/−1892, −907/−725 and −115/+97) and two modules in introns 1 and 2 (at positions +1006/+1689 and +3704/+3916, Fig. 1A and Supplementary Fig. S1). The upstream −907/−725 sequence includes the SURE primary enhancer previously described in rat (Banerjee-Basu and Buonanno, 1993; Calvo et al., 1996; Nakayama et al., 1996) and the −115/+97 fragment contains the basal promoter and exon 1. In the mouse *f*TnI gene, three conserved modules were identified in the upstream region (at positions −1153/−972, −904/−682 and −262/+125), as well as two conserved fragments in introns 1 and 2 (at positions +251/+522 and +952/+1212, Fig. 1B and Supplementary Fig. S2). The +251/+522 sequence located in intron 1 includes the primary enhancer FIRE reported to be involved in muscle specificity in the quail (Hallauer et al., 1993; Hallauer and Hastings, 2002; Yutzey et al., 1989).

Each one of the primary enhancers, SURE and FIRE, contain in their sequences a fully conserved MEF2 binding site. Secondary enhancers do not contain such conserved sites (see Supplementary Figs. S1 and S2).

Thus, as previously reported for the *Drosophila* muscle genes, sequence comparison revealed a modular organisation, whereby regulatory sequences lie in the putative 5′ flanking and in the intronic regions of both mouse skeletal TnI genes (Marco-Ferreres et al., 2005; Mas et al., 2004).

Primary and secondary enhancers from mouse slow and fast Troponin I genes act synergistically to activate reporter gene expression in C2C12 myotubes

To test whether the conserved upstream and intronic regions identified play a role in quantitative transcriptional regulation of TnI genes, we performed classical transcription assays. In order to avoid the loss of functional interactions, we analysed the conserved 5′ flanking and intronic regions in the mouse *s*TnI and *f*TnI genes, preserving all the conserved elements as in our previous studies in *Drosophila* (see Fig. 2, Materials and methods and Mas et al., 2004). Although the size of the fragments differed from those reported in previous studies, we have used the same terminology to avoid confusion (Banerjee-Basu and Buonanno, 1993; Hallauer and Hastings, 2002; Nakayama et al., 1996). Therefore, the primary enhancers will be referred to as SURE for *s*TnI and FIRE for *f*TnI, and the potential new secondary enhancers will be named SIRE and SIRE2 for *s*TnI and FURE and FIRE2 for *f*TnI (Fig. 2). To test the predicted regulatory activity and functional interactions between these primary and secondary enhancers, we analysed the transcriptional activity of the different regulatory elements on their own and in the SURE–SIRE and FURE–FIRE combinations.

Transient transfection assays were performed in which these constructs were used to drive the expression of a LacZ reporter gene in C2C12 myoblasts (Fig. 2). C2C12 cells are capable of differentiating into myotubes *in vitro* upon withdrawal of growth factors (Yaffe and Saxel, 1977). In these assays, reporter gene expression was analysed in myoblasts and myotubes through X-Gal staining, as well as by measuring β-galactosidase activity and its accumulation in Western blots (Figs. 3 and 4). When each fragment of the *s*TnI gene was transfected on its own, only SURE was able to drive LacZ expression in differentiated myotubes (Fig. 3). Nevertheless, while a few SIRE transfected cells displayed X-Gal staining (Fig. 3A), no β-galactosidase protein could be detected by Western blotting and

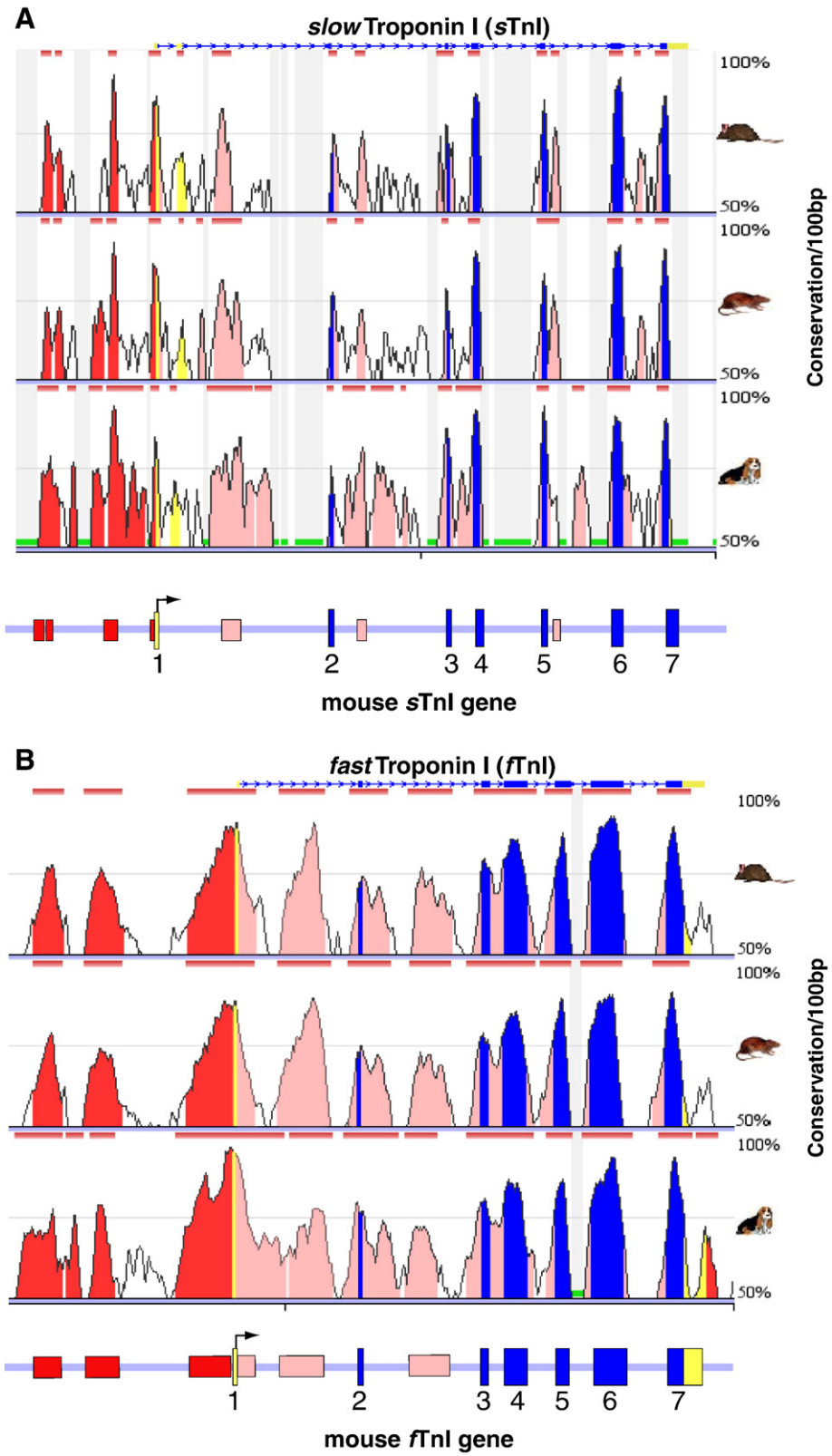


Fig. 1. Evolutionary conserved regions in the *s*TnI (A) and *f*TnI (B) genes. ECR Browser output. The *x*-axis of graphs represents the positions in the human genome and the *y*-axis the percentage identity between the human and the aligned genome. Red peaks represent evolutionary conserved regions (ECR) located in intergenic regions, the pink peaks represent ECRs in intronic regions, the yellow peaks represent ECRs in the non-translated regions and the blue peaks represent ECRs in the coding exons. The parameters used for comparison were fragments >100 bp in length and with >70% identity. In the lower part of each panel, the gene organisation schemes are presented using the same colour code as in the upper panels.

there was no enzyme activity in either SIRE or SIRE2 transfected cells (Figs. 3B and C). As expected, none of the tested fragments could drive LacZ expression in myoblasts (Fig. 3B). Interestingly, the SURE–SIRE fragment drove very high β-galactosidase expression in differentiated myotubes. Thus, the upstream region appears to interact synergistically with the SIRE fragment to increase reporter

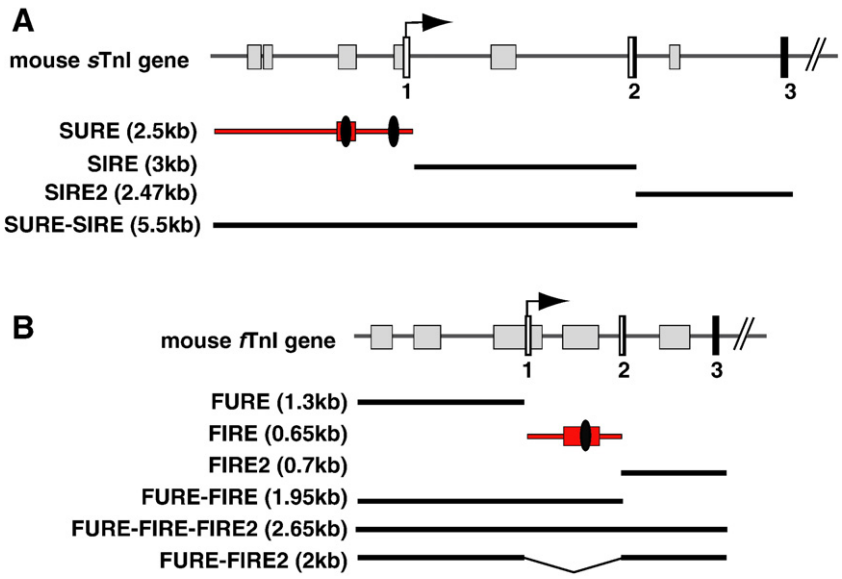


Fig. 2. Reporter gene constructs of mouse TnI skeletal muscle genes. The fragments of the mouse *s*TnI (A) and *f*TnI gene (B) analysed were cloned into a vector derived from pBluescript, upstream of the β-globin minimal promoter and the LacZ gene. Grey boxes indicate non-coding ECRs, while white and black boxes represent the non-coding and coding exons respectively. The core (red box) of both primary enhancers (red lines) is indicated. Black ovals represent MEF2 binding sites.

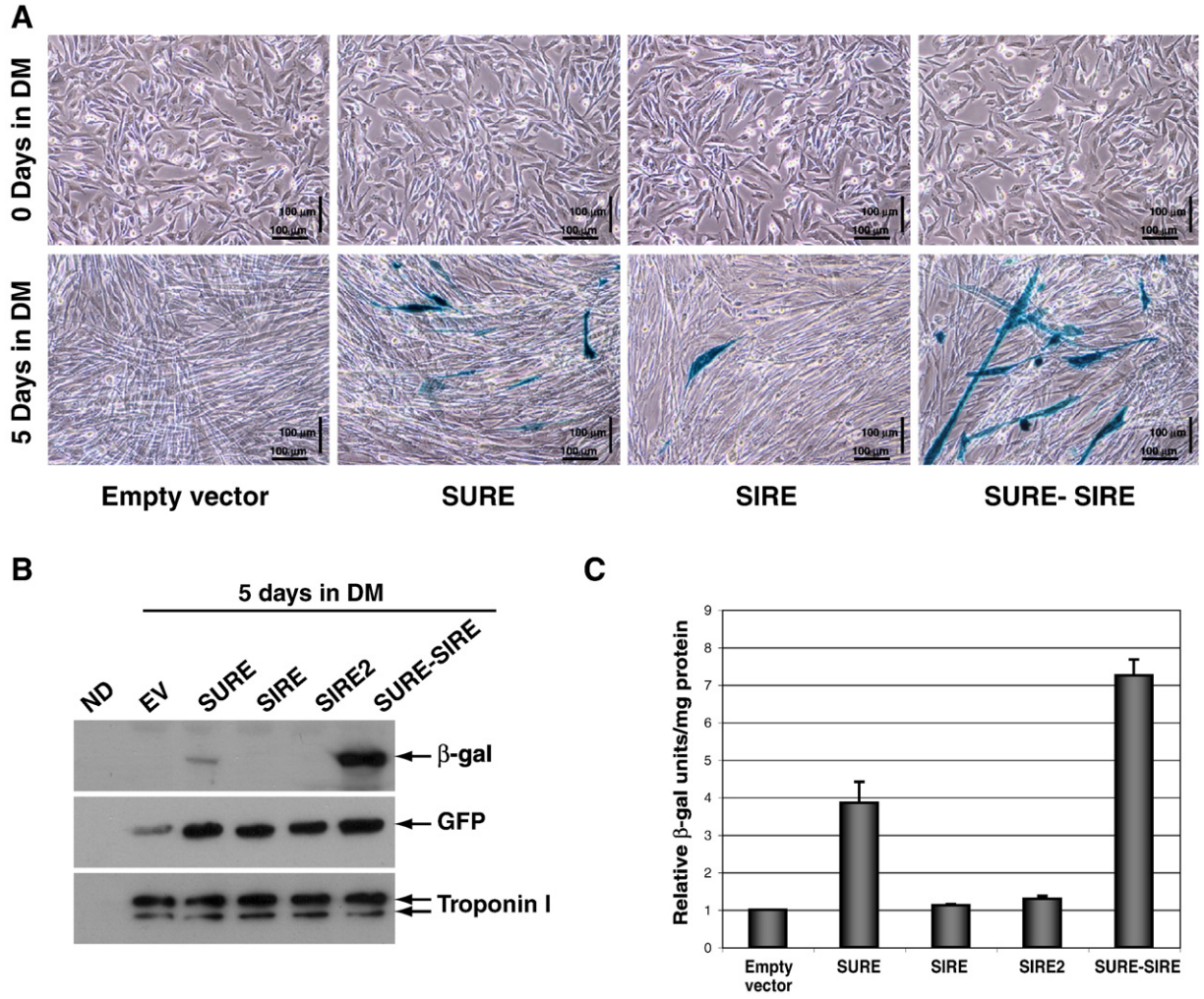


Fig. 3. SURE and SIRE act synergistically to produce high levels of reporter gene expression in C2C12 myotubes. (A) Detection of LacZ expression by X-Gal staining of C2C12 cells transfected with SURE, SIRE and SURE–SIRE constructs. Cells were incubated with X-Gal for at least 4 h. DM, differentiation medium. (B) Determination of β-galactosidase levels in Western blots of extracts from transfected C2C12 myotubes. The levels of GFP and Troponin I used to normalise for transfection efficiency and total protein loading are shown. ND, undifferentiated cells; EV, empty vector. (C) Analysis of β-galactosidase activity in transfected C2C12 myotubes. β-galatosidase activity was normalised to the luciferase activity and to the protein concentration (see Materials and methods). The results are the means ± SE from 7 different experiments (*p*-value between SURE and SURE¬SIRE < 0.0001).

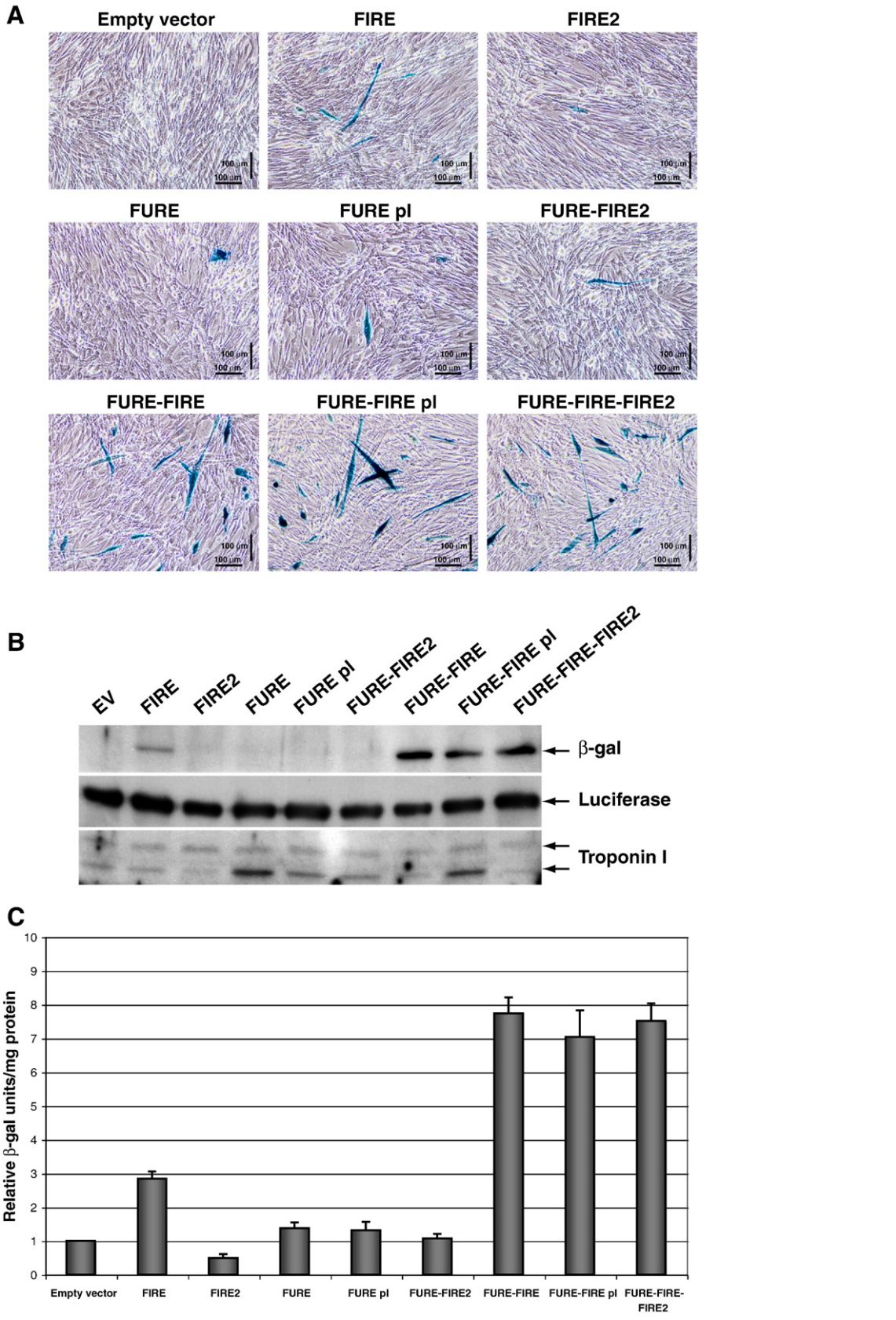
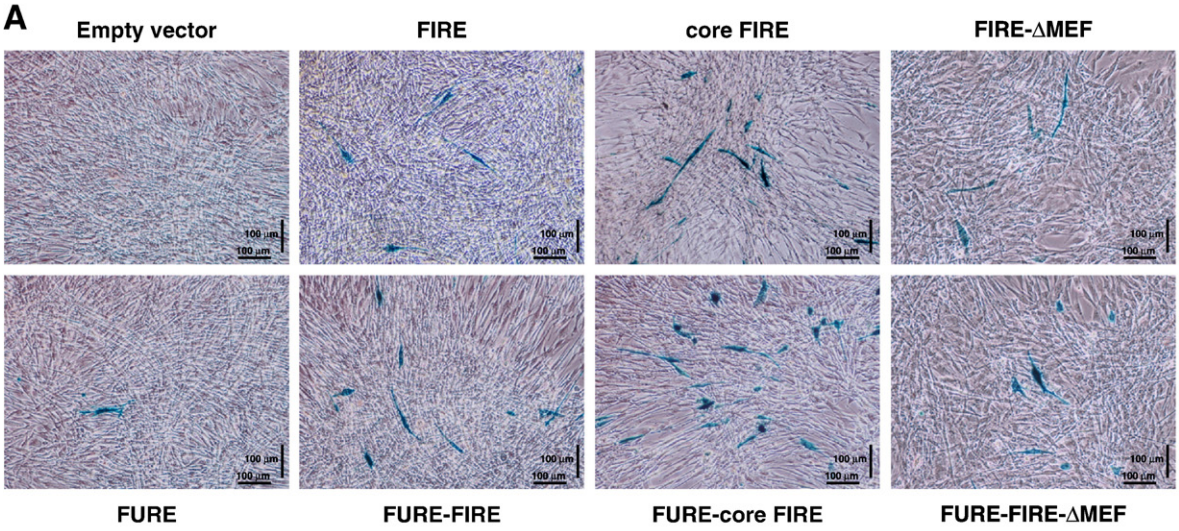
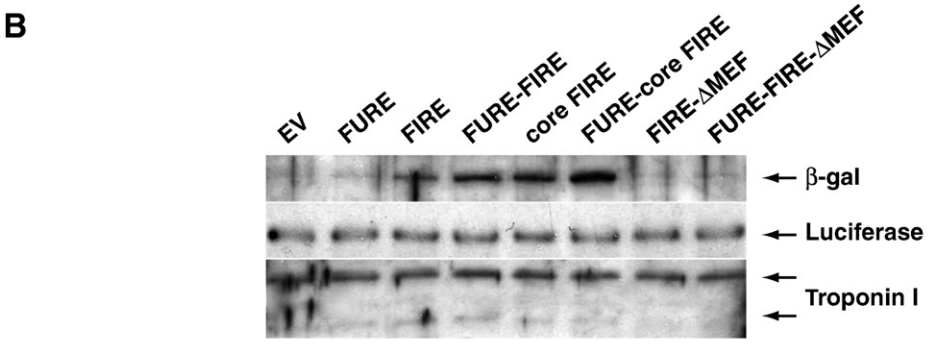


Fig. 4. FURE and FIRE co-operate to produce high levels of reporter gene expression in C2C12 myotubes. (A) Detection of LacZ expression by X-Gal staining of C2C12 cells transfected with the FIRE, FIRE2, FURE, FURE promoterless (pl), FURE–FIRE2, FURE–FIRE, FURE–FIRE pl and FURE–FIRE–FIRE2 constructs. Cells were incubated with X-Gal for at least 4 h. (B) Determination of β-galactosidase levels in Western blots of extracts from transfected C2C12 myotubes. The levels of Luciferase and Troponin I used to normalise for transfection efficiency and total protein loading are shown. A gradient from 4% to 12% SDS-PAGE was used. (C) Analysis of β-galactosidase activity in transfected C2C12 myotubes. The β-galactosidase activity was normalised to the luciferase activity and to the protein concentration (see Materials and methods). The results are the means ± SE from 5 different experiments.

gene expression by approximately 3-fold (Fig. 3C). Hence, while the SIRE fragment is unable to drive LacZ gene expression itself, it contains elements that co-operate with SURE to regulate the levels of transcription.

By contrast, when the *f*TnI gene was analysed the LacZ activity was exclusively observed in myotubes when the FIRE construct, containing the previously described primary enhancer, drove reporter gene expression (Fig. 4). As for *s*TnI, none of the other fragments tested were able to drive LacZ expression in myotubes, e.g. FURE and FIRE2. Hence, FURE and FIRE co-operated synergistically to drive very strong *f*TnI gene expression, as did SURE and SIRE in the *s*TnI gene (almost 3 times higher: Figs. 4B and C).





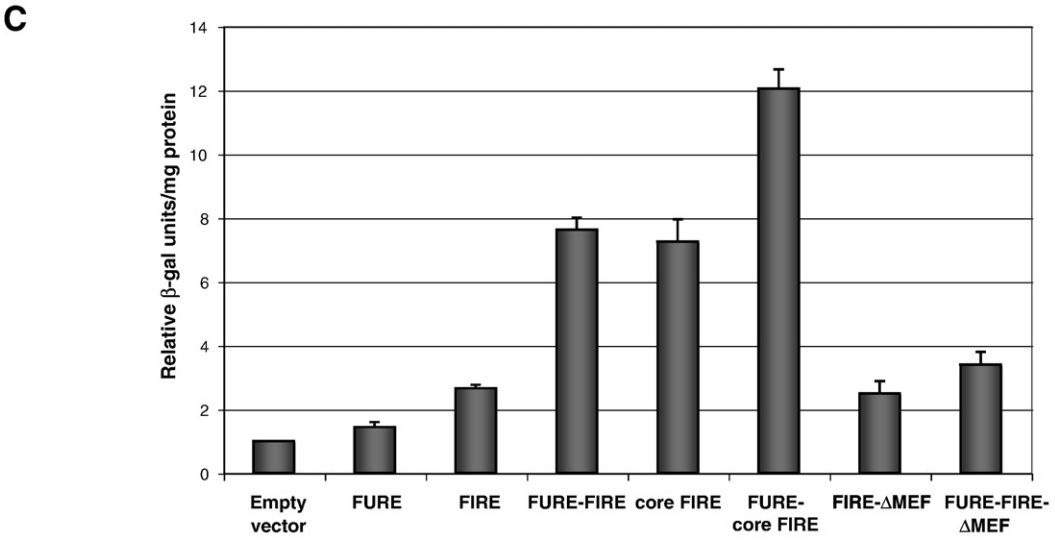


Fig. 5. MEF2 binding site on the FIRE enhancer is essential for the synergism. (A) Detection of LacZ expression by X-Gal staining of C2C12 cells transfected with the FURE, FIRE, core FIRE, FURE–FIRE, FURE–core FIRE, FIRE-ΔMEF, and FURE–FIRE-ΔMEF constructs. These constructs lack the endogenous promoter of *f*TnI. Cells were incubated with X-Gal for at least 4 h. (B) Determination of β-galactosidase levels in Western blots of extracts from transfected C2C12 myotubes. The levels of Luciferase and Troponin I used to normalise for transfection efficiency and total protein loading are shown. (C) Analysis of β-galactosidase activity in transfected C2C12 myotubes. The β-galactosidase activity was normalised to the luciferase activity and to the protein concentration (see Materials and methods). The results are the means ± SE from 4 different experiments.

Since some of the fragments analysed contained the endogenous promoter, we decided to investigate any possible interference between the endogenous and the β-globin promoters present in the plasmids. We examined a set of FURE and FURE–FIRE constructs in which the endogenous promoter was deleted, and no difference was observed in the levels of reporter gene expression with respect to the constructs containing the endogenous promoter (Fig. 4). Similar results were obtained for the *slow* TnI gene when the same experiments were carried out (data not shown). To examine whether the size of the fragments analysed influenced transcriptional activity, we also generated two new constructs in which we added the FIRE2 fragment, of a similar size to FIRE (Fig. 2), to FURE and FURE–FIRE constructs. FURE–FIRE2 construct showed similar levels of LacZ expression to that of FURE alone, even though its size was similar to that of FURE–FIRE. To complete our analysis and to further address whether the length of the enhancer influenced the level of reporter gene expression, we generated the construct FURE–FIRE–FIRE2. Significantly, the reporter gene expression driven by this construct was equivalent to that observed with FURE–FIRE (Fig. 4).

The synergism is fully dependent on the conserved MEF2 binding site present on the primary enhancers

We have demonstrated a clear co-operativity and synergism between two separate regulatory elements (upstream and intronic enhancer elements) in mouse C2C12 cells (Figs. 3 and 4). In this context, the function of the conserved region in FIRE, which has already been shown to be a true enhancer, namely core FIRE (Yutzey et al., 1989), and that of the MEF2 binding site present on it still remained to be determined (Supplementary Fig. S2). To address these questions, the core enhancer was used to drive expression of the Lac Z reporter gene in C2C12 myoblasts alone and in combination with the promoterless FURE element (Fig. 5). As expected, the conserved region was able to synergise with the FURE region. Interestingly, even though the core FIRE drives a much higher activity as compared with that driven by the full intron when assayed alone, the synergism among FURE and core FIRE is clearly shown. Compare lanes in Fig. 5B.

Secondary enhancers from skeletal TnI genes do not harbour any MEF2 binding site but each one of the primary enhancers holds an entirely conserved MEF2 site (see Supplementary Figs. S1 and S2). To test the real contribution of the MEF2 binding sites to quantitative transcriptional regulation of TnI genes, we examined the transcription levels driven by a FIRE element in which the MEF2 binding site has been deleted (FIRE-ΔMEF). The absence of this site in the FIRE primary enhancer totally abolished the synergism between FURE and FIRE elements (Fig. 5). Even more, the FIRE-ΔMEF element was still transcriptionally active when assayed alone. Taken together, these results clearly demonstrate not only that the interaction is dependent on the core FIRE, but that MEF2 transcription factor is a key player in the cooperative interaction between both primary and secondary enhancers.

Fibre-type specificity is conferred by the co-operation of primary and secondary enhancers

The relevance of the synergism effect and fibre-type specificity in mammalian skeletal muscles still remained to be tested *in vivo*. To address this question, we studied the relative expression and the fibre-type specificity of the different *s*TnI and *f*TnI gene constructs *in vivo* by electroporating them into adult rat muscles (SURE–SIRE, FURE–FIRE and each element separately: see Materials and methods). Due to their small size, mouse muscles are very difficult to electroporate efficiently and therefore, we decided to use the rat as a closely related model animal.

To study the expression driven by the different elements in specific muscle fibre-types, we examined LacZ levels in transfected fibres of the Soleus (a slow-twitch oxidative muscle, mainly composed by type 1 slow fibres) and of the EDL muscle (a fast-twitch glycolytic muscle, mainly composed of 2A, 2B and 2X fast fibres). In order to readily identify the electroporated fibres, a GFP expression plasmid was co-transfected with the constructs. Electroporated muscles were examined 7 days after transfection and serial cross-sections of the Soleus and EDL muscles were stained with X-Gal to identify the fibres expressing the reporter gene. In addition, they were immunostained with specific antibodies against GFP to detect transfected fibres, or with specific antibodies against slow type 1 or fast types 2A or 2B MHC isoforms to identify the different fibre types (Schiaffino and Reggiani,

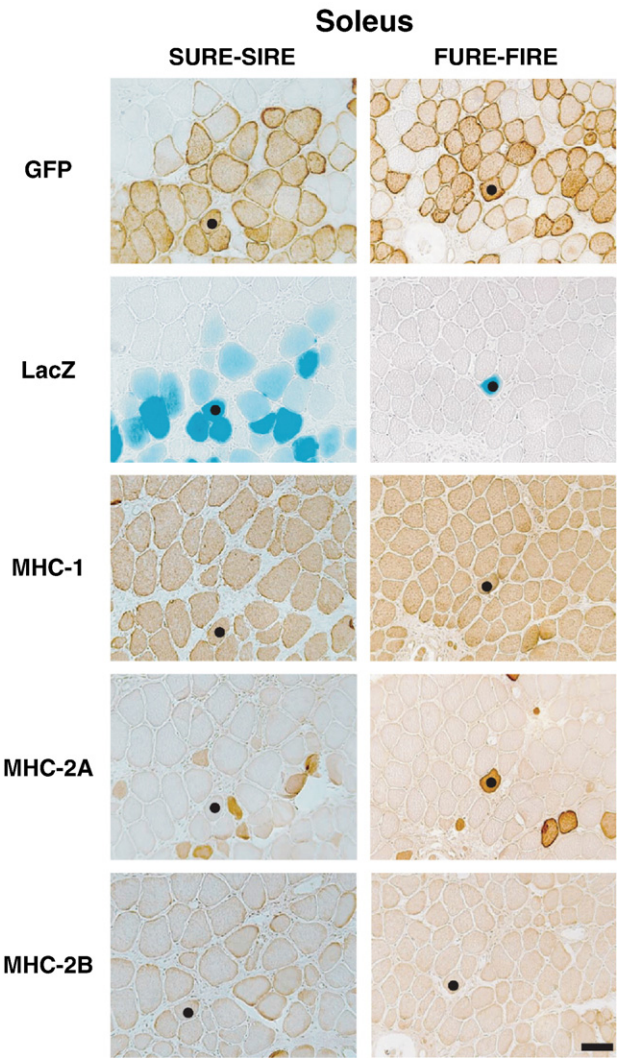
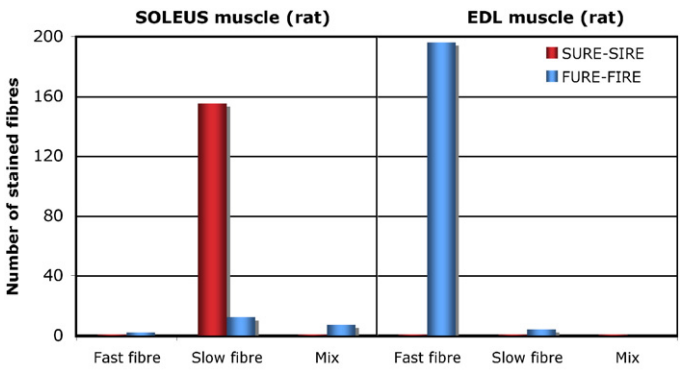


Fig. 6. Both SURE–SIRE and FURE–FIRE confer fibre-type specificity in rat Soleus muscles *in vivo*. Adult rat Soleus muscles were co-transfected with constructs harbouring either, SURE–SIRE or FURE–FIRE, and a GFP expression plasmid to normalise for transfection efficiency. Serial transverse cryosections of transfected muscles were analysed for GFP and LacZ expression and processed for immunohistochemistry with monoclonal antibodies specific to slow, 2A and 2B MHC isoforms (Schiaffino et al., 1989). SURE–SIRE transfected type 1 fibres were intensely stained for LacZ while no fast fibres were stained. Except for an individual fibre, FURE–FIRE transfected type 1 fibres were not LacZ stained. Since this fibre was positive for MHC-1 and MHC-2A antibodies, we speculate a possible transition between slow and fast fibres or the presence of mixed fibres. Note that all fibres in Soleus are positive for slow myosin and importantly, LacZ is only expressed by a few transfected nuclei in an individual fibre. Therefore, LacZ staining develops in a gradient along the fibre. The different intensities observed between individual fibres are due to the position of the sections with respect to the gradient of staining. Black circles identify the same myofibre in the different pictures. Scale bar, 50 μm.

Table 1
Co-operation of upstream and intronic enhancer elements confers fibre-type specificity.



	SOLEUS muscle		EDL muscle	
	SURE-SIRE	*FURE-FIRE*	*SURE-SIRE*	*FURE-FIRE*
Fast fibre-2A	0	2	0	91
Fast fibre-2B	0	0	0	105
Slow fibre-1	155	12	0	4
Mix	0	7	0	0
GFP-fibers	199	255	142	452

Graphic representation of the number of stained fibres when either Soleus (left panel) or EDL (right panel) rat muscles were transfected with SURE–SIRE (red) or FURE–FIRE (blue).

1996). Fibres that did not display any of these three makers were assumed to be fast 2X fibres (Hallauer and Hastings, 2002).

Transfection of constructs harbouring SURE–SIRE resulted in strong LacZ staining of type 1 fibres, the main fibre population present in the Soleus muscle (Fig. 6, left panel). In fact, all fibres in the Soleus muscle of the animals analysed expressed the slow isoform of MHC, whereas fast MHC isoforms were not identified in any fibre expressing LacZ (Table 1). The slow-type specificity of this construct was confirmed by the lack of LacZ staining when it was transfected into the EDL muscle (Fig. 7, right panel, and Table 1). Interestingly, when either SURE or SIRE alone drove transgene expression, the levels of LacZ expression were much lower and could only be detected by histochemistry in the Soleus muscle transfected with SURE. Indeed, LacZ-expressing fibres were only evident after longer incubation times (24 h, versus 2 h in the case of transfection with SURE–SIRE, data not shown).

In agreement with these observations, a small amount of β-galactosidase protein was detected in Western blots when SURE drove LacZ transcription, but only in the Soleus muscle (Fig. 8). By contrast, the presence of both SURE (primary enhancer) and SIRE (secondary enhancer) together produced a more than 10-fold increase in the amount of β-galactosidase protein compared to that generated by SURE alone. Our results reveal a strong synergism between both enhancers in adult skeletal muscle.

Similarly, when FURE–FIRE drove reporter gene transcription, a very different pattern of LacZ expression was observed in rat EDL and Soleus muscles. In accordance with the higher fast fibre content, much higher LacZ expression levels were detected in the EDL muscle, while as expected LacZ expression was significantly lower in Soleus (Fig. 8). Indeed, the X-Gal stained fibres corresponded to the fast-twitch fibres containing 2A, 2B and/or 2X MHC (Figs. 6 and 7 and Table 1), and like endogenous TnI, all three fast fibre types expressed similar levels of LacZ (Fig. 7). The LacZ expression driven by isolated FURE (secondary enhancer) and FIRE (primary enhancer) was significantly lower than that of the FURE–FIRE construct in either the EDL or Soleus muscles (data not shown), confirming the strong synergy between both enhancers *in vivo* and

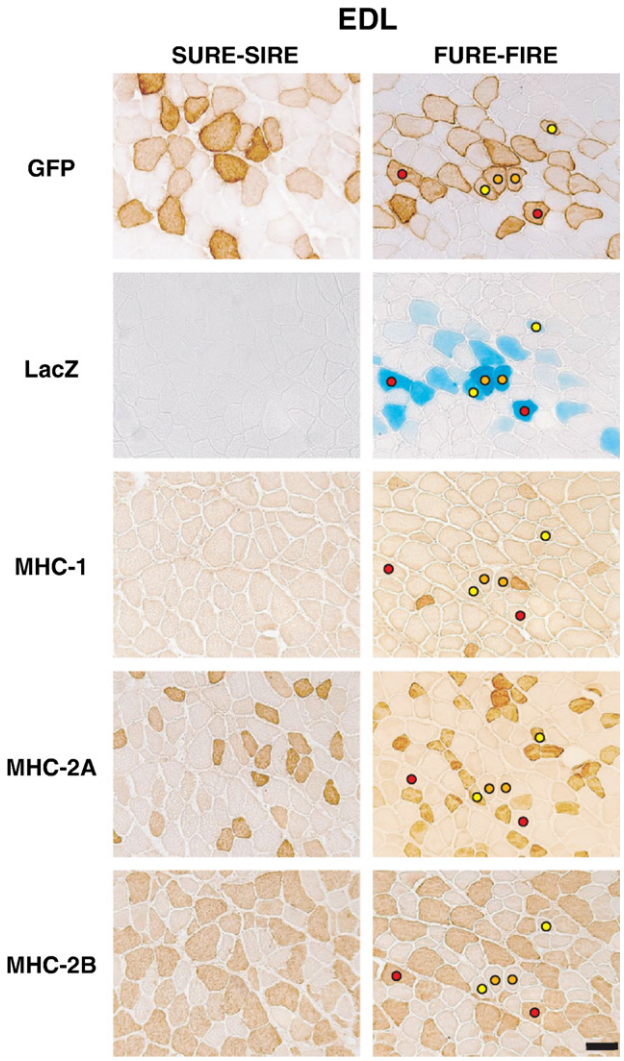


Fig. 7. Both SURE and SIRE, and FURE and FIRE are required for specificity in rat EDL muscles *in vivo*. Adult rat EDL muscles were co-transfected with constructs harbouring either SURE–SIRE or FURE–FIRE, and a GFP expression plasmid to normalise for transfection efficiency. Serial transverse cryosections of transfected muscles were analysed for LacZ and GFP expression, and processed for immunohistochemistry with monoclonal antibodies specific to the slow, 2A and 2B MHC isoforms (Schiaffino et al., 1989). FURE–FIRE transfected type 2 (fast) fibres were intensely stained for LacZ while no slow-type fibres were stained. Moreover, all three types of fast fibres were stained with the same intensity. In the converse experiments, SURE–SIRE transfected type 2 fibres were not LacZ stained. Fibres that were not positive for any of the MHC markers were assumed to be 2X fast fibres (orange circles). The 2A and 2B fibres are marked with yellow and red circles, respectively, and the same myofibres are identified in the different pictures. Scale bar, 50 μm.

in agreement with the results obtained for *s*TnI. In the case of FURE–FIRE and FIRE, minimal levels of LacZ expression were detected in a few slow fibres of Soleus muscle after very long incubation times (24 h, Table 1 and data not shown). A possible transition between slow and fast fibres, or the presence of mixed fibres, may explain this observation.

Together, our results reveal that the expression pattern driven by SURE–SIRE or FURE–FIRE is specific to slow and fast fibres, respectively. In other words, the concerted action of both primary and secondary enhancers is needed to attain high levels of β-galactosidase expression and to confer complete fibre-type specificity. In this sense, it is particularly relevant that the three types of fast fibre (A, B and X) only express similar levels of LacZ when its expression is driven by FURE–FIRE (see coloured circles in Fig. 7). Our results indicate that like *Drosophila* muscle genes, the upstream and intronic

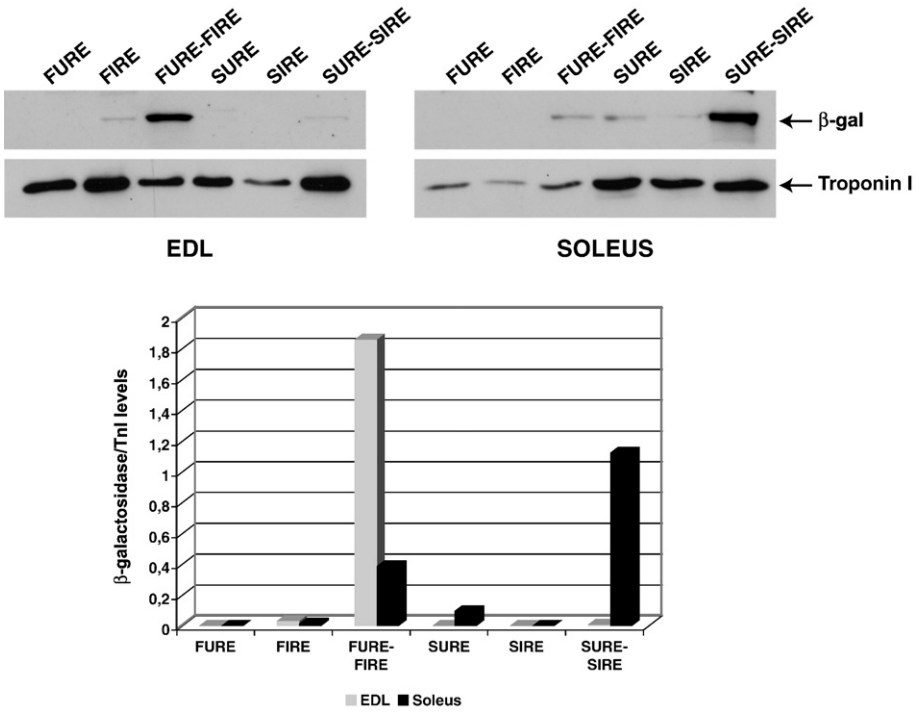


Fig. 8. SURE and SIRE, and FURE and FIRE act co-operatively in rat skeletal muscles. Determination of β-galactosidase levels in Western blots of extracts from transfected Soleus and EDL muscles. Rat muscles were transfected with FURE, FIRE, FURE–FIRE, SURE, SIRE and SURE–SIRE constructs. To normalise for the total protein loading, Troponin I was determined in each sample.

regulatory regions, must co-operate to produce the precise protein levels required to maintain complete fibre-type specificity, thereby representing a conserved mechanism.

Cardiac TnI expression is fine-tuned by the co-operation of primary and secondary enhancer elements

To extend our analysis to the whole TnI family, we studied the *cardiac* TnI gene that is exclusively expressed in heart muscle. The purpose of this study was to determine whether a similar mechanism to that of skeletal TnI genes operates in the transcriptional regulation of this *cardiac* isoform. Using transgenic mice, it has been shown that *c*TnI expression in the heart is controlled by a 356 bp (−230/+126) element, the primary enhancer, located in the upstream region close to the promoter (Di Lisi et al., 1998). This element is responsible for strong activation of the *c*TnI/LacZ transgene, both at early developmental stages and in the adult animal. Hence, we looked for conserved potential regulatory regions in the first introns of *c*TnI and this in silico analysis identified three highly conserved sequences encompassing introns 1 to 4, and the proximal end of intron 5 (positions +127 to +1363, Fig. 9). This newly identified potential regulatory region or secondary enhancer was termed CIRE, in accordance with the nomenclature used herein, whereas the primary enhancer will be referred to as CURE.

To test whether the conserved intronic region identified plays a role in quantitative transcriptional regulation, we performed classical transcription analysis experiments on each element alone. Moreover, we assessed the putative interaction between the previously described CURE and our CIRE element by analysing the transcriptional activity of the CURE–CIRE combination. Transient transfection assays of LacZ reporter gene expression in the H9C2 cardiomyocyte cell line were performed with these three constructs, resulting in very low levels of LacZ expression in all cases. Indeed, the levels of expression observed were no higher than those observed with the empty vector (data not shown). Since the primary enhancer CURE has previously been shown to drive very high levels of LacZ in transgenic animals (Di Lisi et al., 1998), we decided to perform the same analysis using primary rat cardiomyocytes.

As expected, when single upstream or downstream fragments of the *c*TnI gene were transfected into primary cardiomyocytes, only CURE was able to drive high levels of LacZ expression (Fig. 9). Very few CIRE transfected cells displayed X-Gal staining and β-galactosidase expression was very low in Western blots (Fig. 9). Surprisingly, CURE–CIRE also drove lower LacZ expression than CURE alone (Fig. 9). Thus, the downstream region appears to interact with CURE to restrict reporter gene expression. Hence, like *s*TnI and *f*TnI, the secondary enhancer contains elements that co-operate with CURE to regulate the levels of transcription.

Discussion

In multi-cellular organisms, complex regulatory mechanisms are required to ensure tight control of tissue-specific gene expression in diverse environments. These mechanisms can be categorised as transcriptional, post-transcriptional and post-translational regulation, although transcription is probably the most important regulatory checkpoint in the overall expression of a gene. Here, we have identified new conserved enhancer elements in the mouse *s*TnI, *f*TnI and *c*TnI genes, SIRE, FURE and CIRE, respectively, and we show that these elements function as secondary enhancers. In collaboration with the previously described primary enhancers, SURE, FIRE and CURE (Banerjee-Basu and Buonanno, 1993; Calvo et al., 2001; Di Lisi et al., 1998; Hallauer et al., 1993; Hallauer and Hastings, 2002; Rana et al., 2005), these secondary enhancers control the global regulation of each one of these genes. In conjunction with our earlier data (Garcia-Zaragoza et al., 2008; Marco-Ferreres et al., 2005; Mas et al., 2004), these results suggest that this is a regulatory mechanism evolutionary conserved from flies to mammals.

In contrast to *Drosophila*, each of the paired enhancers controlling muscle TnI genes plays a different function. One of the two enhancers, the primary enhancer, harbours the activities needed for fibre-type determination and for transcriptional activation. The secondary

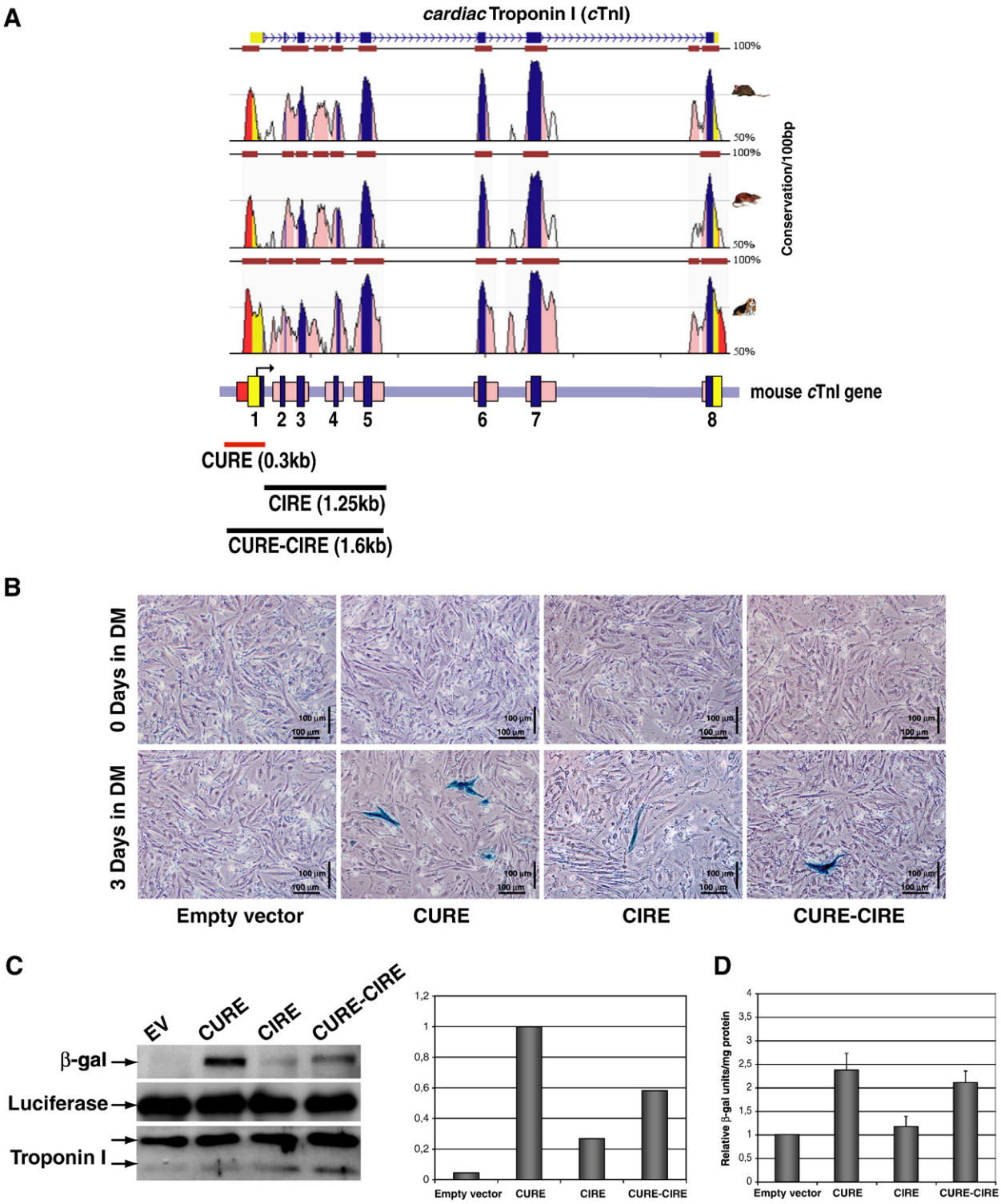


Fig. 9. Co-operation between CURE and CIRE regulates the expression of *c*TnI. (A) Evolutionary conserved regions in the *c*TnI gene (ECR browser output), gene organisation and representation of the analysed fragments. The same colour code as Fig. 1 is displayed. (B) Detection of LacZ expression by X-Gal staining of primary cardiomyocytes transfected with CURE, CIRE and CURE–CIRE constructs (DM, differentiation medium). Cells were incubated with X-Gal for at least 4 h. (C) Determination of β-galactosidase levels in Western blots of cell extracts from transfected cardiomyocytes differentiated for 3 days. EV, empty vector. The levels of Luciferase and Troponin I used to normalise for transfection efficiency and total protein loading are shown. Graphic representation of the Western blot data from the ImageJ program. (D) Analysis of β-galactosidase activity in transfected cardiomyocytes differentiated for 3 days. The β-galactosidase activity was normalised to the luciferase activity and to the protein concentration (see Materials and methods). Results are the means ± SE from 4 different experiments containing 3 replicates of each construct.

enhancers, although silent when tested independently, play an essential role in improving the quality of transcriptional regulation for fibre-type specific determination, and in achieving the correct expression levels required for muscle function. In fact, when the primary and secondary *f*TnI and *s*TnI enhancers are present, reporter expression is almost 3 times higher than that driven by the primary enhancer alone when assayed in C2C12, and at least 10 times higher when assayed *in vivo*. Moreover, the secondary enhancer of the *c*TnI

gene modulates the very high expression directed by the primary one. Thus, as in *Drosophila*, the overall molecular mechanism underlying the correct spatio-temporal muscle gene expression at appropriated levels implies synergistic interactions between the two separate enhancers in each gene.

As described previously, the three individual primary enhancers, SURE, FIRE and CURE, function in transgenic slow and fast skeletal and heart muscles (Banerjee-Basu and Buonanno, 1993; Calvo et al., 2001; Di Lisi et al., 1998; Hallauer et al., 1993; Hallauer and Hastings, 2002; Rana et al., 2005). These results are consistent with the idea that each of the three enhancers displays inherent activity and the basic information to activate fibre-specific expression. However, alone they are not fully capable of reproducing the *in vivo* expression patterns. In transgenic animals, the FIRE primary enhancer was previously shown to drive specific expression exclusively in fast fibres, although this expression varied among the different types of fibre and it was higher in 2B and significantly lower in 2A (Hallauer and Hastings, 2002). Using similar approaches, we demonstrate clear differences in fibre-type specificity between the construct harbouring FURE–FIRE and that which exclusively harbours FIRE. In this sense, it is especially relevant that all three types of fast fibres, A, B and X, only express similar levels of LacZ when its expression is driven by both primary and secondary enhancers together. Thus, the presence of both enhancers, FURE and FIRE, is required to recapitulate the endogenous expression observed for the *f*TnI gene in Soleus and EDL muscles.

The presence of small amounts of the reporter in FURE–FIRE transfected rat Soleus and SURE–SIRE transfected rat EDL correlates well with the fibre composition of both muscles and therefore, it most probably represents the expression driven in the few fast/slow fibres present in Soleus or EDL muscles, respectively. The LacZ expression observed in transverse sections of muscles and differentiated myotubes electroporated with either the SURE or FIRE constructs is very low, reflecting the incapacity of these fragments to drive the high levels of expression required for proper sarcomere formation and function. The relatively low levels of reporter protein detected in Western blots of SURE in Soleus and FIRE in EDL muscles further confirm these results. The differences in expression levels are very significant when compared to those driven by the combination of the URE and IRE enhancers.

In support of our hypothesis, studies aimed at understanding the regulatory mechanism controlling muscle creatine kinase expression identified a highly conserved regulatory region of 900 bp within the first intron of the MCK gene (MR-1). MR-1 exhibits positive transcriptional activity and it depends on the presence of the proximal promoter of the MCK gene. The activity of upstream and downstream regulatory regions was analysed in transgenic mice resulting in different transcriptional effects depending on the fibre-type (Johnson et al., 1989). Moreover, using bioinformatic methods, we predict that the mammalian TnT and TnC genes will have a similar organisation of potential regulatory enhancers (Supplementary Fig. S3).

Muscle enhancers are short-range enhancers, which means that they influence and control transcription of genes in their local surroundings. It is interesting to note that the primary and secondary enhancer positions in each mouse TnI gene are not conserved. While in *s*TnI and *c*TnI genes the secondary enhancer is located in an intronic region, in the *f*TnI gene it lies at the 5′ flanking sequence. From an evolutionary point of view, this enhancer distribution correlates well with previous studies carried out in the TnI gene family that show *s*TnI and *c*TnI to be more closely related to one another than to *f*TnI (Cleto et al., 2003; OOta and Saitou, 1999; Warkman and Atkinson, 2002). On the other hand, comparisons between our data from mouse TnI, TnT and TnC genes and those from the *Drosophila* muscle genes studied previously indicate certain variability in the location of the intragenic regulatory element of the pair. Although in most cases this region is located within intron 1, it can also be found in introns 2 or 3 as is the case of TnT, *c*TnI or *Drosophila* paramyosin genes (Marco-Ferreres et al., 2005; Marin et al., 2004; Mas et al., 2004).

A key transcription factor involved in regulating the transcription of specific genes during skeletal muscle formation and differentiation is MEF2. An active and important MEF2 binding site has been found in primary enhancers of mouse skeletal TnI genes that is largely conserved during evolution (Supplementary Figs. S1 and S2), as well as in *Drosophila* muscle enhancers (Arbeitman et al., 2002; Arredondo et al., 2001; Cripps et al., 2004; Elgar et al., 2008; Kelly et al., 2002; Lin et al., 1996; Marin et al., 2004; Ranganayakulu et al., 1995). However, the secondary enhancers from mammals analysed here do not have conserved MEF2 sites, despite their largely conserved fragments. In these genes, MEF2 plays an essential role in mediating the interaction between active primary and secondary enhancers, which is required for accurate muscle transcription in mammals. By contrast, in *Drosophila* both enhancers contain MEF2 binding sites to activate transcription (Arredondo et al., 2001; Garcia-Zaragoza et al., 2008; Marin et al., 2004; Mas et al., 2004).

Interestingly, recent work in *Drosophila* has shown that changes in regulatory elements are an important mechanism to generate animal diversity during evolution (Jeong et al., 2008). Moreover, a genome wide study of Dorsal target genes has revealed that a large proportion of them possess primary and secondary enhancers (Hong et al., 2008), and that both enhancers contain Dorsal binding sites. Phylogenetic comparisons have suggested that secondary enhancers in Dorsal target genes are evolving more rapidly than the primary ones (Hong et al., 2008). Indeed, secondary muscle enhancers in mammals lack conserved MEF2 sites. We hypothesised that an ancestral TnI gene had MEF2 sites in both primary and secondary enhancers as it has in *Drosophila*. Posterior duplication of that ancestral gene would have given rise to the fibre-specific forms, which kept MEF2 sites in both enhancers. Throughout evolution, secondary muscle enhancers would have lost MEF2 sites, gaining novel binding sites along with new regulatory functions as the fine-tuning of tissue-specific transcription to ensure precise and reproducible patterns of gene expression. Thus in *f*TnI, the upstream enhancer evolved as a secondary one loosing the MEF2 site while this site was maintained in *s*TnI upstream enhancer. The term “shadow enhancer” was proposed for “remote secondary enhancers mapping far from the target gene and mediating activities overlapping to the primary enhancer.” Shadow enhancers have been shown to be active and largely redundant when compared to the primary enhancer, yet they are thought to provide stability in gene expression throughout the evolution (Hong et al., 2008). By contrast, secondary enhancers identified in TnI genes are not active and their role is to modulate the activity of the primary enhancer. Taking this into account, we hypothesise that secondary enhancers from TnI genes might have evolved from shadow enhancers equivalent to those identified in *Drosophila*. In *f*TnI, the FURE element might have arisen from a shadow enhancer while for *s*TnI and *c*TnI, the two more closely related TnI genes (Cleto et al., 2003; OOta and Saitou, 1999; Warkman and Atkinson, 2002), the intronic element would have evolved from a shadow enhacer. Therefore, we propose extending the concept of the term “shadow enhancer” to those secondary enhancers that fine-tune the effects of primary enhancers, enabling them to perform new regulatory functions.

In summary, our results reveal the existence of an evolutionary conserved mechanism to control precise quantitative expression and fibre specificity in each muscle, and maybe in different physiological situations. Thus, we show the existence of a secondary enhancer in all three TnI genes, and that the concerted action of primary and secondary enhancers is required for the correct tissue-specific expression of these genes. Hence, we propose a conserved mechanism of transcriptional regulation in which the synergistic action of two or maybe more enhancer elements is responsible for both correct spatio-temporal and quantitative gene expression. This mechanism

may not be exclusive to muscle tissues but it may also represent a general mechanism to fine-tune transcriptional regulation in any other tissue.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.10.006.

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