

Myh7b/miR-499 gene expression is transcriptionally regulated by MRFs and Eos

Fan Yeung, Eunhee Chung, Martin G. Guess, Matthew L. Bell and Leslie A. Leinwand*

Department of Molecular, Cellular, and Developmental Biology and Biofrontiers Institute, University of Colorado, Boulder, Colorado 80309, USA

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ABSTRACT

The sarcomeric myosin gene, *Myh7b*, encodes an intronic microRNA, miR-499, which regulates cardiac and skeletal muscle biology, yet little is known about its transcriptional regulation. To identify the transcription factors involved in regulating *Myh7b/miR-499* gene expression, we have mapped the transcriptional start sites and identified an upstream 6.2 kb region of the mouse *Myh7b* gene whose activity mimics the expression pattern of the endogenous *Myh7b* gene both *in vitro* and *in vivo*. Through promoter deletion analysis, we have mapped a distal E-box element and a proximal Ikaros site that are essential for *Myh7b* promoter activity in muscle cells. We show that the myogenic regulatory factors, MyoD, Myf5 and Myogenin, bind to the E-box, while a lymphoid transcription factor, Ikaros 4 (Eos), binds to the Ikaros motif. Further, we show that through physical interaction, MyoD and Eos form an active transcriptional complex on the chromatin to regulate the expression of the endogenous *Myh7b/miR-499* gene in muscle cells. We also provide the first evidence that Eos can regulate expression of additional myosin genes (Myosin 1 and β -Myosin) via the miR-499/Sox6 pathway. Therefore, our results indicate a novel role for Eos in the regulation of the myofiber gene program.

INTRODUCTION

Myosin is the major contractile protein of cardiac and skeletal muscle cells. Myosin heavy chain (MyHC) genes act broadly to control muscle gene expression and performance through a network of intronic microRNAs (1). As a member of the sarcomeric MyHC family, *Myh7b* and its intronic microRNA, miR-499, are expressed in skeletal muscle, heart and, surprisingly, in a subset of cells in the brain (2–4). Frequently, the gene undergoes non-productive splicing, resulting in a RNA that does

not encode a functional protein while at the same time preserving the expression of miR-499 (3). *Myh7b/miR-499* expression is regulated by thyroid hormone, adrenergic agonists, myocardial infarction (3) and by the α - and β -Myosin microRNAs: miR-208a and -b (1). Currently, the only known function of *Myh7b* was reported by Rubio *et al.* in which the knockdown of *Myh7b* dramatically disrupted synapse structure, function and surface α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor content (2).

Unlike *Myh7b*, miR-499 has been shown to play an important role in both cardiac and skeletal muscle biology. In heart, miR-499 is strongly associated with cardiac differentiation (5,6). Indeed, overexpression of miR-499 in mouse embryonic stem cells enhances their differentiation into cardiomyocytes with a concomitant upregulation of the cardiac transcription factor MEF2C and cardiac MyHC genes in the embryoid bodies (5). Furthermore, miR-499 was shown to prevent cardiomyocyte apoptosis by targeting calcineurin-mediated Drp1 activation and consequent mitochondrial fission (7).

In skeletal muscle, miR-499 is involved in the specification of fiber type by targeting a collection of transcriptional repressors which promotes a fast to slow myofiber type switch (1). One transcription factor integral to this regulatory pathway is Sox6, a member of the SoxD family of transcription factors. These factors are defined by the presence of the SRY-related HMG box domain, which mediates sequence-specific DNA binding. Recently, Quiat *et al.* (8) demonstrated that Sox6 directly represses the transcription of multiple myosin and sarcomeric genes by binding to conserved *cis*-elements in their promoters. In Sox6-null mice, expression of these sarcomeric genes was indeed de-repressed, suggesting Sox6 regulates muscle fiber type specification by coordinately regulating multiple sarcomeric proteins. Despite the important roles of *Myh7b/miR-499* in muscles and brain, very little is known about the mechanisms by which its expression is regulated. Therefore, the purpose of the current study is to characterize the regulatory region of *Myh7b* and identify transcription factors that are crucial for *Myh7b/miR-499* expression.

*To whom correspondence should be addressed. Tel: +303 492 7606; Fax: +303 492 8907; Email: leslie.leinwand@colorado.edu

Numerous studies have indicated that the muscle-specific transcription factors collectively known as myogenic regulatory factors (MRFs) play a major role in the regulation of MyHC gene expression (9,10). MRF family members, such as MyoD, Myogenin, Myf5 and MRF4, are basic helix-loop-helix (bHLH) proteins that interact with E-box DNA elements located in the promoter/enhancer region of many muscle-specific genes (11). Genetic studies have indicated that the MRFs play distinct but overlapping roles in a mutually reinforcing transcriptional network to activate myogenesis appropriately (12). In general, MyoD and Myf5 serve as myogenic specification factors, while Myogenin functions downstream and is essential for muscle differentiation (13).

The Ikaros gene, which encodes a hematopoietic-specific zinc finger protein, is one of the central regulators of lymphocyte differentiation (14). By means of alternative splicing, it encodes at least eight Ikaros isoforms (15). All isoforms contain C-terminal zinc fingers required for protein dimerization and interactions, and six of the isoforms contain N-terminal zinc fingers for binding the DNA consensus site 5'-GGGA-3' (15,16). Ikaros has been shown to associate with gene activation (17), potentiation (18), priming (19) and transcriptional repression (20). Loss of functional experiments suggests that it is required at the earliest stage of B-cell and T-cell specification during fetal development (21). Interestingly, Ikaros 4, also known as Eos, is expressed in skeletal muscle, heart, brain and liver in addition to hematopoietic tissues (16,22). However, its function remains poorly understood.

In this study, we found that the Myh7b gene uses multiple transcription start sites in heart but only one in skeletal muscle. We cloned a 6.2 kb upstream promoter region of the mouse Myh7b/miR-499 gene for the purpose of identifying transcription factors that are crucial for its expression and regulation. We showed that the activity of the 6.2 kb promoter region mimics the endogenous Myh7b gene expression profile *in vitro* and *in vivo*. Through detailed promoter analysis, we identified a distal E-box and a proximal Ikaros *cis*-element within the promoter that are critical for its activity. We demonstrated that MRFs, MyoD, Myf5 and Myogenin bind to the E-box *cis*-element while Ikaros 4 (Eos) binds to the Ikaros motif, and through physical interaction between MyoD and Eos, they form an active transcriptional complex on the Myh7b chromatin to regulate its expression. Furthermore, our results provide the first evidence that Eos, through its regulation of miR-499 can indirectly modulate Sox6 levels; as a result, it is capable of coordinately regulating expression of additional myosin genes (Myh1 and Myh7) via Sox6. Therefore, the current study indicates a novel role for the lymphoid transcription factor, Eos, in the regulation of the myofiber gene program through the miR-499/Sox6 pathway.

MATERIALS AND METHODS

Cell culture, reagents and plasmid constructs

C₂C₁₂ myoblasts, HEK-293 T and NIH3T3 were cultured in dulbecco's modified eagle's medium (DMEM)

supplemented with 10% fetal bovine serum and glutamine/penicillin/streptomycin (PSG) (Invitrogen). To induce differentiation into myotubes, 50% confluent C₂C₁₂ myoblasts were cultured in DMEM with 2% horse serum and PSG. Neonatal rat ventricular myocytes (NRVMs) were isolated and cultured from neonatal Sprague-Dawley rats as previously described (23). Mouse MM14 skeletal muscle cells were cultured as described previously (24). Antibodies used were M2 Flag, α -Actin, Eos, Sox6 (Sigma F1804, A2103, SAB2103090, HPA003908, respectively), hemagglutinin (HA) (Covance), Ikaros (Active Motif 39355), p65 (Millipore 06418), Histone H3 (Cell Signaling 5275s), MyoD, Myogenin, Myf5 and MRF4 (Santa Cruz sc760, sc576, sc302, sc784, respectively). The Sox6 expression plasmid was previously described (3). A 6.2 kb genomic region upstream of the first exon of the mouse Myh7b gene was cloned from C₂C₁₂ genomic DNA with Supermix II (Invitrogen) into pGL3-basic (Promega) to generate m7bp-6.2/*luc* construct. All truncation, deletion or mutation constructs were created by inverse polymerase chain reaction (PCR) with iproof polymerase (Biorad). MyoD, Myogenin, Myf5 and MRF4 plasmids were a generous gift from Drs X. Liu (University of Colorado) and E. Olson (UT Southwestern). Phenylephrine (PE) and 3,3', 5-tri-iodo-L-thyronine (T3) were purchased from Sigma (P6126, T2752).

RNA extraction, RT-PCR and real-time PCR analysis

Total RNA was isolated using Tri Reagent (Molecular Research, Inc.) according to the manufacturer's protocol. One to five micrograms of RNA were used for cDNA synthesis with Superscript II reverse transcriptase (Invitrogen). Changes in miR-499 expression were assessed in reference to snoRNA202 using Taqman microRNA assays (Applied Biosystems), and analyzed using the comparative threshold cycle (*Ct*) method. Real-time PCR primer sequences for mouse Myh1 are: 5'-caggtcaacaagctgcgggtg-3'; 5'-gatcttcacatttgcctac-3'; for mouse Myh7 are: 5'-tccttacttgcctaccctc-3'; 5'-cttctcagacttccgcag-3'; for mouse Myh7b are: 5'-taaggcgcgccgagccgcgggaagacctcagcag-3'; 5'-taaggcgcgcc cctgcctcagggcttacc-3' (product = 1130 bp); for mouse 18S 5'-gccgtagaggtgaaattcttg-3', 5'-cttctcgtctgtcctctt-3'; for mouse actin are: 5'-atatcgtcgcgtgtgctc-3'; 5'-aggatggcgtgagggagagc-3'.

Glutathione S-transferase-pull down assays, immunoprecipitation and western blot

³⁵S-labeled Eos was *in vitro* transcribed and translated using the TnT[®] T7 Quick Coupled Transcription/Translation system from Promega. Production of recombinant glutathione S-transferase (GST)-MyoD proteins in *Escherichia Coli* and *in vitro* GST pull-down assays were performed as previously described (25). Cells were lysed in radioimmunoprecipitation assay buffer and protein concentration was determined by Pierce 660 nm protein assay. Immunoblotting was performed using the NOVEX (Invitrogen) system. Briefly, 30–50 μ g proteins were separated on 4–12% Tris-Bis polyacrylamide gel electrophoresis gels and transferred onto nitrocellulose

membranes. Primary antibodies were used at 1:200–1000 dilution and secondary antibodies (Jackson Lab) were used at 1:5000 dilution in blocking solution. For immunoprecipitation, primary antibodies (2 µl of anti-M2 Flag antibody) were mixed with pre-cleared lysates for an hour at 4°C before the addition of 20 µl protein G agarose. After rocking overnight at 4°C, the agarose beads were extensively washed the next day and followed by immunoblot analysis.

Transfection, luciferase assay and siRNA

For transfection, C₂C₁₂ and NIH3T3 cells were transfected in suspension. 293T cells were plated at 80% confluency 1 day before transfection. Plasmids were transiently transfected using TransIT-LT (Mirus) according to the manufacturer's protocol. Thirty-six hours post-transfection, cells were harvested for luciferase and β-galactosidase assays (26). NRVMs were transfected with electroporation with the primary myocyte nucleofector kit (Amaxa). All transient transfection data are expressed as relative luciferase units (RLU), which is defined as luciferase activity normalized to internal control CMV/β-galactosidase activity for transfection efficiency. RLU is expressed as the mean ± standard error of at least three independent experiments.

MyoD and Myogenin siRNA were synthesized with Silencer[®] siRNA Construction Kit (Ambion) according to manufacturer's protocol. siRNA oligos for MyoD are: sense 5'-aatgataaatcgattgggctctgtctc-3', antisense 5'-aacccaatgcgatttaccctgtctc-3'; Myogenin: sense 5'-aacaagatccactgcaaatgcctctgtctc-3', antisense 5'-aagcatttgcagtggatcttgctgtctc-3'. Myf5 and Eos siRNA pools were purchased from Santa Cruz and siCONTROL Non-Targeting siRNA smart pool was from Thermo Fisher. In siRNA experiments, cells were transfected with 100 nM siRNA using oligofectamine (Invitrogen). When transfecting cells with both plasmid DNA and siRNA, plasmid DNA was transfected 24 h after siRNA transfection.

Linker-scanning mutagenesis and ChIP analysis

For linker-scanning mutagenesis, D450-TATA or P500/luc was used as DNA templates for inverse PCR with primers containing the GAL4 sequence (cggagtactgtctccg). ChIP analysis was performed as previously described (27). Briefly, each 10-cm plate of C₂C₁₂ cells at 60% confluency was used for four antibodies pull down. Primers for the D450 region were 5'-ggaacaactgaaggacaggaga-3', 5'-ccaccaccacccaggggctgg-3' (product 150 bp); for the P500 region were 5'-attttatggctcctttactgg-3', 5'-ctgttgaaggctcagcaaa-3' (product 300 bp); for -3600 bp were 5'-accaccagtgcagatctct-3', 5'-tgcattggctgagctaggg-3' (product 200 bp) and for mouse GAPDH promoter were 5'-accaggaggctcagctcc-3', 5'-tcagttcggagcccacacgc-3' (product 250 bp).

Nuclear extracts and electrophoretic mobility shift assay

Nuclear protein extractions and electrophoretic mobility shift assays (EMSA) were performed as described previously (26). Oligos (Invitrogen) were dissolved in annealing

buffer (10 mM Tris, 1 mM EDTA, 50 mM NaCl) and annealed by heating up to 95°C and slowly cooling to room temperature. The oligo sequences used as probes or competitors are as follows: distal region (DR), 5'-gatgcacagcagctgccacgttccagcccgggca-3'; DR-mut-Ebox, 5'-gctgctgccgattccagcccgggca-3'; proximal region (PR), 5'-aggagataaaagggaatttgcctttgtgtctctgt-3'; PR-mut-Ikaros, 5'-cctttgcctttgtgtctctgtgg-3'; Ikaros, 5'-tcagctttgggaataccctgtca-3'; SP-1, 5'-attcgatcggggcggggcgagc-3'; Ebox, 5'-ctagaccacaacacctgtgcctt-3'; Ets, 5'-gggctgctgaggaagtataagaat-3'. The double-stranded probes were end-labeled with [γ -³²P] adenosine triphosphate by using T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA). 100 000 cpm of labeled probe and 10 µg of nuclear extracts were incubated with binding buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 4% glycerol, 1 µg of poly(deoxyinosinic-deoxycytidylic) (poly(dI-dC)) (Amersham Pharmacia Biotech), and 1 mM KCl at 30°C for 30 min. The samples were subjected to electrophoresis at room temperature on a 4% non-denaturing polyacrylamide gel in 0.5× Tris/Borate/EDTA at 35 mA for 2 h. For antibody supershift experiments, 2–4 µg of antibody was pre-incubated with nuclear extracts for 30 min at room temperature before the addition of the probe. In competition experiments, 100× of unlabeled competitor oligos were pre-incubated with nuclear extracts for 30 min at 30°C before the addition of the probe.

DNase I protection assay and 5'-rapid amplification of cDNA ends

D450-B and P500-C were labeled by Klenow (New England Biolabs) according to current protocols. The binding reactions for DNase I footprinting were described previously (26). About 100 000 cpm of labeled probe and 10 µg of nuclear extracts were incubated in 13 µl of buffer containing 12.5 mM hydroxyethyl piperazineethanesulfonic acid, pH 7.9, 12.5% glycerol, 5 mM MgCl₂, 70 mM KCl, 0.2 mM EDTA, 60 mM β-mercaptoethanol, 0.5 mg/mL bovine serum albumin and 200 ng of poly(dI-dC). After 30 min at 30°C, 1–2 µl of DNase I (Promega) was added to the reactions. The cleavage reactions were terminated after 1 min by addition of 100 µl of stop buffer containing 400 mM sodium acetate (pH 5.2), 0.2% sodium dodecyl sulfate, 10 mM EDTA, 50 µg/ml yeast tRNA, and 50 µg/ml proteinase K (Invitrogen). The mixtures were incubated at 50°C for 15 min, extracted with phenol/chloroform, and precipitated with ethanol. Precipitates were dissolved in formamide loading buffer and analyzed on 6% sequencing gels.

5'-rapid amplification of cDNA ends (RACE) was performed on 1 µg total RNA from mouse heart and soleus muscle using the GeneRACER kit (Invitrogen) as per the manufacturer's instruction. The mouse gene specific primer and a nested primer near the 5'-end of the coding sequence used in the PCR reaction are as follows: mouse Myh7b RACE: 5'-ggtagccctggcggaggtagcaggctgatt-3'; mouse Myh7b nested RACE: 5'-ccatcatggtggcga-gaggtgcaaaa-3'.

Hydrodynamic limb vein injection and *in vivo* bioluminescence imaging

The hydrodynamic limb vein (HLV) injection technique was performed as described by Hagstrom *et al.* (28). Six- to ten-month-old male C57BL/6 mice were anesthetized with 1–3% isoflurane (MWI, Meridian ID). After shaving and cleaning the right hind limb, a small latex tourniquet was tightened around the upper part of the quadriceps and secured with a hemostat. A small incision was then made above the ankle to expose the distal great saphenous vein. A 30-gauge needle, linked to a syringe by polyethylene tubing, was then inserted 0.5 cm into the great saphenous vein in the retrograde direction. About 100 µg/conconstruct of endotoxin-free m7b-6.2/luc or minTATA was co-injected with a control plasmid (mKate) in 1 ml of phosphate buffered saline at 7 ml/min using a programmable syringe pump (KD Scientific). Two minutes after injection, the tourniquet and needle were removed and the incision was closed with 6-0 suture. After 7 days, *in vivo* luciferase activity was imaged and quantified using the IVIS-100 imaging system (Caliper Life Sciences, Hopkinton, MA). Briefly, mice were again anesthetized with 1–3% isoflurane. mKate fluorescence was first measured and used as an internal control for injection efficiency, followed by intraperitoneal injection of 100 µl of 30 mg/ml luciferin. After 15 min, bioluminescent signal was collected for 1 min. Luciferase and mKate

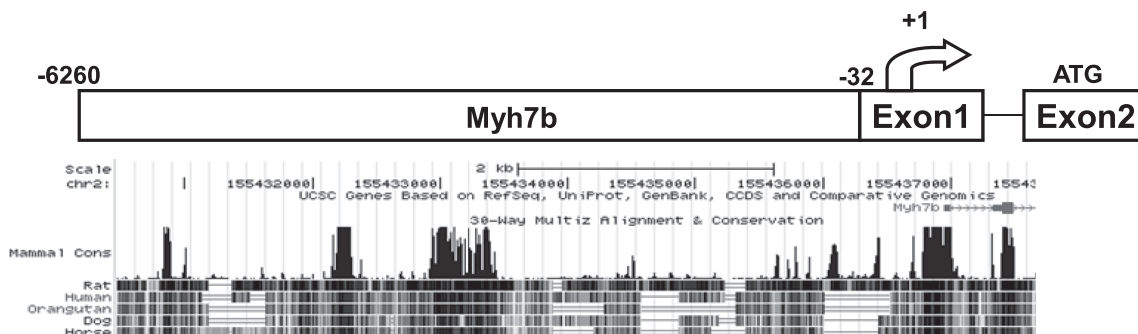
signals were then quantified using region of interest (ROI) analysis. The same region size was used for all animals. The *in vivo* luciferase activity was normalized to the mKate level, and *P* values were calculated by Student's *t* test.

RESULTS

Transcription start sites of the Myh7b/miR-499 gene differ in heart and skeletal muscle

Since there is no canonical TATA box immediately upstream of the translation start site (ATG), we sought to identify the Myh7b promoter by experimentally determining the transcriptional start site (TSS) of the mouse Myh7b gene. 5' RACE was performed on mRNA isolated from mouse cardiac and skeletal muscle (soleus). While multiple TSSs were identified in cardiac tissue, there was only a single unique TSS (+1) identified in the soleus muscle. In all cases, the TSSs are located in exon 1, upstream of the predicted translational start site (Figure 1, bottom panel).

Based on the 5'-RACE results and sequence conservation across species, we cloned a 6.2 kb genomic region upstream of Myh7b exon 1 into a luciferase reporter vector (m7b-6.2/luc) to identify the regulatory regions involved in the transcriptional control of Myh7b/miR-499 expression in skeletal muscle. The 6.2 kb region, which covers –6260 to



Mouse 5' RACE

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CTGTGGCCAG GCTGGGGGGG CTGTGACACG TGGAGATTGC TGACATAGCT CTCCTTTGCT GACCTTGACA
-32 ACAGCAAATAA AAGGGGTAGC AGTGCTCCGC +1 TCCCTGTCCT CACCGTGGTG CCAGaGCCCC CTGCCTAGGA
CTGCTTTGAG TTGCTGCCCC GGTTTTCAGG TCCTCCTTTT GCACCTCTCG CCACCATGAT GGATATGAGT
GAACTTGGAG AATCAGCCTG CTACCTCCGC CAGGGCTACC AGGAAATGAT GAAGGTGCAC ACTGTCCCAT
GGGATGgTAA GAAACGGGTC

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Figure 1. Myh7b/miR-499 has different transcription start sites in heart and skeletal muscle. Top: Schematic of the location of the 6.2 kb Myh7b promoter region relative to the transcription start site (+1) and the translation start site (ATG). Middle: UCSC Genome Browser graphical map of the mouse 6.2 kb Myh7b promoter indicates multiple high homology regions (denoted by peaks) across species. Bottom: 5'-RACE analysis of the mouse Myh7b transcripts revealed striking differences in position of the TSSs in skeletal and cardiac muscle. While multiple TSSs were identified for Myh7b transcripts in cardiac muscle as denoted by bolded nucleotides, a single TSS was identified for the mouse soleus (as indicated by +1). All TSSs identified from the striated muscle are located in exon 1 upstream of the putative translational start site (underlined). Exon boundaries are indicated by lowercase letters.

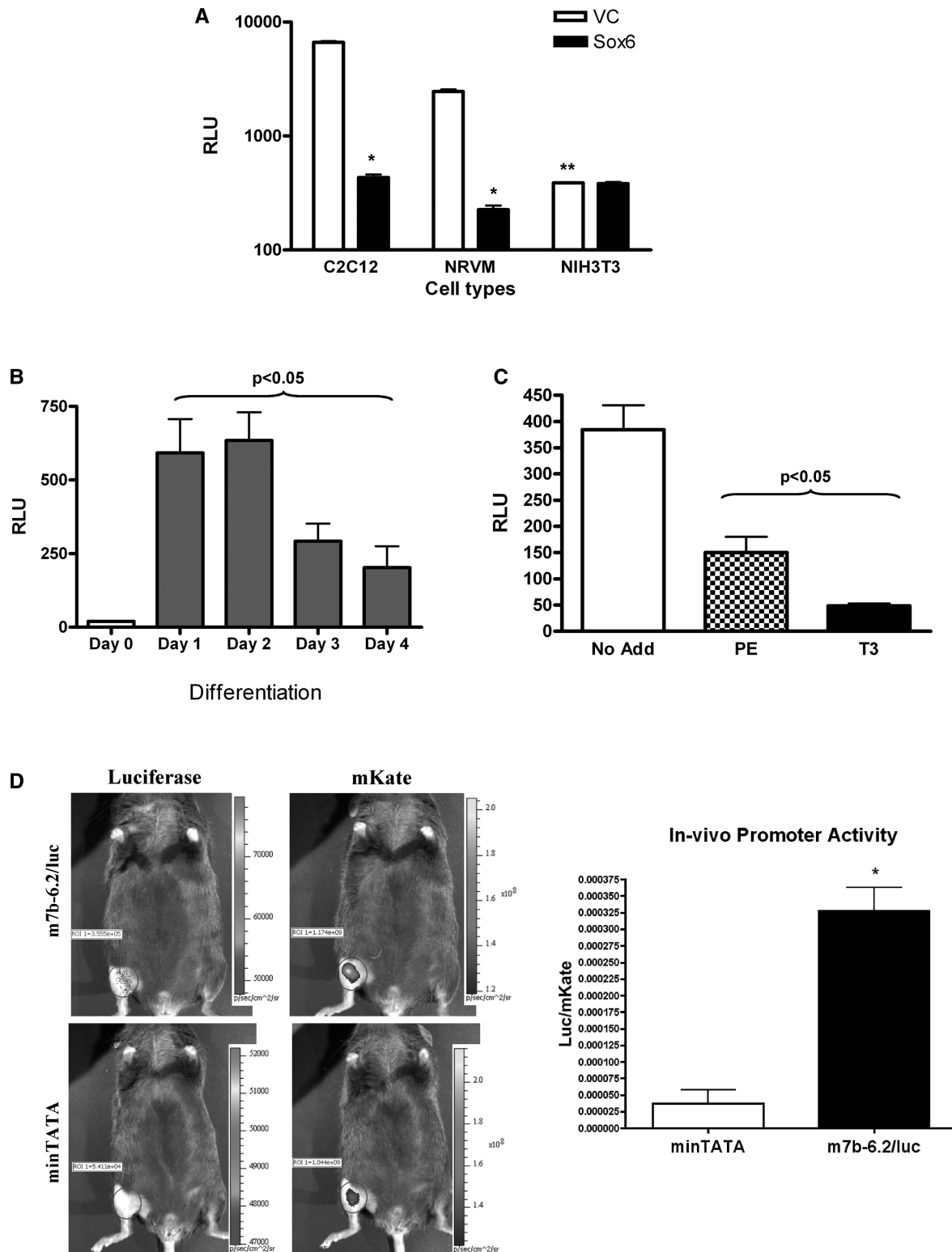


Figure 2. A 6.2kb upstream promoter region of the Myh7b gene mimics endogenous Myh7b gene expression patterns. (A) The 6.2kb Myh7b promoter shows cell type-specific activity in cardiac and skeletal muscle cells. C₂C₁₂, NRVM and NIH3T3 cells were transiently transfected with m7b-6.2/luc, CMV/ β -gal and vector control (VC) or pcDNA-Sox6. *Significantly different from VC, $P < 0.05$; **significantly different from C₂C₁₂, $P < 0.05$. (B) For differentiation experiments, C₂C₁₂ myoblasts were transiently transfected with m7b-6.2/luc and CMV/ β -gal, transferred to differentiation medium 24 h post-transfection and harvested at the indicated time points for luciferase and β -gal assays. (C) NRVMs were transfected as in (B). Cells were either left untreated (No add), treated overnight with PE (20 μ M) or thyroid hormone (T3, 100 nM). (D) *In vivo* imaging of the m7b-6.2/luc construct activity in skeletal muscle. Bioluminescent images of a representative individual mouse co-injected with either m7b-6.2/luc or the minimal TATA promoter luciferase control (minTATA) and the internal control (CMV-mKate). Quantification of photons emitted is displayed as Luc activity normalized to mKate activity in the graph. Data presented are means of two mice \pm SD. * $P < 0.01$.

–32 bp (Figure 1, top panel) relative to the skeletal muscle TSS (+1), contains multiple high homology regions across various species, as indicated in the UCSC Genome Browser conservation graph (Figure 1, middle panel).

Activity of the 6.2 kb upstream promoter region of Myh7b mimics endogenous Myh7b expression patterns

To investigate whether the 6.2 kb 5' flanking region of the Myh7b gene mimics the relative expression levels of the endogenous Myh7b genes, luciferase activities of the m7b-6.2/luc construct were assessed in various cell lines. Figure 2A (white bars) shows that the m7b-6.2/luc construct is significantly more active in primary NRVMs and skeletal muscle cells (C₂C₁₂) than in fibroblasts (NIH3T3). Since studies from our group and van Rooij *et al.* have demonstrated that overexpression of the transcription factor Sox6 can repress Myh7b/miR-499 expression (1,3), we also investigated whether ectopic Sox6 expression could repress the transcriptional activity of the 6.2 kb Myh7b promoter. Figure 2A (black bars) shows that Sox6 represses m7b-6.2/luc activity only in NRVM and C₂C₁₂ cells, but not in NIH3T3 cells, suggesting that Sox6 represses m7b-6.2/luc activity in a cell-type specific manner.

We previously showed that expression of endogenous Myh7b/miR-499 is activated during C₂C₁₂ differentiation and repressed by stimuli such as the pro-hypertrophic agonist PE and thyroid hormone (T3) in primary myocytes (3). The results shown in Figure 2B and C indicate that m7b-6.2/luc activity is also upregulated in differentiated C₂C₁₂ cells and repressed by PE and T3 in NRVMs. Therefore, our data show that the 6.2 kb Myh7b promoter region is regulated similarly to the endogenous Myh7b gene. Although C₂C₁₂ cells are an excellent model for studying transcriptional regulation in skeletal muscle cells, cultured myoblasts or myotubes cannot adequately mimic the complexity of skeletal muscle *in vivo*. Thus, we performed *in vivo* analyses of m7b-6.2/luc activity. The HLV injection technique was used to determine whether m7b-6.2/luc exhibits strong activity in fully mature muscle fibers in mice. Figure 2D demonstrates that, when compared to the minimal TATA promoter control, m7b-6.2/luc shows significantly higher activity in skeletal muscle. Together our data strongly suggest that the 6.2 kb Myh7b promoter activity mimics the endogenous Myh7b expression pattern and is sufficient to drive reporter activity in skeletal muscle both *in vitro* and *in vivo*.

Mapping of important cis-regulatory elements in the Myh7b promoter

To dissect out the critical *cis*-regulatory elements involved in the transcriptional control of Myh7b/miR-499 expression in skeletal muscle, we used C₂C₁₂ cells as an *in vitro* model system for detailed deletion analyses of the promoter. Various deletion and truncation constructs were generated by inverse PCR using m7b-6.2/luc as the template. As depicted in Figure 3A, a 2.6 kb terminal deletion of the m7b-6.2/luc construct results in a significant decrease in promoter activity, suggesting that a crucial *cis*-element resides in the distal region of the promoter.

Further deletion was performed on the 2.6 kb distal region (Figure 3B) and a 450 bp *cis*-element (D450) located at –4860/–4410 bp appears to be essential for m7b-6.2/luc activity. In addition, results from internal deletion experiments (Figure 3A, construct E4) show that a 500 bp DNA fragment (P500) located at –510/–32 bp within the proximal promoter region is also important for m7b-6.2/luc activity. We then inserted the proximal P500 *cis*-element (–510/–32 bp) upstream of a luciferase reporter vector to generate P500/luc and the distal D450 fragment (–4860/–4410 bp) upstream of a minimal TATA box promoter linked to a luciferase reporter gene to generate D450-TATA for further deletion studies. As shown in Figures 3C, 150-bp regulatory elements were identified within D450 (Site B, –4710/–4560 bp) and P500 (Site C, –191/–32 bp).

We then performed DNase I footprinting assays to precisely map the potential transcription factors binding sites within these 150-bp regions. As indicated in Figure 4A, in the absence of nuclear extracts (Lanes 2, 3, 5 and 6), both regions showed uniform DNA ladders at different DNaseI concentrations. Footprints were observed only in the presence of C₂C₁₂ nuclear extracts (Lanes 1 and 4) at positions –4664/–4582 bp within D450 and –182/–120 bp within P500 regions, suggesting the binding of protein factors to those sequences.

Based on the information from the DNase I protection assays, we next used linker-scanning mutagenesis to determine the nucleotides within the protected regions that are crucial for Myh7b promoter activity. Briefly, a 17-bp fragment in the protected regions was replaced systematically with a GAL4 binding site to preserve the DNA spacing within the regions. Four mutants were generated for D450-TATA (G1–G4) and three mutants (G1–G3) for P500/luc. They were then transiently transfected into C₂C₁₂ cells and their activities compared to their respective wild type controls. As shown in Figure 4B, when the DR site (–4667/4650 bp) within D450-TATA is replaced by the GAL4 sequence in the D450-G2-TATA mutant, there is a tremendous drop in promoter activity. Correspondingly, GAL4 replacement of the PR site (–182/–165 bp) within the P500-G2 mutant results in a significant loss of promoter activity when compared to the non-mutated control (Figure 4B). Therefore, our data suggest that the DR and PR sites are positive regulatory elements which are essential for D450-TATA and P500/luc activities. To identify potential transcription factor binding motifs within these two sites, the sequences were queried using the GeneACT transcription factor binding site database. The analysis revealed that DR contains a putative E-box motif while PR contains a potential Ikaros binding motif.

E-box and Ikaros binding motifs are essential for Myh7b promoter activity

To experimentally determine which transcription factors bind to the DR and PR sites, EMSA was performed with C₂C₁₂ nuclear extracts to investigate the mobility pattern of the binding factors. Two protein–DNA complexes associate with the DR site (Figure 5A), and they are competed

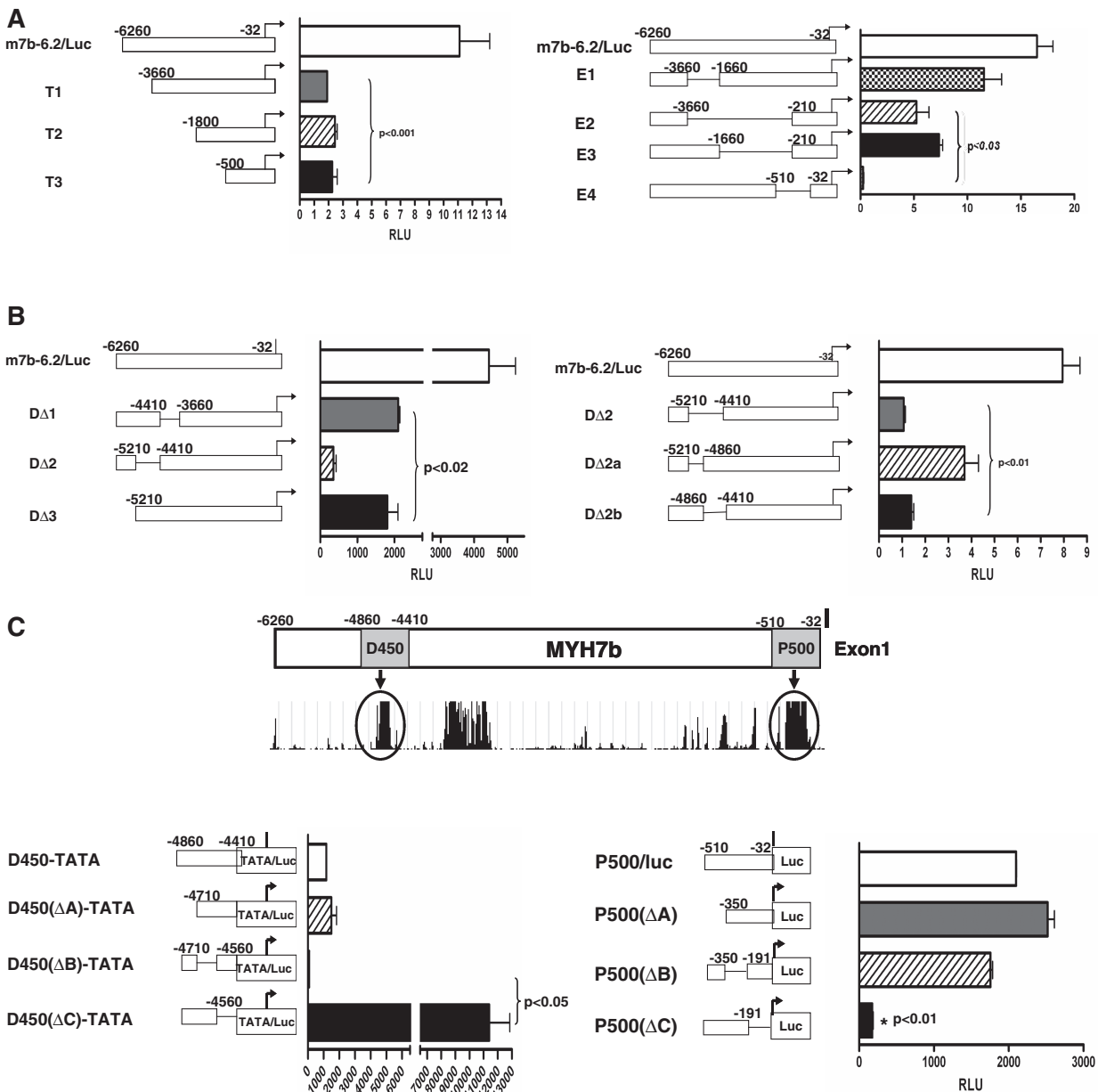


Figure 3. Mapping of the critical *cis*-elements in the Myh7b promoter. (A) Deletion studies indicated a distal and a proximal region within the Myh7b promoter that are important for promoter activity. Indicated deletion or truncation constructs of m7b-6.2/luc were co-transfected with CMV/ β -gal into C₂C₁₂ cells. Activities of the constructs were shown as luciferase activity normalized to β -gal activity (RLU). (B) Fine deletion of the 2.6 kb distal region of m7b-6.2/luc identified a 450 bp DNA fragment that is responsible for promoter activity. Transient transfections were performed as in (A). (C) D450 and P500 regions contain critical *cis*-elements for Myh7b promoter activity. Top: The positions of D450 and P500 are shown in the context of the 6.2 kb promoter while the graphical map from the UCSC Genome Browser is shown below with the corresponding regions circled, indicating D450 and P500 share high homology across multiple species. D450 was cloned upstream of a minimal TATA box (D450-TATA) and P500 was inserted upstream in the pGL3 basic vector (P500/luc).

away by specific competitor DR (Lane 9), but not by the non-specific competitor SP-1 (Lane 6). Since there is an E-box motif within the DR sequence, we investigated whether the E-box is important for the DR–protein complexes to form. As shown in Figure 5A, a generic un-labeled E-box sequence partially competed away the DR–protein complexes (Lane 7), and when the E-box motif within DR is mutated, DR can no longer act as a specific competitor to inhibit the formation of the

DR–protein Complexes (Lane 8). Moreover, DR–protein complexes are more abundant in differentiated myotube nuclear extracts (Lane 11) than in un-differentiated myoblast nuclear extracts (Lane 12) and are not observed when 293 T nuclear extracts are used (Lane 10), indicating that DR binding factors are muscle cell-enriched. Furthermore, antibody super-shift experiments show that the DR-binding factors can be super-shifted by a Myf5 antibody (Lane 2), whereas MyoD or Myogenin antibodies

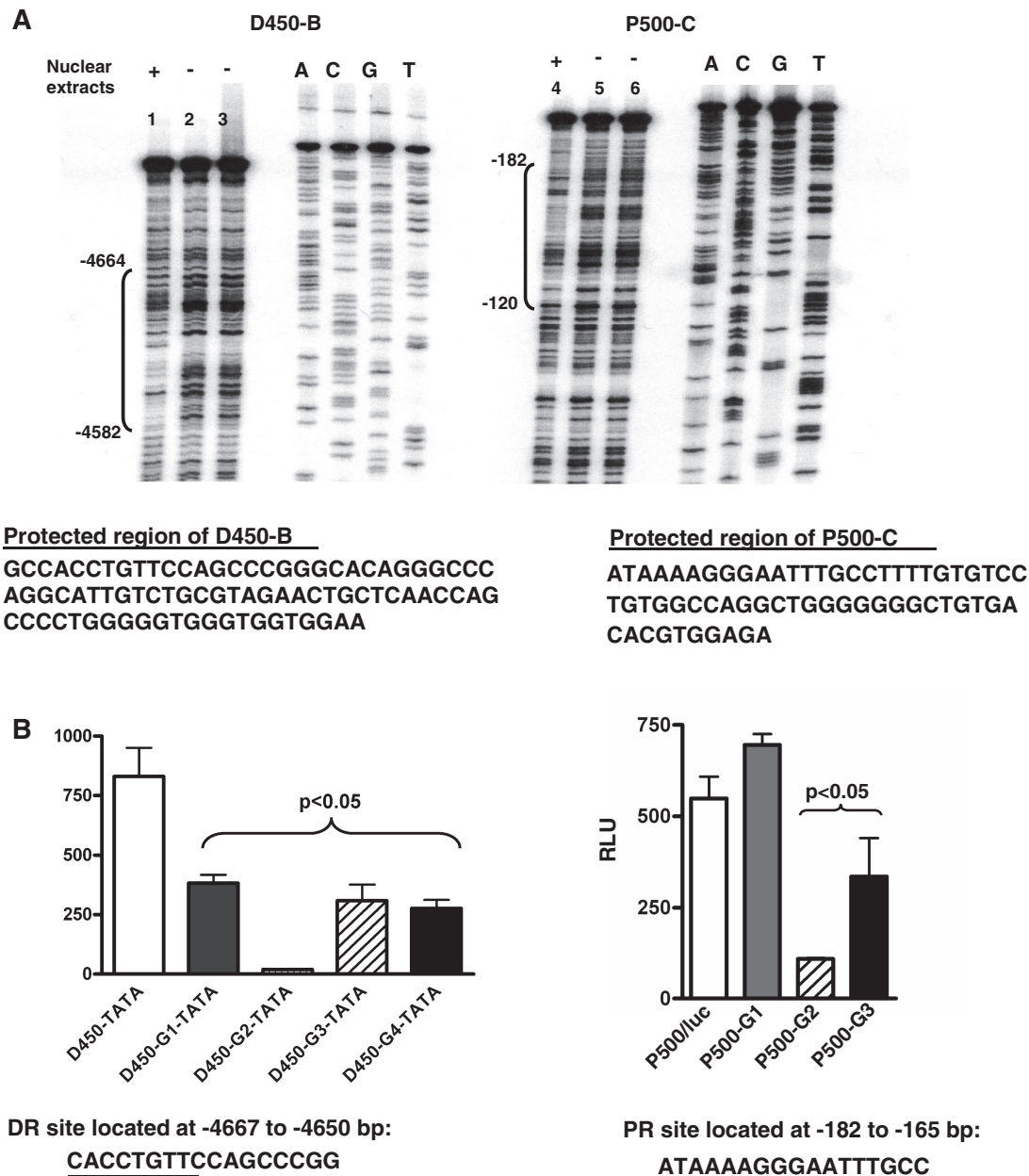
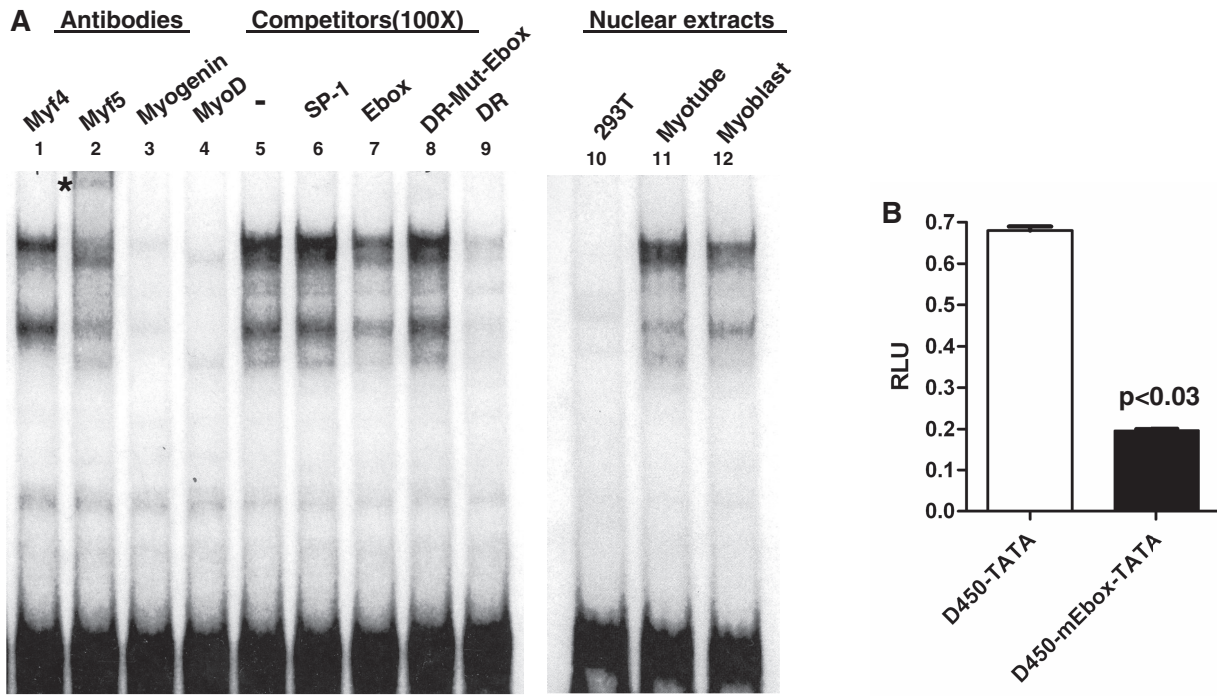


Figure 4. Identification of critical nucleotides in D450 and P500. (A) DNase I footprinting assays were performed with the 150 bp fragments containing either Site B of D450 (D450-B, -4710/-4560) or Site C of P500 (P500-C, -191/-32). Lanes 2, 3, 5 and 6 were naked DNA controls with different concentrations of DNase I in the absence of C₂C₁₂ myoblast nuclear extracts indicating even DNA ladders. The footprints were observed (brackets) in D450-B and P500-C only in the presence of C₂C₁₂ nuclear extracts (Lanes 1 and 4). The corresponding sequencing reactions are shown to the right of the footprinting assays. The sequences of the protected regions are shown beneath the autoradiogram. (B) Linker-scanning mutagenesis identified DR and PR as the important nucleotides for D450-TATA and P500/luc activities, respectively. A 17 bp fragment in each of the protected regions was replaced systematically with a GAL4 binding site to generate a panel of mutants. The sequences of the PR site and DR site within the respective G2 mutants are shown beneath the graphs. The potential E-box and Ikaros motifs are underlined.

effectively block the DR-binding factors from associating with the DR probe, hence the disappearance of the bands (Lanes 3 and 4). These results strongly suggest that the MRF family members, MyoD, Myogenin and Myf5, present in C₂C₁₂ nuclear extracts, associate with the DR site and together they could potentially regulate Myh7b promoter activity.

To validate our *in vitro* EMSA results, we first mutated the E-box motif in D450-TATA and determined how the

mutation affected the activity of D450-TATA. As shown in Figure 5B, when the critical nucleotides for MRF binding are mutated, D450-TATA activity is reduced to 30% of the wild type activity. Next, we co-expressed MyoD, Myogenin or Myf5 with D450-TATA or D450-G2-TATA in C₂C₁₂ cells (Figure 5C). Our data show that overexpression of MyoD most strongly upregulates D450-TATA activity (>500 fold), followed by Myf5 and Myogenin. The activation is specific,



DR: gatgcacagcagctgccacctgttccagcccgggca
 DR-Mut-Ebox: gatgcacagcagctgcTGccGAttccagcccgggca

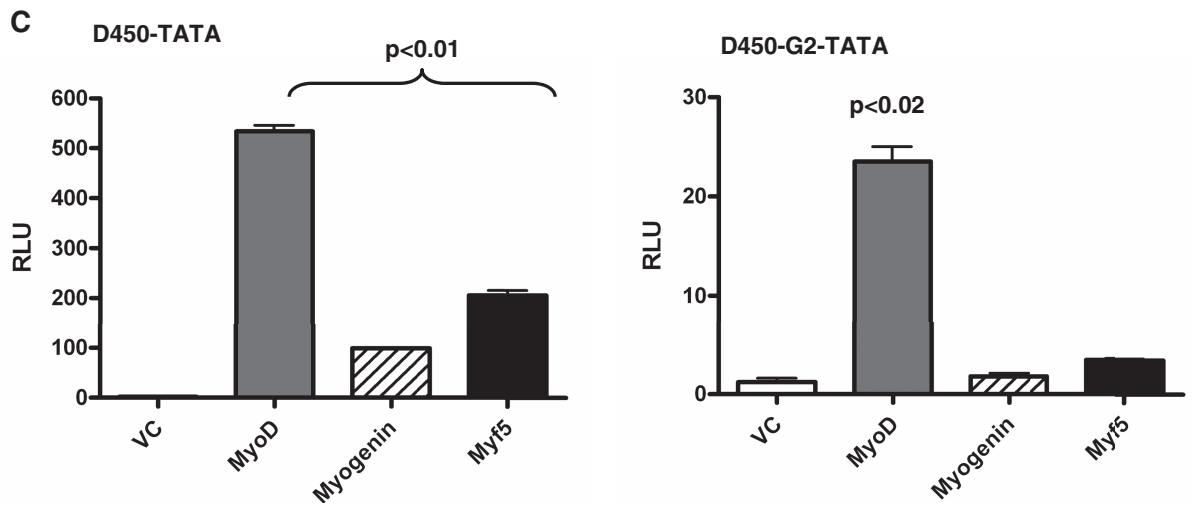
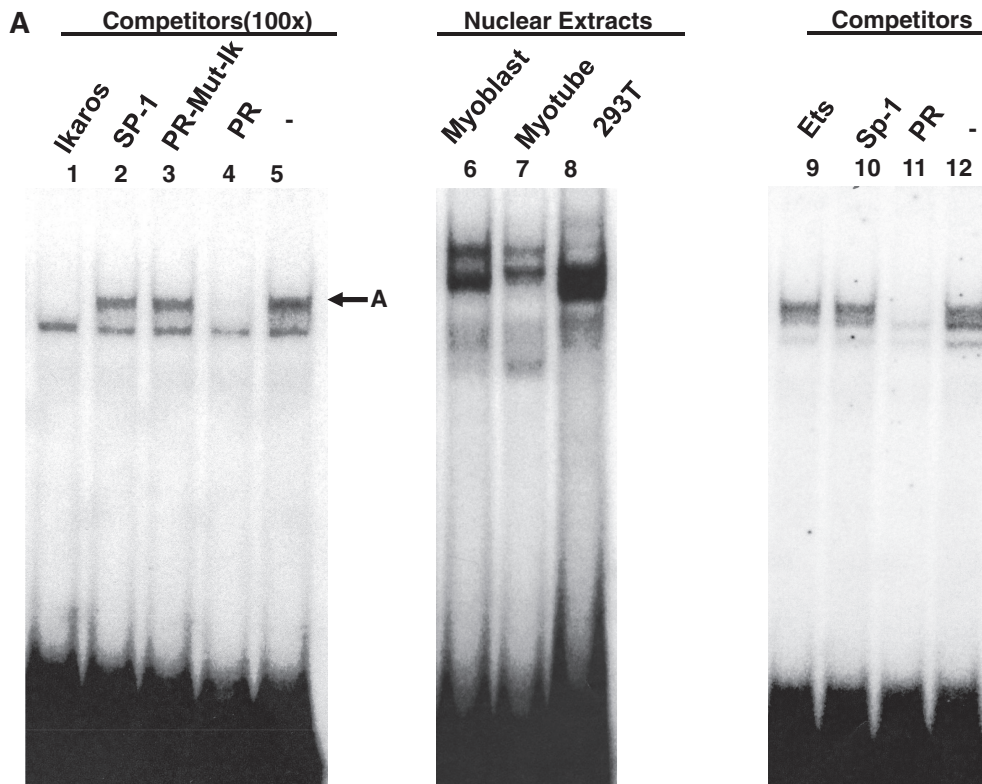


Figure 5. The E-box motif is crucial for D450 transcriptional activity. **A,B:** EMSA and site-directed mutagenesis indicated that the E-box is essential for D450-TATA activity. C₂C₁₂ nuclear extracts (Lanes 1–9 and 12) and the DR probes were used in EMSA. The E-box motif is underlined in the DR probe sequence. DR-mut-Ebox competitor contains nucleotide mutations as indicated in capital letters. D450-mEbox-TATA contains the same mutations as the DR-mut-Ebox probe. **C:** Overexpression of MRFs activates the distal E-box in the Myh7b promoter. One microgram of D450-TATA or D450-G2-TATA was co-transfected with 0.1 μg of MyoD, Myf5, Myogenin or empty vector (VC) into C₂C₁₂ cells.

because when the E-box motif is replaced by a GAL4 linker in the D450-G2-TATA construct, Myogenin and Myf5 fail to activate the construct, while the activation by MyoD is greatly attenuated. Therefore, both our EMSA and transient transfection data show that the

MRFs, MyoD, Myogenin and Myf5 bind to the E-box within the DR site to regulate the activity of the D450 region.

EMSAs were also performed with the PR sequence within P500 (Figure 6A), which shows two protein–DNA



PR: aggagataaaaggggaattgcctttgtgtcctgt
PR-mut-IK: aggagataaaagggCctttgtgtcctgt
Ets: gggctgctgaggaagtataagaat

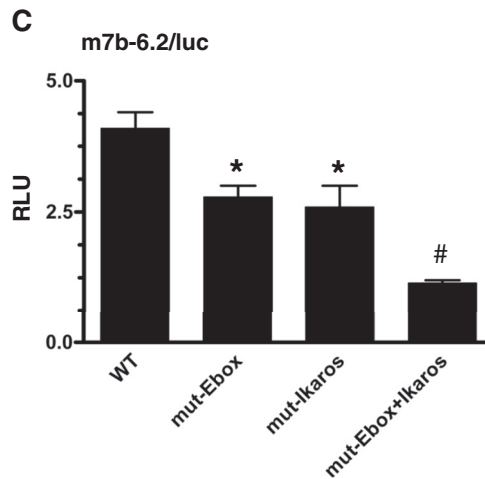
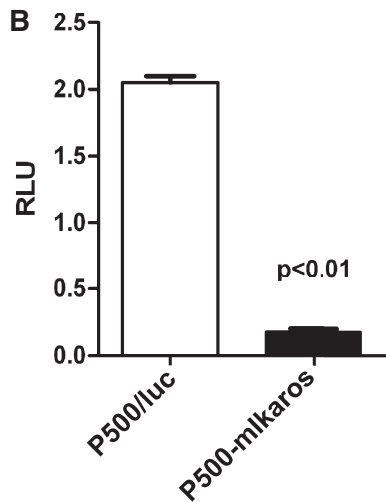


Figure 6. Eos associates with the Ikaros motif within P500 to regulate Myh7b promoter activity. (A and B) EMSA and site-directed mutagenesis indicated the Ikaros site is important for P500 activity. C₂C₁₂ nuclear extracts (Lanes 1–7, 9–12) and the PR probes were used in the EMSA experiments. The Ikaros motif is underlined in the PR probe sequence. The PR-mut-Ik competitor contained the indicated point mutations (capital letters). Multiple point mutations were introduced into the Ikaros site within P500/luc as indicated in PR-mut-Ik to generate P500-mIkaros. C₂C₁₂ were transiently transfected with either P500/luc or P500-mIkaros. (C): Wild-type m7b-6.2/luc construct or m7b-6.2/luc constructs containing point mutations in the distal E-box (mut-Ebox), proximal Ikaros site (mut-Ikaros) or both sites (mut-Ebox + Ikaros) were transiently transfected into C₂C₁₂ cells. *Significantly different from the wild-type (WT) promoter, $P < 0.05$; #significantly different from WT, mut-Ebox and mut-Ikaros, $P < 0.05$. (D) Ikaros family members were overexpressed in 293 T cells together with the m7b-6.2/luc and CMV/ β -gal. The RLU of the vector control (VC) group was set to 1 and the activities of the various overexpression groups are presented relative to the VC. (E) Mutation of the Ikaros site within P500/luc abolished the effects of Eos overexpression. One microgram of P500-mIkaros was co-transfected with either 0.1 μ g of VC or Flag-Eos plasmids into C₂C₁₂ cells. (F) EMSA super-shift experiment using Eos antibody and control antibody p65. *Indicates the disappearance of Complex A.

(continued)

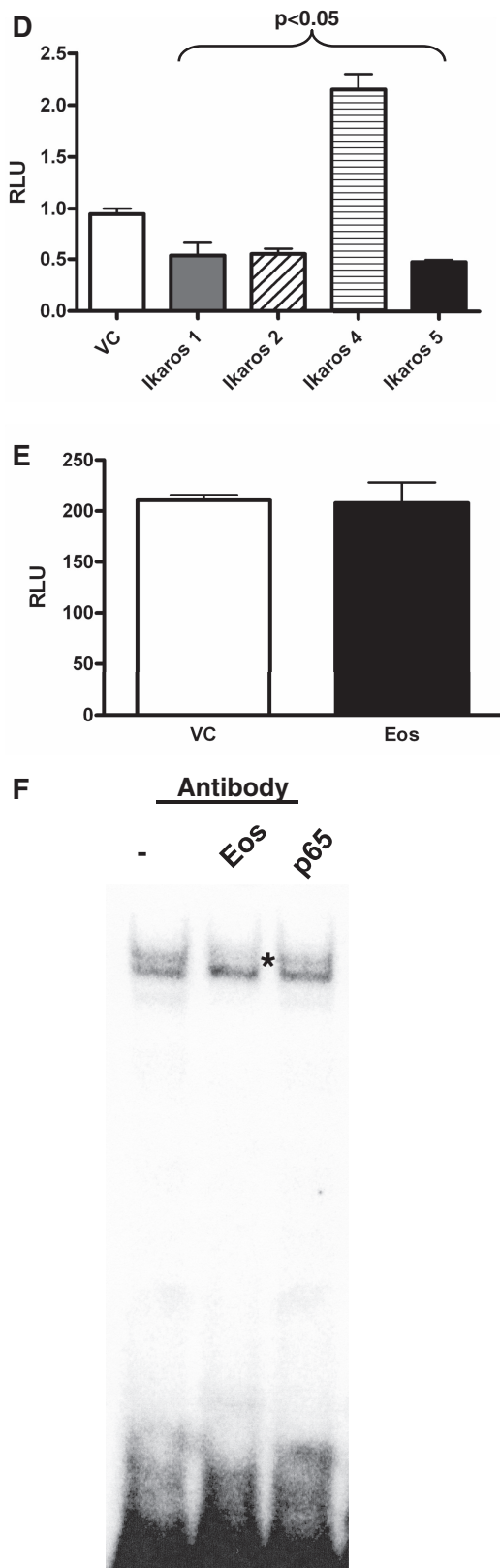


Figure 6. Continued.

complexes associated with the PR site. However, only one appears to be specific (Complex A), for it was competed away by the specific competitor PR (Lane 4), and not affected when non-specific competitor SP-1 was used (Lane 2). Moreover, Complex A seems to be more abundant in myoblast nuclei (Lane 6) than in myotube nuclei (Lane 7), and a different mobility pattern is observed when 293T nuclear extract was used (Lane 8). Since PR contains an Ikaros motif, we then tested the importance of the Ikaros site within the PR sequence for protein binding. As shown in Figure 6A Lane 1, an Ikaros consensus sequence effectively competed away Complex A, while mutation of the Ikaros motif within PR site eliminated its ability to block the formation of Complex A (Lane 3). Furthermore, since the Ets transcription factor binding site (GGAA) shares homology with the Ikaros site (GGGAA), we also included it as a competitor in the EMSA experiments. Figure 6A (Lane 9) indicates that unlike the specific competitor PR (Lane 11), the Ets competitor did not compete away Complex A.

Next, we mutated the Ikaros site within P500/luc to determine whether the Ikaros site is essential for P500/luc activity. Figure 6B shows that point mutations of the Ikaros site reduced P500/luc activity to 10% of wild-type activity. In the context of the 6.2 kb promoter, point mutations of the individual distal E-box or the proximal Ikaros site downregulated the wild type promoter activity to ~60%, while mutations of both sites further decreased the promoter activity to ~28% of wild-type activity (Figure 6C).

Using a published C_2C_{12} microarray dataset (29), we found that Ikaros 1, 2, 4 and 5 are expressed in C_2C_{12} cells, and upon differentiation Ikaros 4 and 5 RNA levels go down. Since our EMSA data (Figure 6A) suggest that the level of the PR binding factor decreased when differentiated C_2C_{12} nuclear extracts were used and Perdomo *et al.* showed that Ikaros 4 (Eos) is the only Ikaros member that is highly expressed in skeletal muscle (16), Eos appeared to be a good candidate for the PR binding factor.

To determine the role of individual Ikaros members in the regulation of Myh7b promoter activity, we cloned Ikaros 1, 2, 4 and 5 from C_2C_{12} cDNA and overexpressed them individually with m7b-6.2/luc in cells. As shown in Figure 6D, among the four Ikaros members, only the overexpression of Eos activates the Myh7b promoter activity ≥ 2 fold in cells while the other members of Ikaros actually repressed (40–60%) the m7b-6.2/luc activity. Furthermore, when the Ikaros site is mutated within the promoter, Eos can no longer activate the promoter (Figure 6E), suggesting that the Ikaros site is required for Eos activation. We have also performed EMSA experiments to determine whether Complex A can be super-shifted by an antibody specific to Eos. As shown in Figure 6F, the Eos antibody effectively blocked Complex A formation while the control antibody (p65) did not. Thus, our results strongly suggest that Ikaros 4-Eos binds to the Ikaros motif within the proximal PR sequence to activate Myh7b promoter activity in cells.

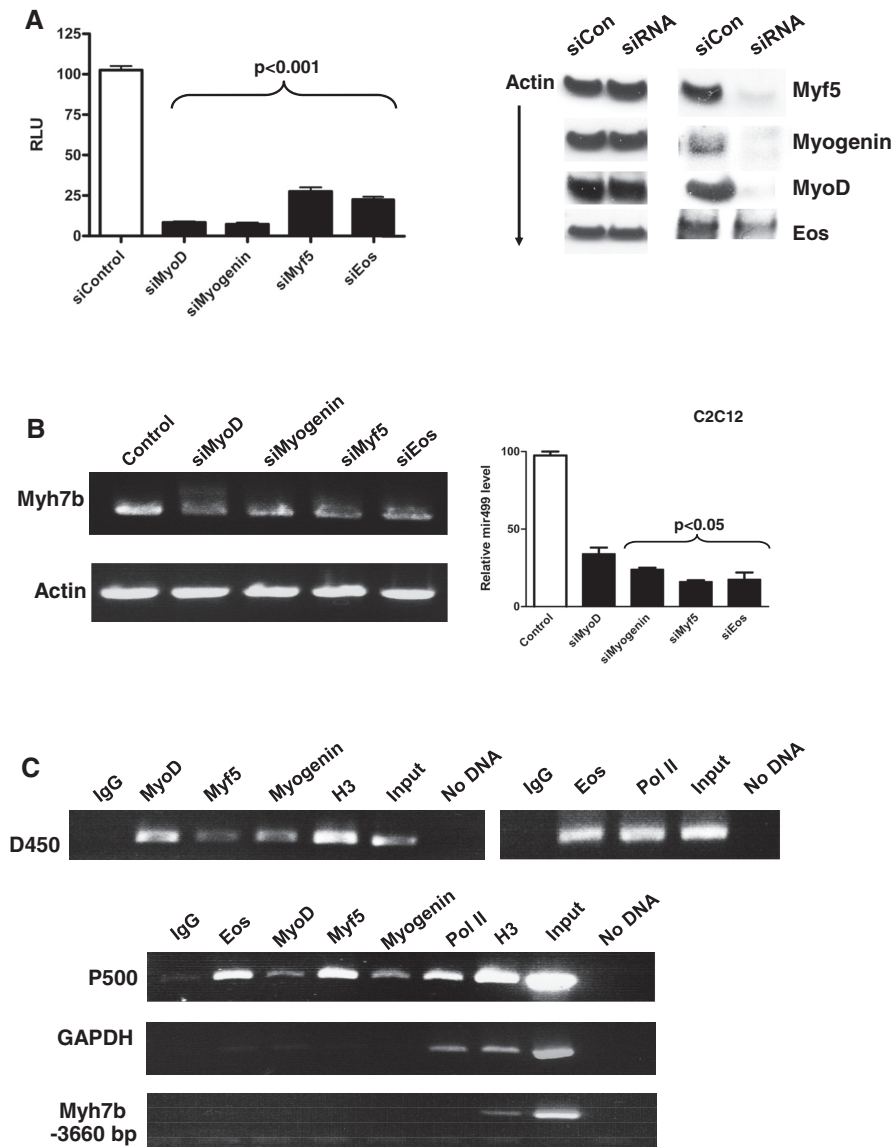


Figure 7. MRFs and Eos regulate Myh7b/miR-499 expression. (A) Myh7b promoter requires MRFs and Eos for activity. Indicated siRNA was transfected into C₂C₁₂ cells for 24 h followed by m7b-6.2/luc plasmid transfection. The RLU of m7b-6.2/luc in the presence of respective siRNAs is depicted relative to the non-targeting siRNA control, which was set as 100. Western blot analysis of C₂C₁₂ cells demonstrated efficient knockdown of the target proteins by the siRNAs. Endogenous α -actin protein levels are shown as controls. (B) Eos and MRFs are essential for the endogenous expression of Myh7b/miR-499 in muscle cells. Indicated siRNA knockdowns were performed in C₂C₁₂ cells. After RNA isolation, Myh7b and miR-499 levels were determined by SYBR green qPCR and Taqman microRNA assays, respectively. α -Actin was used as an internal control for Myh7b PCR and Sno202 was used as the internal control for miR-499 expression. (C) MRFs and Eos are chromatin-bound on D450 and P500 regions of the Myh7b promoter. ChIP assays were performed with the indicated antibodies on the Myh7b promoter. IgG served as the no antibody control, no DNA served as the PCR negative control, Myh7b -3660 bp and Gapdh served as a non-specific gene control.

MRFs and Eos regulate Myh7b/miR-499 expression

Next, endogenous MyoD, Myf5, Myogenin or Eos was knocked down individually by siRNA to investigate their roles in the regulation of Myh7b promoter activity and endogenous Myh7b/miR-499 expression. As shown in Figure 7A, siRNA targeting MyoD or Myogenin reduced m7b-6.2/luc activity most, followed by Eos and Myf5 siRNA. Similarly, expression levels of Myh7b and miR-499 were significantly reduced when MyoD, Myf5, Myogenin or Eos were knocked down (Figure 7B).

Hence, our data indicate that MyoD, Myogenin, Myf5 and Eos are essential for Myh7b promoter activity and endogenous expression levels in C₂C₁₂ cells.

To determine whether these transcription factors associate with the Myh7b promoter region at the chromatin level, ChIP assays were performed. Figure 7C shows that MyoD, Myogenin, Myf5 and Eos antibodies pulled down both D450 and P500 regions in C₂C₁₂ cells. Furthermore, RNA polymerase II was recruited to the complex on the chromatin which together with acetylated-Histone 3 suggests that the DNA region is transcriptionally active.

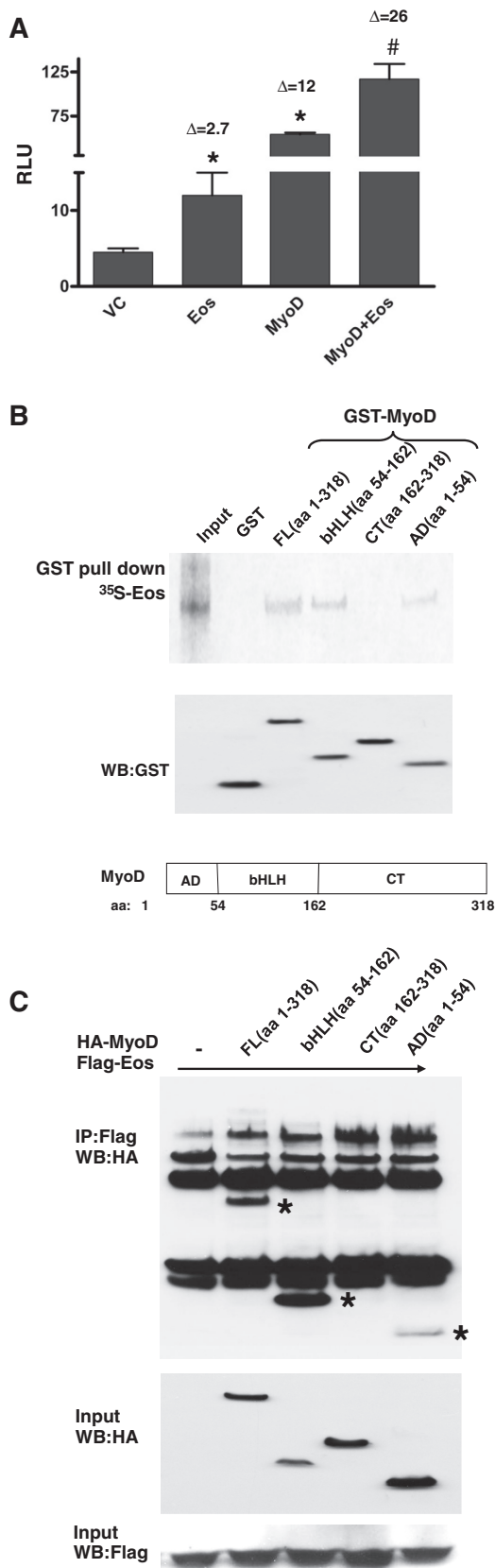


Figure 8. MyoD interacts with Eos through its bHLH and AD domains. (A) MM14 muscle cells were transfected with expression constructs encoding Eos or MyoD, either individually or together. *Significantly different from vector control (VC), $P < 0.05$; #significantly different from

Since the MRF and Eos antibodies pulled down both the distal E-box and proximal Ikaros regions, we also included two negative controls to eliminate the possibility of non-specific pull-down by the antibodies. The Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter control is used to demonstrate that the MRF and Eos antibodies pulled down the Myh7b gene specifically. The -3660 bp region control of the Myh7b promoter is used to show that the recruitment of these transcription factors to the Myh7b promoter depends on the presence of the distal E-box and proximal Ikaros motifs, since this region does not contain any E-box or Ikaros sites. The lack of chromatin-associated MyoD, Myogenin, Myf5 and Eos on either of the negative control genomic regions strongly suggests that these transcription factors specifically associate with the distal E-box and proximal Ikaros motifs on the Myh7b chromatin.

Eos interacts with MyoD through two distinct domains

The fact that the Eos antibody pulls down the D450 region which does not contain an Ikaros site and that the MRF antibodies also pulls down the P500 region which does not contain any E-box motif suggests that these two groups of transcription factors may physically interact with each other on chromatin to activate transcription. To test this hypothesis, we overexpressed MyoD, Myogenin or Myf5 with or without Eos in muscle cells to determine whether they synergistically activate the Myh7b promoter. Among the three MRFs, only MyoD could activate the Myh7b promoter synergistically with Eos (Figure 8A). Next, we performed GST pull-down assays with ³⁵S-labeled Eos and recombinant GST-MyoD to determine whether they interact with each other *in vitro*. As shown in Figure 8B, in addition to the full length, both the activation domain (AD) and the bHLH domain of MyoD also pulled down Eos, suggesting that MyoD interacts with Eos through the AD and bHLH domains. The *in vitro* interactions were confirmed with Co-IP assays by overexpressing Flag-tagged Eos and hemagglutinin (HA)-tagged MyoD in 293 T cells. Similar results were obtained from the Co-IP experiments (Figure 8C) in which Eos effectively pulled down the AD, bHLH domains and full-length MyoD. Collectively, our data strongly suggest that Eos physically interacts with MyoD; however, it is not clear whether the interaction is direct or mediated through a bridging factor.

The role of Eos in the regulation of myosin genes

To determine whether there is a broader role for Eos in endogenous muscle gene regulation, Eos was knocked

VC, Eos and MyoD, $P < 0.05$, Δ indicates fold increase compared to VC. (B) ³⁵S-labeled Eos and recombinant GST-MyoD as well as different domains of MyoD (AD = activation domain, bHLH = basic helix-loop-helix, CT = C-terminal) were used for *in vitro* GST pull-down assays and analyzed by autoradiography. Inputs were determined by immunoblotting with a GST antibody to show that the same amounts of recombinant proteins were used in the reactions. (C) IP-western with 293 T cells overexpressing Flag-Eos and HA-MyoD as indicated. Cell lysates were immunoprecipitated (IP) with a Flag antibody, followed by immunoblotting (WB) with a HA antibody. Inputs were analyzed by WB using indicated antibodies.

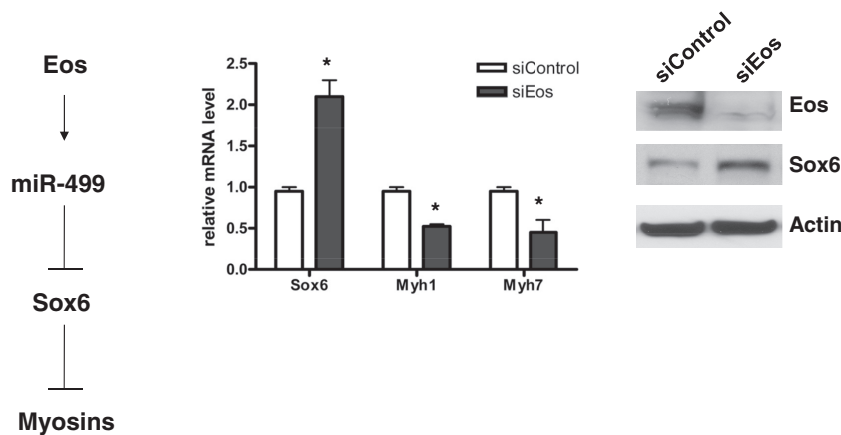


Figure 9. The role of Eos in myosin gene regulation. Indicated siRNA knockdowns were performed in C_2C_{12} cells. mRNA levels of Myh1, Myh7 and Sox6 were determined by qPCR. 18S was used as an internal control for q-PCR. Protein levels of Sox6 and Eos were analyzed by western blot with α -actin as internal control. *Significantly different from the siRNA control (siControl), $P < 0.05$.

down by siRNA and the mRNA levels of multiple myosin genes were determined by real-time PCR. As indicated in Figure 7B, the knockdown of Eos protein levels led to the downregulation of miR-499 levels, and as a result, both the protein and RNA levels of the miR-499 target Sox6 went up (Figure 9). The fact that Sox6 is capable of repressing multiple myosin genes by directly binding to their promoter regions (8) suggests that the upregulation of Sox6 could lead to the downregulation of myosin gene expression. Therefore, we hypothesized that Eos can regulate myosin gene expression through the regulation of the miR-499/Sox6 pathway. Our results show that when Eos protein expression is decreased, both Myh1 and Myh7 mRNA levels are significantly reduced (~50% and ~45%, respectively). This agrees with published data demonstrating that Myh1 and Myh7 are both targeted by Sox6 repression through direct chromatin association (8) but indirectly by Eos, since no apparent Ikaros motifs were found in the proximal region of the promoters. Therefore, our study strongly suggests that Eos can coordinately regulate multiple myosin genes (Myh1, Myh7 and Myh7b) either directly through the regulation of the promoter or indirectly through the miR-499/Sox6 pathway.

DISCUSSION

The transcriptional regulation of eukaryotic genes is a complex process that is regulated by a cohort of transcription factors. The regulatory regions of genes require a specific combination and co-operation of various transcription factors unique to each cell lineage at different stages of differentiation to achieve gene expression in an efficient and specific manner. Myh7b is a more recently discovered myosin that encodes miR-499. In spite of the diverse functions of miR-499 in multiple biological systems (1,5,7,30,31), very limited analysis has been performed on the Myh7b promoter (3,7), as a result little is known regarding the transcription factors involved in the regulation of Myh7b/miR-499 in muscle cells. The present study is the first extensive Myh7b promoter analysis for

the purpose of identifying trans-activators controlling expression of the gene and its intronic microRNA. We have cloned a 6.2 kb promoter region of Myh7b gene that is capable of driving muscle-specific reporter gene activity both *in vitro* and *in vivo* and the activity of the promoter recapitulates the endogenous expression of Myh7b/miR-499 in response to various stimuli. Work presented here identifies two critical regulatory elements, an E-box and an Ikaros motif, involved in the regulation of Myh7b promoter activity in C_2C_{12} cells. Through EMSA, we identified MyoD, Myogenin and Myf5 as the transcription factors which bind the E-box, while Ikaros 4-Eos associates with the Ikaros motif. ChIP analyses further suggest that these two different classes of transcription factors interact with each other on the Myh7b chromatin. Interestingly, Figure 7A and B show that knocking down any one of the four individual transcription factors is sufficient to significantly decrease Myh7b promoter activity and endogenous Myh7b/miR-499 expression, suggesting that MRFs and Eos not only bind to their respective consensus sites within the Myh7b promoter, but also require one another to form an enhanceosome complex (32) on the chromatin to recruit co-activators and general transcription factors to activate Myh7b transcription. Therefore, the delicate interplay and co-ordination of MyoD, Myf5, Myogenin and Eos could specifically fine tune the expression level of Myh7b/miR-499 in cells.

Several key pathways including the MRF family of transcription factors have been implicated in the transcriptional control of muscle-specific genes including the MyHCs. The results from the current study complement our previous findings that E-boxes and their cognate binding factors (MRFs) are essential in modulating the tissue-specific activity of multiple MyHC promoters (9,10), including Myh7b in muscle. Results from Figure 5A demonstrate that (i) the muscle-specific expression of Myh7b (Figure 2A and D) is tightly regulated by MRFs that are only present in C_2C_{12} nuclei (Lanes 11 and 12) and not in 293 T nuclei (Lane 10); (ii) our EMSA data further indicate that the DR-protein complexes (Lane 11) are more abundant in C_2C_{12} myotube

nuclear extracts than myoblasts (Lane 12), which implies that DR binding proteins are upregulated during differentiation. Interestingly, among the three MRFs, Myogenin is the only essential immediate early gene that is activated by MyoD during myogenesis; therefore, differentiated myotubes are known to have increased expression of Myogenin. Additionally, results from Figure 5C also show that when the E-box motif is replaced by a GAL4 linker, ectopic expression of Myogenin can no longer activate D450-TATA activity, suggesting that Myogenin may be the transcription factor which directly associates with the E-box within the distal region while MyoD and Myf5 are tethered to the DNA by complexing with Myogenin.

One of the major findings in this study is the discovery of a novel role of a member of the Ikaros family, Eos, in the regulation of the expression of multiple myosin genes. At present there is no information on the function of Eos in muscle; our results are the first to suggest that Eos plays a role in regulating muscle contractile proteins via the miR-499/Sox6 pathways. The fact that Eos interacts with a potent myogenic transcription factor, MyoD (Figure 8B and C), and regulates Sox6 levels through miR-499 (Figures 7B and 9) indicate its potential function in the regulation of the myofiber gene program. Further efforts to elucidate the role of Eos in muscle could provide additional insights into the myogenic program.

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