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Transcriptional Regulation of the Type I Myosin Heavy Chain Promoter in Inactive Rat Soleus

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ABSTRACT

Chronic muscle inactivity with spinal cord isolation (SI) decreases expression of slow type I myosin heavy chain (MHC) while increasing expression of the faster MHC isoforms, primarily IIx. The purpose of this study was to determine if type I MHC down-regulation in the soleus of SI rats is regulated transcriptionally and to identify cis elements or regions of the rat type I MHC gene promoter involved in this response. One week of SI significantly decreased in vivo activity of the –3500, -408, -299, -215, and –171 type I MHC promoters. The activity of all tested deletions of the type I MHC promoter, relative to the human α skeletal actin promoter, were significantly reduced in the SI soleus except for activity of –171 promoter which increased. Mutation of the βe3 element (-214/-190) in both the –215 and –408 promoters and deletion of this element (-171 promoter) attenuated type I down-regulation with SI. Gel mobility shift assays demonstrated a decrease in transcription enhancer factor 1 (TEF-1) binding to the βe3 element with SI despite an increase in total binding to this region. These results demonstrate that type I MHC down-regulation with SI is transcriptionally regulated and suggest that interactions between TEF-1 and the βe3 element are likely involved in this response.

βe3 DNA regulatory element, transcription enhancer factor-1, spinal cord isolation, chronic muscle inactivity

INTRODUCTION

Chronic decreases in neuromuscular activity strongly influence myosin heavy chain (MHC) expression in rodent skeletal muscle (for reviews see 27, 36). Both decreased neuromuscular activation and unloading as occurs with spinal cord transection (37) and reduced loading or weight bearing as induced by hindlimb suspension (simplified herein as unloading) (14, 23) decrease the relative expression of slow type I MHC while increasing the relative expression of the faster MHC isoforms in the normally slow rat soleus. While these models decrease overall muscle activity and load, some residual neuromuscular activation remains, which could influence muscle gene expression. Specifically, MHC expression could be altered by any remaining activation-associated factors despite the dramatic reduction in load and activity. While denervation results in complete inactivity, it also removes non-activity dependent neurotrophic factors, which may be involved in the regulation of MHC plasticity (32, 33). Consequently, it has been difficult to develop a suitable model to study the effects of complete inactivity on MHC gene regulation while maintaining an intact motoneuron-muscle connection, i.e. without direct interruption of potential activity-independent neurotrophic factors.

Spinal cord isolation (SI) provides a unique model in which the hindlimb muscles are silenced for prolonged periods by functionally isolating the lumbar region of the spinal cord (6, 25, 28). This model eliminates supraspinal, infraspinal, and peripheral afferent input to the isolated cord segment while maintaining motoneuron-muscle connectivity. Thus, SI provides a model that removes both neuromuscular activation and loading while the motoneuron continues to exert activity-independent neurotrophic effects on the inactive muscles (6, 28).

We recently reported that SI results in a time-dependent decrease in type I MHC in the rat soleus, which is primarily offset by an increase in fast IIx MHC (16). These MHC shifts were characterized by an early loss in type I MHC as evidenced by the rapid muscle atrophy and concomitant decreases in muscle protein and MHC content occurring over the first 8 days of SI. The loss of nearly half of the type I MHC protein in the soleus after only 8 days of inactivity is the result of negative balance between protein synthesis and degradation. This imbalance may be due to alterations in transcriptional/ translational and/or post-translational processes. In a previous study (16), analysis of the time course of changes in type I MHC protein and mRNA expression in the soleus of SI rats revealed that the changes at the mRNA level preceded changes at the protein level. Furthermore, over the complete SI time course ranging from 4 to 90 days, there was a tight coupling between changes at the mRNA and protein levels suggesting that pre-translational processes dominated MHC isoform shifts in the chronically inactive soleus. However, the question remains as to whether these pretranslational processes are transcriptional; therefore, the present study further characterizes the molecular mechanisms mediating down regulation of type I MHC expression in inactive slow muscle. It is hypothesized that the molecular remodeling of the soleus associated with SI is regulated in part at the transcriptional level via interaction of specific transcription factors with cis regulatory elements located on muscle gene promoters. Furthermore, inactivity is likely associated with changes in the nuclear milieu that renders it unfavorable for transcription of slow muscle genes.

Within the MHC gene family, slow type I MHC expression has been studied most extensively in both cardiac and skeletal muscle, and the gene has been isolated from several mammalian species including human (21, 30), rat (22), mouse (11, 42), and rabbit (8). In vivo studies involving transgenic mice (26), have provided evidence that type I MHC promoter activity is muscle specific. Reporter activity was detected primarily in cardiac and slow skeletal muscles. In contrast, no reporter activity was detected in pure fast muscles (masseter) or non-muscle tissue of transgenic animals harboring the type I MHC promoter fused to the CAT reporter gene. Further, in vivo studies using direct muscle gene injection demonstrated that the type I MHC promoter is 25 fold more active in the slow soleus as compared to the fast tibialis anterior (TA) (9) which corresponds to endogenous gene expression. Based on transient transfection assays in cardiac and muscle cell culture (5, 7, 17, 18, 31, 38), several highly conserved positive and negative cis regulatory elements have been identified within the most proximal region of the type I MHC promoter (within 350 bp of the transcription start site). For example, at least 3 positive elements that are necessary for muscle-specific expression have been identified: βe2 (-285/-269), CACC Box (-245/-233) and βe3 (-210/-188) (38). A negative element, βe1 (-330/-300) has also been identified (5). In addition, the upstream distal region (-3500/-2500) was found to be required for optimal promoter activity in both cardiac and slow skeletal muscles (9, 45).

Although several positive and negative regulatory elements have been identified and found necessary for type I MHC promoter activity under normal conditions, their role in modulating type I MHC gene expression under altered physiological condition is unclear. Studies have implicated the βe1 repressor region in type I MHC down-regulation in response to skeletal muscle unloading (9, 23), while all three positive elements have been implicated in the adaptation to skeletal muscle overloading (39, 43).

While transcriptional regulation of the type I MHC gene has been demonstrated under altered loading states, regulation of this gene has never been studied under conditions of complete neuromuscular inactivity. It remains to be elucidated whether decreases in type I MHC expression with chronic inactivity in slow muscles are mediated transcriptionally and whether transcriptional down-regulation would result from increased activity of a repressor element or decreased activity of an enhancer element. Consequently, SI provides an excellent model to begin to establish the functional significance of these positive and negative elements of the type I MHC promoter during periods of complete muscle inactivity. These findings will provide an excellent baseline with which to compare Type I MHC regulation in other models of decreased muscle activity and/or neurotrophic influence such as hindlimb unloading and denervation.

The purpose of the present study, therefore, was to test the hypothesis that down-regulation of type I MHC expression in the soleus of SI rats is regulated at the transcriptional level, using the in vivo direct gene transfer approach. A second goal was to identify cis-acting regulatory sequences on the rat type I MHC gene promoter necessary for its down-regulation in response to chronic inactivity. We found that one-week of SI results in a significant decrease in type I MHC promoter activity in the inactive rat soleus and that important cis DNA regulatory elements mediating this response are contained within 215 bp of the proximal promoter. Within this fragment, the βe3 element is likely involved in the type I MHC down-regulation, as both deletion and mutation of this element attenuated the decrease in promoter activity associated with SI.

METHODS

Experimental Design and Animal Procedures.

These experiments were conducted over a period of approximately 1 year, and consisted of repeating the following experimental design. For each tested promoter construct, adult female Sprague-Dawley rats (145 ± 10 g) were randomly assigned to normal control (NC) and SI groups (n=8 animals/group). Rats were anesthetized and underwent surgery for intramuscular plasmid injection into both left and right soleus muscles as described previously (9). A skin incision was made to expose the soleus, and 20 μl of phosphate buffered saline (PBS) containing plasmid DNA mixtures were injected into the muscles using a 29-gauge needle attached to a 0.5 ml insulin syringe. Plasmid mixtures consisted of the rat type I MHC test promoter (molar equivalent to 10 μg of –3.5kb type I MHC-pGL3 promoter construct) and of a fixed amount (3.29 μg) of the human skeletal α-actin reference promoter construct (HSA2000-pRL). After plasmid injection, the rats in the SI group were subjected to complete spinal cord transections at both mid-thoracic and upper sacral levels along with bilateral deafferentation between the transection sites as described previously (10, 29). Additional rats were assigned to NC (n=12) and SI (n=24) groups and used to obtain soleus for muscle nuclei extraction for use in the DNA gel mobility shift assays (see below). The care and maintenance of the SI rats has been described in detail previously (10, 29). All experiments were 7 days in duration. After 7 days, the animals were euthanized and the soleus muscle was quickly removed, frozen in isopentane cooled by liquid nitrogen and stored at -80°C until subsequent analysis. This study was approved by the Animal Use Committee at the University of California, Los Angeles, and followed the American Physiological Society Animal Care Guidelines.

Plasmid Constructs

The test promoter consisted of the rat type I MHC gene promoter linked to the firefly luciferase reporter gene in the pGL3 vector (Promega). A series of deletions of the type I MHC promoter were tested in this study, namely –3500, -408, -299, -215, and –171 bp promoter fragments all extending to position +34 at the 3’-end relative to the transcription start site (TSS). These promoter constructs were the same as those used previously by Wright et al (44). The –408 and -215 type I MHC promoter constructs with βe3 mutation (-408 βe2/βe3 mutant) were also tested in the NC and SI soleus. Mutations of the target promoter sequence consisted of changing ACC to **CGG** in the βe3 element (17, 38), and GTG to **TGT** in the βe2 element (38), and were designed to disrupt specific transcription factor binding sites (see ref (44) for details on mutagenesis). The reference promoter consisted of the human skeletal α-actin gene extending from –2000 to +250 bp relative to TSS which was linked to the renilla luciferase reporter gene in the pRL vector (Promega).

In addition, a type IIb MHC promoter construct was injected in NC and SI soleus to examine the promoter response of a gene up-regulated in response to SI (16). This experiment would also rule out the possibility of global transcriptional down-regulation with SI. The test plasmid consisted of a mouse IIb MHC promoter fragment extending from -1400 bp to +13 bp relative to the TSS, and linked to a firefly luciferase reporter gene (pGL3 basic, Promega). The IIb and human skeletal α-actin promoter fragments were the kind gift of Dr. Steve Swoap, Williams College, Williamstown, MA, and their activity in rat muscle corresponds to relative expression of the endogenous gene (35). Plasmids were amplified in E-coli cultures and purified by anion exchange chromatography using disposable columns (Endofree Maxiprep, Qiagen). Plasmids were suspended in sterile PBS and the concentration was determined by UV absorbance at 260 nm, using the conversion factor of 50 μg/ml per OD unit. Plasmid preparations were examined by ethidium bromide staining after agarose gel electrophoresis to verify their supercoiled nature and that they were free of genomic DNA and cellular RNA.

Reporter Gene Assay

Each soleus was homogenized in 1 ml of ice-cold passive lysis buffer (Promega) supplemented with protease inhibitors (0.2 mM AESBF, 5 μg/ml aprotinin, 5 μg/ml leupeptin) and the homogenate was centrifuged at 10,000 g for 10 min at 4° C. The supernatant was reserved to measure reporter gene activity using the Promega Dual Luciferase Assay kit, which is designed for sensitive detection of both firefly and renilla luciferase activities within a single extract aliquot. Five μl of supernatant was used in the assay and light output was integrated over 10 seconds and expressed as relative light units (RLUs) using a luminometer (Analytical Luminescence). Background activity levels, based on measurements of non-injected tissue for both luciferases, were determined and subtracted from the activities measured in plasmid-injected tissue.

Rationale for the Use of Skeletal α-Actin as a Reference Promoter

In the present study, activity of the type I MHC promoter linked to a firefly luciferase reporter was tested in the SI soleus using reporter gene assays following direct muscle gene transfer. This approach is predicated on the assumption that reporter gene expression (firefly luciferase activity) is directly proportional to the activity of the promoter linked to the 5' upstream region. The method of gene transfer (whether in vivo or in cell culture) is associated with variability in DNA uptake by the cells, which makes it difficult to interpret changes in reporter gene activity. Consequently, a reference promoter is co-injected with the test promoter and used as an internal control for variable gene transfer efficiency. The reference promoter drives expression of a different reporter gene, which allows independent measurement of its activity along with that of the test promoter. The reference gene promoter should not interact with test promoter activity and should ideally not change in response to experimental manipulations. Skeletal α-actin is a good reference promoter for studies utilizing muscle gene promoters for the following reasons: 1) Both skeletal α-actin and MHC are sarcomeric genes that are intrinsically active in muscle cells; thus, the transfection of exogenous promoters of these genes into muscle cells is less likely to disturb the nuclear milieu balance and cause competition for nuclear factors as observed after transfection with strong viral promoters (SV40 and CMV). 2) Both skeletal α actin and MHC are muscle specific genes that are not expressed in non-muscle cells; thus the possibility of confounding expression in surrounding non-muscle cells is unlikely. 3) Both firefly luciferase and renilla luciferase reporter expression can be assayed in a single aliquot with similar detection sensitivity and range (Promega Dual Luciferase assay protocol). As evidence of the validity of α-actin for normalizing gene transfer efficiency in muscle, Giger et al. (9) reported a strong correlation between firefly and renilla activity following co-injection of type I MHC pGL3 and human α actin pRL in the normal control soleus.

While there is strong evidence supporting the skeletal α-actin promoter as a reference in normal control muscle, its use as reference promoter in manipulated muscle needs further justification. We present reasons that strongly validate the use of skeletal α actin as a reference promoter in the soleus of SI rats, especially when comparing the responsiveness of two or more different promoters. The actin promoter has been shown to be responsive to altered loading conditions (2, 3), and based on mRNA analysis, after 8 days of SI, expression of both total MHC and skeletal α-actin mRNA relative to 18S ribosomal RNA in the soleus was reduced by ~74 % and 72% respectively (Haddad, Baldwin manuscript in preparation). This is not surprising considering the fact that both MHC and actin are basic muscle structural genes and their expression should be tightly regulated when the muscle undergoes atrophy or hypertrophy.

In the present experiments, the response of the skeletal α-actin promoter (SI vs NC) was expected to be relatively constant across all experiments because it was subjected to the same experimental manipulation (7 days of SI). For all 8 experiments in this project (8 rats/group/construct), experimental procedures and personnel were identical, and soleus atrophy was homogeneous (see Table 1), suggesting comparable actin responsiveness across experiments. As expected, the actin promoter response to SI was not significantly different among all experiments with an average decrease of ~90% (data not reported).

Unlike the actin promoter which responded to SI in a predictable and comparable manner, responses of the different type I MHC promoters could vary depending upon the presence or absence of regulatory elements. In gene transfer assay systems, measured reference and test reporter activities depend on plasmid uptake and possible variations must be accounted for in the interpretation of the results. When both actin and MHC promoter constructs are co-injected into the muscle, their uptake efficiency is thought to be similar. Thus, normalizing type I MHC test promoter activity to actin eliminates differences in plasmid uptake across experiments utilizing different MHC promoters. With this normalization, the results are interpreted as changes in test promoter activity relative to actin rather than absolute changes per se. Consequently, by using this approach (normalization to actin) the behavior of different test promoter constructs could be evaluated to define critical regulatory regions of the promoter.

Plasmid Uptake

Plasmid DNA uptake amounts can be used to normalize reporter activity without use of a reference plasmid; however, this approach does not account for possible alterations in post-transcriptional processes induced by the experimental manipulation. During the course of this study, it was clear that SI caused a dramatic decrease in both test and reference promoter activities. Consequently, the possibility existed that plasmid uptake efficiency was less in SI muscles, which would cause this decrease in both test and reference promoter activity. To gain insight on plasmid uptake in this model, tissue plasmid content at the time of analysis for reporter activity was determined using PCR.

Total tissue DNA (genomic and plasmid) was extracted from the resulting tissue pellet following centrifugation of the soleus homogenate in the reporter gene assay protocol described above. DNA extraction was performed using the Quiagen QIAamp DNA Mini Kit according to manufacturer's protocol for tissue extraction. DNA was eluted in 200 μl of nuclease-free water.

Plasmid detection and quantification was based on PCR amplification. PCR primers to detect pGL3 plasmid were targeted specifically to the firefly luciferase cDNA and their sequence shares no identity to any of the rat genomic DNA sequence. The primers used to detect the pGL3 plasmid are as follows and they generated a 380 bp PCR product:

5' pGL3 Primer (1163-1181): CGGGCGCGGTCGGTAAAGT

3' pGL3 Primer (1542-1524): AACAACGGCGGCGGGAAGT

The numbers in parentheses refer to the base position within the pGL3 basic sequence (Genebank; Accession# U47295). The FLuc cDNA gene is located from 88 to 1737 bp (Promega Technical Reference).

Five μl of the DNA template was amplified using PCR (final concentrations): 1X PCR buffer, 20 μM of each dNTP, 1 μM primers, 2.8 mM $MgCl_2$, and 0.75 units Taq polymerase. Amplification was carried out in a Stratagene thermocycler (Stratagene, La Jolla, CA) and was started with an initial denaturation step at 96°C for 3 min, followed by 1 min at 96°C, 45 s at 59°C, and 50 s at 72°C for 25 cycles, and a final elongation step for 3 min at 72°C. Under these

PCR conditions, the signal was in the linear range of a semilog plot of the yield vs. number of PCR cycles. Samples were run in duplicate with appropriate negative and positive controls. PCR products were separated on 2% agarose gels containing ethidium bromide. Pictures of the gels were obtained under UV light with Polaroid 55 film to obtain both a negative and positive gel image. The negatives were scanned (Molecular Dynamics Personal Densitometer) and band intensity was determined with the Image Quant 5.0 software package (Molecular Dynamics) using volume integration.

Gel Mobility Shift Assays

Skeletal muscle nuclei were isolated and extracted according to the method described previously by Blough et al. (1). Nuclear extract was prepared from NC and SI soleus muscles (n=6/group, each ‘n’ consisted of a pool of 4 muscles for control, and 8 muscles for SI, ~200 mg/pool) and stored at –80° C. Double-stranded oligonucleotides consisting of 24 bp from the Type I MHC gene promoter spanning specific regulatory elements. The βe3 oligonucleotide sense strand sequence and βe3 mutant sense strand sequence are given in Table 2. After strand annealing, double-stranded probes were end labeled with ^{32}P using T4 polynucleotide kinase (Promega).

For the binding reaction, 20 μg nuclear protein was pre-incubated for 10 minutes on ice with 15 ng nonspecific homologous DNA (poly dIdC) in a buffer containing 50 mM KCl, 20 mM HEPES pH 7.9, 1 mM DTT, 1 mM $MgCl_2$, 1 mM EDTA, 7.5% glycerol, and 0.025% BSA. At the end of this pre-incubation, approximately 200,000 cpm of 5’ end labeled probe was added and reactions (20 μl total volume) were incubated at room temperature for 30 min. For the supershift experiments, 0.5 μg of TEF-1 antibody (BD Transduction Laboratories, Los Angeles, CA) was added to the reactions after 20 min of incubation with the probe and incubated at room temperature for an additional 20 min. Reactions were terminated by adding 2 µl loading buffer (10% ficoll, 0.01% xylene cyanol and 0.01% bromophenol blue). Immediately, the samples were loaded on a 7% polyacrylamide gel and electrophoresed at 250 V for two hours in 0.5X TBE under non-denaturing conditions. Gels were dried and exposed to a phosphorimager storage screen for 24 hours. Gel images were obtained by laser scanning of the storage screen with a phosphorimager (Molecular Dynamics). For competition experiments**,** 100-fold molar excess of unlabeled probes were included in the pre-incubation described above (i.e. before addition of the labeled probe). Band intensity was quantified with the Image Quant 5.0 software package (Molecular Dynamics).

Specificity of the TEF-1 antibody used in the gel supershift assays was confirmed using western blots and supershifts with a double-stranded DNA probe which is unrelated to the TEF-1 binding site such as BNP-GATA (41). In western blots using soleus nuclear extract, along with HeLa nuclear extract and a positive control provided by the commercial source of TEF-1 antibody (BD Transduction), TEF-I antibody immuno-reacted with a single specific band corresponding to 53kDa. (data not shown).

Statistical Analysis

Statistical analysis was performed with Graphpad Prism 3.0 statistical software. Values are reported as means ± SE. An unpaired, two-tailed t-test was used to detect differences between NC and SI groups. For multiple comparisons, one-way ANOVA with Newman-Keuls post-hoc tests were performed. $P<0.05$ was accepted as the level of statistical significance.

RESULTS

Body and Muscle Weights

Animals assigned to separate experiments were matched in initial body weight which were 139 ± 1 g and 140 ± 1 g for NC and SI groups, respectively. At the termination of the 7 day experiment, body weight increased to 166 ± 1 g in control rats, and decreased to 122 ± 1 g in SI rats. SI resulted in similar changes in body weight and muscle weight relative to control across all experiments (Table 1). The average body weight of SI rats was 26% smaller, absolute soleus muscle weight was 60% smaller, and normalized soleus weight to body weight was 46% smaller than in NC rats (Table 1). All these differences were highly significant ($P<0.0001$).

Deletion Analysis of the Type I MHC Promoter

The validity of the direct gene injection technique to study MHC regulation in intact skeletal muscle has been previously demonstrated in our laboratory (9). Transfected type I and IIb MHC promoter activities, based on reporter assays, correlated well with muscle-fiber type specific expression of the corresponding endogenous genes.

In the normal control soleus a "full-length" –3500 type I MHC promoter demonstrated the greatest activity confirming the existence of a distal enhancer in the upstream region of the promoter (Fig. 1). In addition to the –3500 type I MHC promoter, four shorter promoter fragments were tested for their responsiveness to SI. All of the deletion fragments tested (-408, -299, -215, and –171) were sufficient to activate reporter activity in the soleus of NC rats above background levels; however, activity was further decreased as additional upstream sequences were deleted. Activity of the –408 promoter was reduced 75% ($P<0.001$), while –171 promoter activity was reduced 95% ($P<0.001$) relative to the 3500 promoter (Fig. 1).

Normalized activity of the full length –3500 type I promoter as well as the -408, -299, and -215 deletion fragments was significantly decreased in the soleus of SI rats compared to NC rats (Fig. 1). The -3500 promoter exhibited the greatest responsiveness to SI, decreasing ~90% relative to NC (Fig. 1), while the –215 promoter showed the least, decreasing ~39%.

In contrast to the –408, -299, and –215 promoters, -171 promoter activity normalized to the α-actin reference promoter was significantly increased in response to SI (Fig. 1). This apparent increase in -171 promoter activity with SI is the result of normalization to actin and should not be interpreted as promoter activation. In fact, uncorrected absolute –171 type I FLuc activity was still significantly decreased in response to SI (Fig. 2) (by 80% relative to NC). However, this decrease in activity was smaller than all other constructs (95-98% decrease), and smaller than the decrease in actin-promoter activity (~90% across all experiments). The increase in normalized 171 promoter activity in SI was the result of a smaller fractional decrease in 171 promoter activity ([SI-NC]/NC) as compared to the fractional decrease in actin promoter activity. This higher ratio can be interpreted as attenuated responsiveness of the test promoter. This reduced responsiveness to SI of the -171 when compared to the -215 promoter suggests that some regulatory elements essential for type I MHC down-regulation with SI are likely located between 215 and 171 bp of the type I promoter. One putative cis DNA regulatory element in this region is the βe3 element (-214/-190) (38) which could confer type I MHC responsiveness to SI. Consequently, to determine the role of the βe3 element in type I MHC down-regulation with chronic inactivity, an additional set of experiments was performed that involved mutation of this element in two promoter fragments.

Mutational Analysis of the Type I Promoter

Mutation of the βe3 element in the –215 type I promoter decreased activity in the NC soleus confirming the role of the βe3 element as a positive regulatory element in normal expression of the type I MHC gene in slow skeletal muscle (Fig. 3). Mutation of this element in the -215 promoter construct also attenuated type I MHC down-regulation in response to SI. Similar to the results observed with the –171 promoter, activity of the –215 βe3 mutant promoter relative to actin significantly increased in response to SI. Absolute FLuc activity of the 215 βe3 mutant promoter was still decreased 65% in response to SI (6742±1990 to 2274±660 RLU); however, this was smaller than the 96% decrease found with the 215 WT promoter (29360±12082 to 1206±550 RLU). Thus, decreased responsiveness to SI when the βe3 element is mutated suggests that this element is involved in regulating transcriptional down-regulation of type I MHC (Fig. 3).

The same βe3 mutation in the –408 promoter fragment also attenuated type I down-regulation in response to SI (Fig. 3). These results further confirm the importance of the βe3 element in type I down-regulation with SI, since the additional upstream elements in the –408 promoter were unable to rescue type I responsiveness to SI. In an additional promoter fragment, the upstream βe2 (-285/-269) regulatory element was simultaneously mutated with the βe3 element. This second mutation had no additional effect on promoter activity in normal soleus as compared to the single βe3 mutation (Fig. 3) as both activities were similarly lower than the 408 wild type promoter construct. If the βe2 cis regulatory element was involved in the SI response, the responsiveness of the 408 double mutant would be expected to be less than that of the single βe3 mutant. Collectively, these results with the βe2/βe3 mutation demonstrate that βe3, but not βe2, positively regulates the type I MHC promoter in normal soleus and that the 408 and 215 promoters were responsive to SI via the βe3 element, the proximal positive enhancer (17).

Normalization of Reporter Activity to Plasmid Uptake

As discussed in the Methods section, an additional control for pGL3 plasmid uptake normalization was performed in selected experiments. In contrast to actin promoter activity which was significantly decreased in response to SI (Fig. 2), plasmid uptake was slightly greater in the soleus of SI vs. NC rats (Table 3). While normalization to actin reduced reported responsiveness of the type I promoter to SI, normalization to plasmid uptake resulted in type I down-regulation, which was more closely matched to the decrease in FLuc activity than to the ratio of FLuc to RLuc. Specifically, while the normalized type I MHC activity relative to actin decreased 92 and 39% for the –3500 and -215 promoters and increased 109% for the –171 promoter, the activities were decreased 99, 98 and 80% relative to plasmid uptake. These results clearly demonstrate that significant down-regulation of the type I MHC promoter in response to SI was not due to reduced plasmid uptake, but rather to SI-induced transcriptional regulation.

Activity of the Type IIb MHC Promoter

Activity of a –1400 type IIb MHC promoter construct was significantly increased in the soleus of SI rats as compared to NC (Fig. 4). This upregulation is in agreement with the shift in MHC expression in response to SI (16) and demonstrates that the previously reported increases in IIb mRNA with SI are transcriptionally regulated. Further, up-regulation of the type IIb promoter demonstrates that SI does not cause a global decrease in transcription.

Gel Mobility Shift Assays

The findings that: a) mutation of the βe3 element in both the –215 and –408 promoter fragments, and b) deletion of this element (-171 fragment) decreased type I responsiveness to SI, strongly suggest that this element is involved in the SI response.

Thus, gel mobility shift assays were used to examine interactions of the βe3 element with nuclear proteins extracted from the soleus of NC and SI rats (Fig. 5). Although DNA –protein interactions were resolved into several complexes on the gel, only one complex, based on competition with different DNA probes, was clearly specific to the βe3 probe (labeled Sp. C in Fig. 5). This specific band (Sp.C) was competed out by 100 fold molar excess of unlabeled βe3 probe. In contrast, an unlabeled βe3 mutant probe and an unrelated sequence (GATA) were not able to effectively compete out binding to the radiolabeled βe3 probe when incubated at 100 fold molar excess. Densitometric analysis determined that there was a significant increase in binding with the SI nuclear extracts as compared to NC (NC: 235800 ± 38060 vs. SI: 392500 ± 48630 densitometric units; N=6/ea). Although the difference in binding to the βe3 element was relatively small (85% increase with SI vs. NC), it was not due to differences in nuclear protein concentration between groups as binding to probes containing other cis DNA elements (GATA or NFAT) was not different between the two experimental groups (data not shown).

Reactions with an antibody specific to TEF-1 caused a clear supershift of the Sp C. protein-DNA complex into a heavier supershifted complex (SS, Fig. 5). While SI significantly increased nuclear protein binding to the βe3 element by 85%, there was 50% less protein supershifted by the TEF-1 antibody in SI samples (453000 ± 122000 vs. 233000 ± 146000 densitometric units for NC and SI, respectively.

DISCUSSION

Several models of chronic decreased activity, such as denervation, hindlimb unloading, spinal cord transection, and spinal cord isolation (SI) have clearly demonstrated the ability of skeletal muscles to adapt their MHC phenotype to the imposed alterations in activity and load (for review see (27, 36). However, while variable amounts of neuromuscular activity are known to impact muscle fibers in the majority of these models, SI is unique in that it provides a baseline for complete inactivity while preserving potential neural, but non-activity related factors. Using this model, the findings reported herein provide the first evidence that the complete absence of neuromuscular activation and loading significantly decreases in vivo transcription of the type I MHC gene in a slow muscle. In addition, deletion and mutation analyses strongly suggest that the βe3 DNA regulatory element is involved in the type I MHC down-regulation associated with chronic inactivity. As evidenced by the supershift assays, this down-regulation with SI is potentially mediated by decreased binding of the transcriptional enhancer, TEF-1, to the βe3 element. Transcriptional regulation of the MHC genes with chronic inactivity was not confined to the slow type I MHC gene as we also demonstrated a significant transcriptional up-regulation of the fast type IIb MHC gene. Thus, the previously observed time-dependent MHC shifts at the steady-state mRNA and protein levels (i.e. decreases in slow type I MHC and increases in the fast isoforms) (16), are initiated at the level of transcription. Clearly, both neuromuscular activation and loading are essential factors in maintaining normal levels of type I MHC transcription in a slow rat hindlimb muscle.

The significant depression in type I MHC promoter activity occurring within 7 days of SI has a profound effect on muscle MHC protein expression as evidenced by the rapid loss of muscle mass and protein/MHC content occurring over the first 8 days of SI. We recently reported that the soleus had lost nearly half of its type I MHC protein after only 8 days (16). As demonstrated by the present study, this rapid loss was primarily due to transcriptional regulation of the type I MHC gene, rather than post-transcriptional and/or translational regulation. With longer duration SI, the rate of muscle atrophy and MHC protein loss was reduced, and the atrophied muscles then underwent additional remodeling of the myosin phenotype.

This is the first study to demonstrate that decreased type I MHC expression with complete skeletal muscle inactivity is transcriptionally regulated and likely involves the βe3 regulatory element. The enhancer core of the βe3 element has been characterized in cardiac myocytes and is also referred to as the myocyte specific CAT (M-CAT) cis element (17, 19). Evidence that βe3 is an enhancer element in the normal weight bearing soleus is demonstrated herein by the significant decrease in –215 and –408 type I promoter activity when the βe3 element is mutated (Figs. 3). Members of the TEF-1 multigene family bind to this βe3 (M-CAT) element and activate transcription of muscle genes in cardiac myocytes such as type I MHC (17, 18, 34, 40) αMHC (12), and skeletal α–actin (19). Recently, a TEF-1 related protein, RTEF-1, was identified and found to be a target of α1-adrenergic signaling in hypertropied cardiac myocytes (40). Specifically, phosphorylation of a serine residue accounted for a majority of the α1 adrenergic response. Over-expression of TEF-1 activated a –215 type I MHC promoter while over-expression of RTEF-1 activated both the –215 and –3300 type I promoters (34). An intact M-CAT element was necessary for this transactivation of the type I promoter by TEF-1 and RTEF-1.

TEF-1 is also involved in regulating skeletal muscle genes, as 3 days of stretch-overload in the chicken anterior latissimus dorsi increased TEF-1 binding to the α–actin promoter (2). Our supershift assays clearly demonstrate that TEF-1 is also involved in type I MHC regulation in the rat soleus and is responsive to changes in neuromuscular activity (Fig. 5). A TEF-1 antibody supershifted protein complexes binding to the βe3 oligonucleotide in nuclear extracts from both NC and SI soleus. Although total nuclear protein binding to the βe3 oligonucleotide increased 85% with SI, the protein supershifted by the TEF-1 antibody was only 50% of that observed in NC soleus. Thus, chronic inactivity with SI decreased TEF-1 binding, in contrast to muscle overload which was previously shown to increase TEF-1 binding (2). Consequently, one mechanism whereby SI could down-regulate type I MHC is by decreasing TEF-1 binding to the βe3 element as evidenced by a significant reduction in down-regulation when this element was mutated or deleted. Taken together with previous work, TEF-1 may up-regulate muscle specific genes in response to muscle overload (2), while down-regulating these genes in response to chronic inactivity.

Since SI increased nuclear protein binding to the βe3 oligonucleotide while decreasing the amount of TEF-1 binding, this suggests that the residual binding was composed of other transcription factors that increase in response to SI. One possibility is that SI may increase other TEF-1 related factors (e.g. RTEF-1, DTEF-1, ETF). However, this is not likely since this family of transcription factors enhance transcription while chronic inactivity down-regulates type I MHC expression. Another potential transcription factor that may contribute to the increase in nuclear protein binding to the βe3 oligonucleotide is cAMP responsive binding protein (CREB). The βe3 oligonucleotide probe used in the gel mobilty shift assays also contains a putative CREB site (-213/-216; Table 2) adjacent to the TEF binding site (MCAT). Unphosphorylated CREB has been shown to repress activity of the c-jun promoter (20), consequently deletion of this CREB site could remove another potential regulatory site for decreasing type I MHC expression.

In our previous SI time course study (16), we reported de novo expression of type IIb MHC mRNA after only 4 days of SI. These findings are supported by the present observation that one week of SI significantly up-regulates type IIb MHC promoter activity (Fig. 4). Interestingly, while short term inactivity and/or unloading is sufficient to activate transcription of the IIb MHC gene, it is unable to induce expression of IIb protein. For example, in hindlimb unloaded rats, IIb MHC was expressed at the mRNA, but not at the protein level (14). Similarly, 60 days of SI (16), 30 days of denervation (13, 15), or 14 days of tetrodotoxin-paralysis (24) did not induce de novo IIb protein expression despite increases in its mRNA. Taken together, these observations suggest that there is a marked uncoupling between transcriptional and translational events in the up-regulation of IIb MHC in slow muscles during the initial stages of unloading. However, more prolonged inactivity (e.g., 90 days of SI) or decreased activity coupled with other factors such as thyroid hormone status have induced IIb protein expression in the soleus. For example, the combination of hindlimb unloading and triiodothyronine treatment resulted in increases in both IIb mRNA and protein in the rat soleus (4, 14), which was transcriptionally regulated (4).

Collectively, in this report we have used the direct gene transfer approach to characterize the regulation of type I MHC gene expression in response to chronic inactivity. In summary, our results demonstrate that chronic inactivity and unloading of slow rat hindlimb muscles caused a significant down-regulation of type I MHC promoter activity. Deletion and mutation analyses strongly suggest involvement of the βe3 DNA regulatory element in the type I MHC down-regulation associated with SI. Further, decreased TEF-1 binding to the βe3 element likely contributed to this type I MHC down-regulation. Future studies should investigate mechanisms whereby prolonged absence of activation and/or loading alter the amount and binding of TEF-1 to the βe3 element and how these interactions contribute to type I MHC down-regulation with different forms of reduced activity.

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REFERENCES

1. Blough, E, Dineen, B, and Esser, K. Extraction of nuclear proteins from striated muscle tissue. *Biotechniques* 26: 202-4, 206, 1999.

2. Carson, JA, Schwartz, RJ, and Booth, FW. SRF and TEF-1 control of chicken skeletal alpha-actin gene during slow-muscle hypertrophy. *Amer J Physiol* 270: C1624-33, 1996.

3. Carson, JA, Yan, Z, Booth, FW, Coleman, ME, Schwartz, RJ, and Stump, CS. Regulation of skeletal alpha-actin promoter in young chickens during hypertrophy caused by stretch overload. *Amer J Physiol* 268: C918-24, 1995.

4. di Maso, NA, Haddad, F, Zeng, M, McCue, SA, and Baldwin, KM. Role of denervation in modulating IIb MHC gene expression in response to T(3) plus unloading state. *J Appl Physiol* 88: 682-9, 2000.

5. Edwards, J, Bahl, J, Flink, J, Milavetz, J, Goldman, S, and Morkin, E. A repressor region in the human beta-myosin heavy chain gene that has a partial postion dependancy. *Biochem Biophys Res Commun* 189: 504-510, 1992.

6. Eldridge, L, Liebhold, M, and Steinbach, JH. Alterations in cat skeletal neuromuscular junctions following prolonged inactivity. *J Physiol* 313: 529-45, 1981.

7. Flink, IL, Edwards, JG, Bahl, JJ, Liew, CC, Sole, M, and Morkin, E. Charaterization of a strong positive cis-acting element of the human beta-myosin heavy chain gene in fetal rat heart cells. *J. Biol. Chem.* 267: 9917-9924, 1992.

8. Friedman, DJ, Umeda, PK, Sinha, AM, Hsu, HJ, Jakovcic, S, and Rabinowitz, M. Characterization of genomic clones specifying rabbit alpha- and beta-ventricular myosin heavy chains. *Proc Natl Acad Sci U S A* 81: 3044-8, 1984.

9. Giger, JM, Haddad, F, Qin, A, and Baldwin, KM. In vivo regulation of the beta-myosin heavy chain gene in soleus muscle of suspended and weight-bearing rats. *Am J Physiol Cell Physiol* 278: C1153-C1161, 2000.

10. Grossman, EJ, Roy, RR, Talmadge, RJ, Zhong, H, and Edgerton, VR. Effects of inactivity on myosin heavy chain composition and size of rat soleus fibers. *Muscle Nerve* 21: 375-89, 1998.

11. Gulick, J, Subramaniam, A, Neumann, J, and Robbins, J. Isolation and characterization of the mouse cardiac myosin heavy chain genes. *J Biol Chem* 266: 9180-5, 1991.

12. Gupta, MP, Kogut, P, and Gupta, M. Protein kinase-A dependent phosphorylation of transcription enhancer factor-1 represses its DNA-binding activity but enhances its gene activation ability. *Nuc Acids Res* 28: 3168-77, 2000.

13. Haddad, F, Arnold, C, Zeng, M, and Baldwin, KM. Interaction of thyroid state and denervation on skeletal myosin heavy chain expression. *Muscle Nerve* 20: 1487-1496, 1997.

14. Haddad, F, Qin, AX, Zeng, M, McCue, SA, and Baldwin, KM. Interaction of hyperthyroidism and hindlimb suspension on skeletal myosin heavy chain expression. *J Appl Physiol* 85: 2227-36, 1998.

15. Huey, KA, and Bodine, SC. Changes in myosin mRNA and protein expression in denervated rat soleus and tibialis anterior. *Eur J Biochem* 256: 45-50, 1998.

16. Huey, KA, Roy, RR, Baldwin, KM, and Edgerton, VR. Temporal effects of inactivty on myosin heavy chain gene expression in rat slow muscle. *Muscle Nerve* 24: 517-26, 2001.

17. Kariya, K, Farrance, IK, and Simpson, PC. Transcriptional enhancer factor-1 in cardiac myocytes interacts with an alpha 1-adrenergic- and beta-protein kinase C-inducible element in the rat beta-myosin heavy chain promoter. *J Biol Chem* 268: 26658-62, 1993.

18. Kariya, K, Karns, LR, and Simpson, PC. An enhancer core element mediates stimulation of the rat beta-myosin heavy chain promoter by an alpha 1-adrenergic agonist and activated beta-protein kinase C in hypertrophy of cardiac myocytes. *J Biol Chem* 269: 3775-82, 1994.

19. Karns, LR, Kariya, KI, and Simpson, PC. M-CAT, CArG, and Sp1 elements are required for alpha1-adrenergic induction of the skeletal alpha-actin promoter during cardiac myocyte hypertrophy. *J Biol Chem* 270: 410-417, 1995.

20. Lamph, WW, Dwarki, VJ, Ofir, R, Montminy, M, and Verma, IM. Negative and positive regulation by transcription factor cAMP response element-binding protein is modulated by phosphorylation. *PNAS* 87: 4320-4, 1990.

21. Leinwand, LA, Saez, L, McNally, E, and Nadal-Ginard, B. Isolation and characterization of human myosin heavy chain genes. *Proc Natl Acad Sci U S A* 80: 3716-20, 1983.

22. Mahdavi, V, Chambers, AP, and Nadal-Ginard, B. Cardiac alpha- and beta-myosin heavy chain genes are organized in tandem. *Proc Natl Acad Sci U S A* 81: 2626-30, 1984.

23. McCarthy, JJ, Fox, AM, Tsika, GL, Gao, L, and Tsika, RW. beta-MHC transgene expression in suspended and mechanically overloaded/suspended soleus muscle of transgenic mice. *Amer J Physiol* 272: R1552-61, 1997.

24. Michel, RN, Parry, DJ, and Dunn, SE. Regulation of myosin heavy chain expression in adult rat hindlimb muscles during short-term paralysis: comparison of denervation and tetrodotoxin-induced neural inactivation. *FEBS Lett* 391: 39-44, 1996.

25. Pierotti, DJ, Roy, RR, Bodine-Fowler, SC, Hodgson, JA, and Edgerton, VR. Mechanical and morphological properties of chronically inactive cat tibialis anterior motor units. *J Physiol* 444: 175-92, 1991.

26. Rindt, H, Gulick, J, Knotts, S, Neumann, J, and Robbins, J. In vivo analysis of the murine beta-myosin heavy chain gene promoter. *J Biol Chem* 268: 5332-8, 1993.

27. Roy, RR, Baldwin, KM, and Edgerton, VR. The plasticity of skeletal muscle: effects of neuromuscular activity. *Exer Sport Sci Rev* 19: 269-312, 1991.

28. Roy, RR, Hodgson, JA, Lauretz, SD, Pierotti, DJ, Gayek, RJ, and Edgerton, VR. Chronic spinal cord-injured cats: surgical procedures and management. *Lab Anim Sci* 42: 335-43, 1992.

29. Roy, RR, Kim, JA, Grossman, EJ, Bekmezian, A, Talmadge, RJ, Zhong, H, and Edgerton, VR. Persistence of myosin heavy chain-based fiber types in innervated but silenced rat fast muscle. *Muscle Nerve* 23: 735-47, 2000.

30. Saez, LJ, Gianola, KM, McNally, EM, Feghali, R, Eddy, R, Shows, TB, and Leinwand, LA. Human cardiac myosin heavy chain genes and their linkage in the genome. *Nucleic Acids Res* 15: 5443-59, 1987.

31. Shimizu, N, Prior, G, Umeda, PK, and Zak, R. Cis-acting elements responsible for muscle-specific expression of the myosin heavy chain beta gene. *Nucleic Acids Res* 20: 1793-1799, 1992.

32. Spector, SA. Effects of elimination of activity on contractile and histochemical properties of rat soleus muscle. *J Neurosci.* 5: 2177-88, 1985.

33. Spector, SA. Trophic effects on the contractile and histochemical properties of rat soleus muscle. *J Neurosci.* 5: 2189-96, 1985.

34. Stewart, AF, Suzow, J, Kubota, T, Ueyama, T, and Chen, HH. Transcription factor RTEF-1 mediates alpha1-adrenergic reactivation of the fetal gene program in cardiac myocytes. *Circ Res* 83: 43-9, 1998.

35. Swoap, SJ. In vivo analysis of the myosin heavy chain IIB promoter region. *Am J Physiol Cell Physiol* 274: C681-C687, 1998.

36. Talmadge, RJ. Myosin heavy chain-isoform expression following reduced neuromuscular activity: potential regulatory mechanisms. *Muscle Nerve* 23: 661-679, 2000.

37. Talmadge, RJ, Roy, RR, and Edgerton, VR. Prominence of myosin heavy chain hybrid fibers in soleus muscle of spinal cord-transected rats. *J Appl Physiol* 78: 1256-65, 1995.

38. Thompson, WR, B. Nadal-Ginard, and V. Mahdavi. A myoD1-independent muscle-specific enhancer controls the expression of the beta-myosin heavy chain gene in skeletal and cardiac muscle cells. *J Biol Chem* 266: 22678-22688, 1991.

39. Tsika, GL, Wiedenman, JL, Gao, L, McCarthy, JJ, Sheriff-Carter, K, Rivera-Rivera, ID, and Tsika, RW. Induction of beta-MHC transgene in overloaded skeletal muscle is not eliminated by mutation of conserved elements. *Am J Physiol* 271: C690-9, 1996.

40. Ueyama, T, Zhu, C, Valenzuela, YM, Suzow, JG, and Stewart, AF. Identification of the functional domain in the transcription factor RTEF-1 that mediates alpha 1-adrenergic signaling in hypertrophied cardiac myocytes. *J Biol Chem* 275: 17476-80, 2000.

41. Vyas, DR, McCarthy, JJ, and Tsika, RW. Nuclear protein binding at the beta-myosin heavy chain A/T-rich element is enriched following increased skeletal muscle activity. *J Biol Chem* 274: 30832-42, 1999.

42. Weydert, A, Daubas, P, Lazaridis, I, Barton, P, Garner, I, Leader, DP, Bonhomme, F, Catalan, J, Simon, D, Guenet, JL, and et al. Genes for skeletal muscle myosin heavy

chains are clustered and are not located on the same mouse chromosome as a cardiac myosin heavy chain gene. *Proc Natl Acad Sci U S A* 82: 7183-7, 1985.

43. Wiedenman, JL, Tsika, GL, Gao, L, McCarthy, JJ, Rivera-Rivera, ID, Vyas, D, Sheriff-Carter, K, and Tsika, RW. Muscle-specific and inducible expression of 293-base pair beta-myosin heavy chain promoter in transgenic mice. *Am J Physiol* 271: R688-95, 1996.

44. Wright, CE, Bodell, PW, Haddad, F, Qin, AX, and Baldwin, KM. In vivo regulation of the beta-myosin heavy chain gene in hypertensive rodent heart. *Am J Physiol Cell Physiol* 280: C1262-76, 2001.

45. Wright, CE, Haddad, F, Qin, AX, Bodell, PW, and Baldwin, KM. In vivo regulation of beta-MHC gene in rodent heart: role of T3 and evidence for an upstream enhancer. *Am J Physiol* 276: C883-91, 1999.

FIGURE LEGENDS

Figure 1. *Deletion analysis of the type I MHC promoter in the soleus of normal control (NC) and spinal cord isolated (SI) rats.*

One week of SI significantly decreased activity of the full length (-3500) and several deletion fragments (-408 to –215) of the type I MHC promoter. Activity is expressed as the ratio of type I MHC to skeletal α-actin promoter activity. Schematic illustration of the type I MHC promoter-reporter depicts several DNA regulatory elements. Values are means ± SE. *, $P<0.05$, NC vs. SI. There were significant differences in promoter activity in NC soleus among all constructs with the exception of 408 vs. 299 and 215 vs. 171 (ANOVA). There were no significant differences in activity among promoters in SI soleus. Inset: Percent changes in type I promoter activity with SI as compared to NC ([SI-NC]/NC).

Figure 2. *Absolute reporter activity in the soleus of normal control (NC) and spinal cord isolated (SI) rats*

(A) Absolute firefly luciferase (FLuc) activity of the type I MHC promoter in NC and SI soleus. Activity of all the deletion constructs significantly decreased in response to SI. Inset depicts the change in firefly luciferase activity with SI relative to NC for each type I MHC promoter construct.

(B) Absolute renilla luciferase (RLuc) activity of the α-actin promoter in NC and SI soleus when co-injected with the various type I MHC promoter constructs. RLuc activity was significantly decreased with SI in all promoter fragments. No significant differences existed among either the NC or SI groups.

Reporter activity is based on measurements from 5 µl of muscle supernatant integrated over 10 second, and corrected to non-injected tissue basal activity (Dual Luciferase Assay, Promega). Reporter activities are expressed in relative light units (RLU). Values are means ± SE. *, $P<0.05$, NC vs. SI.

Figure 3. *Mutational analysis of the -215 (A) and –408 (B) type I MHC promoters in the soleus of normal control (NC) and spinal cord isolated (SI) rats.*

(A) Mutation of the βe3 element or its deletion (-171), significantly decreased activity of the –215 promoter in NC soleus (vs. the wild type [WT], †, $P<0.05$), and decreased promoter responsiveness to SI. Values are means ± SE. *, $P<0.05$, NC vs. SI; †, $P<0.05$, vs. 215 WT.

(B) Mutation of the βe3 element significantly decreased normalized activity of the –408 promoter in NC soleus (vs. the WT) and attenuated promoter responsiveness to SI. Simultaneous mutations of the βe3 and βe2 elements resulted in similar promoter activity in the NC soleus as compared to the βe3 mutation alone and also attenuated responsiveness to SI. Values are means ± SE. *, $P<0.05$, NC **vs.** SI; †, $P<0.05$, vs. 408 WT.

Figure 4. *Activity of the –1400 type IIb MHC promoter in the soleus of normal control (NC) and spinal cord isolated (SI) rats.*

Chronic inactivity significantly increased activity of the fast IIb MHC promoter. Values are means ± SE. *, $P<0.05$, NC vs. SI. Promoter activity is expressed as the ratio of FLuc to RLuc.

Figure 5. *Representative gel mobility shift of muscle extracts from normal control (C) and spinal cord isolated (SI) rat soleus binding to the* ***b****e3 element.*

Several DNA-protein complexes were formed with a gel mobility shift assay when control soleus nuclear extract was reacted with a 24 bp double stranded probe representing the rat βe3 element (lane #1). The two slowest migrating bands are due to nonspecific (NS) binding as they were competed off with 100 fold excess of cold βe3 probe (lane #2), cold mutant βe3 (lane #3) or an unrelated sequence (GATA lane #4). Examination of the gel strongly suggests that the third complex labeled as Sp.C. was due to specific interactions of the βe3 element and muscle nuclear proteins. This specific complex was not competed off with excess cold mutant βe3 (lane #3) or with an unrelated probe (lane #4). Also, the specific complex was absent when nuclear extract was reacted to a mutant βe3 probe (lane #5). A significant 85% increase in specific binding to the βe3 element (Sp.C complex) was seen with extracts from SI (lane #8) soleus as compared to normal control (C2, lane #6). Despite increases in the Sp.C complex with SI nuclear extract, the amount of DNA-protein complexes supershifted (SS) by the TEF-1 antibody was approximately 50% less in SI (lane #9) vs. C (lane #7) soleus.

Table 1. *Body weight, muscle weight, and muscle to body weight ratios in NC and SI groups for individual experiments used in deletion and mutation analysis of the type I MHC promoter.*

	Body Weight (g)			**Soleus Muscle Weight (mg)**			**SW/BW Ratio (mg/g)**		
Type I MHC Promoter	**NC** (N=8/ea)	**SI** (N=8/ea)	**Change (%)**	**NC** (N=16/ea)	**SI** (N=16/ea)	**Change (%)**	**NC** (N=16/ea)	**SI** (N=16/ea)	**Change (%)**
3500	170±4	124±4	-27	71±2	27±1	-62	0.42±0.01	0.22±0.01	-48
408	160±5	119±2	-26	71±1	26±1	-64	0.45±0.01	0.22±0.01	-52
299	164±2	129±3	-21	71±1	30±1	-59	0.44±0.01	0.23±0.01	-48
215	167±3	118±5	-29	75±2	27±2	-65	0.45±0.01	0.23±0.01	-50
171	168±3	121±2	-28	71±2	27±2	-59	0.42±0.01	0.24±0.01	-43
215 b e3	156±3	121±5	-23	68±2	29±1	-58	0.44±0.01	0.24±0.01	-45
408 b e3	164±4	123±2	-25	69±3	27±1	-61	0.42±0.01	0.22±0.01	-48
408 b e2/3	172±7	121±3	-29	72±2	29±1	-60	0.42±0.01	0.24±0.01	-44
Average	166±1	122±1	-26	71±1	28±1	-60	0.42±0.01	0.24±0.01	-46

Data are expressed as means±SE. For all promoter constructs, SI vs. NC was significantly different (unpaired two-tailed t-test). No significant differences were found among all NC or all SI groups (ANOVA).

SW/BW: soleus weight to body weight ratio.

Table 2. *Oligonucleotides used as probes or competitors in the gel mobility shift assays*.

Oligonucleotide	*Sequence*	**Position**
Type I MHC βe3	5’ CATGCCAT**<u>ACC</u>**ACAACAA***TGAC***GC 3’;	-215/-192
βe3 Mutant	5’-CATGCCAT**cgg**ACAACAATGACGC-3’	-215/-192
GATA	5' AATGTAAG**<u>GGATAT</u>**TTTTGCTTCA 3'	-277/-254

Position is relative to transcription start site of the rat Type I MHC promoter.

Bases altered to create mutations are indicated in lower case and bases targeted for mutation are underlined. The putative CREB binding site is indicated in italics. Probes were obtained in both sense and antisense, and were used after annealing to form double stranded oligonucleotides.

Table 3. *Firefly luciferase activity, pGL3 plasmid uptake levels, and firefly luciferase activity relative to pGL3 uptake for the -3500, -215, and -171 Type I MHC promoters in normal control and spinal isolated rat soleus*

	3500		215		171	
	NC	SI	NC	SI	NC	SI
FLuc (RLU)	125295	1786	142943	2397	17215	4076
	±39343	±629	±78440	±570	±4832	±1490
pGL3 (OD units)	61	117	168	192	75	110
	±9	±17	±14	±12	±11	±16
FLuc/pGL3	1857	14	732	12	175	34
	±346	±4	±371	±3	±37	±9

Values are means ± SE. N=7-12 per construct. FLuc, firefly luciferase activity; RLU, relative light units; NC, normal control; SI, spinal isolated

Figure 1

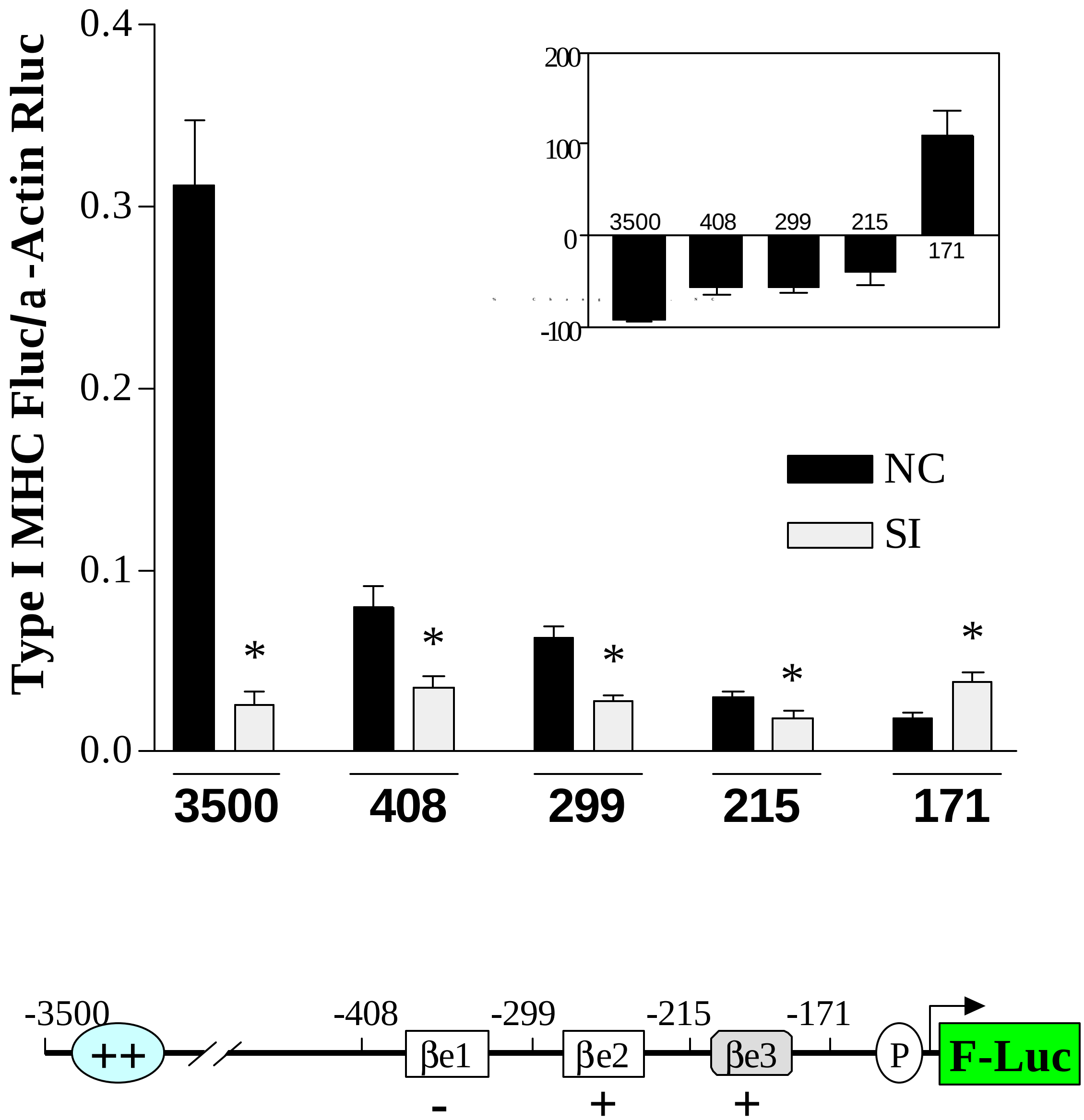

Type I MHC Fluc/a -Actin Rluc
0.4
0.3
0.2
0.1
0.0
200
100
0
-100
3500
408
299
215
171
NC
SI
*
3500
408
299
215
171
-3500
-408
-299
-215
-171
++
βe1
βe2
βe3
P
F-Luc
-
+
+

Figure 2

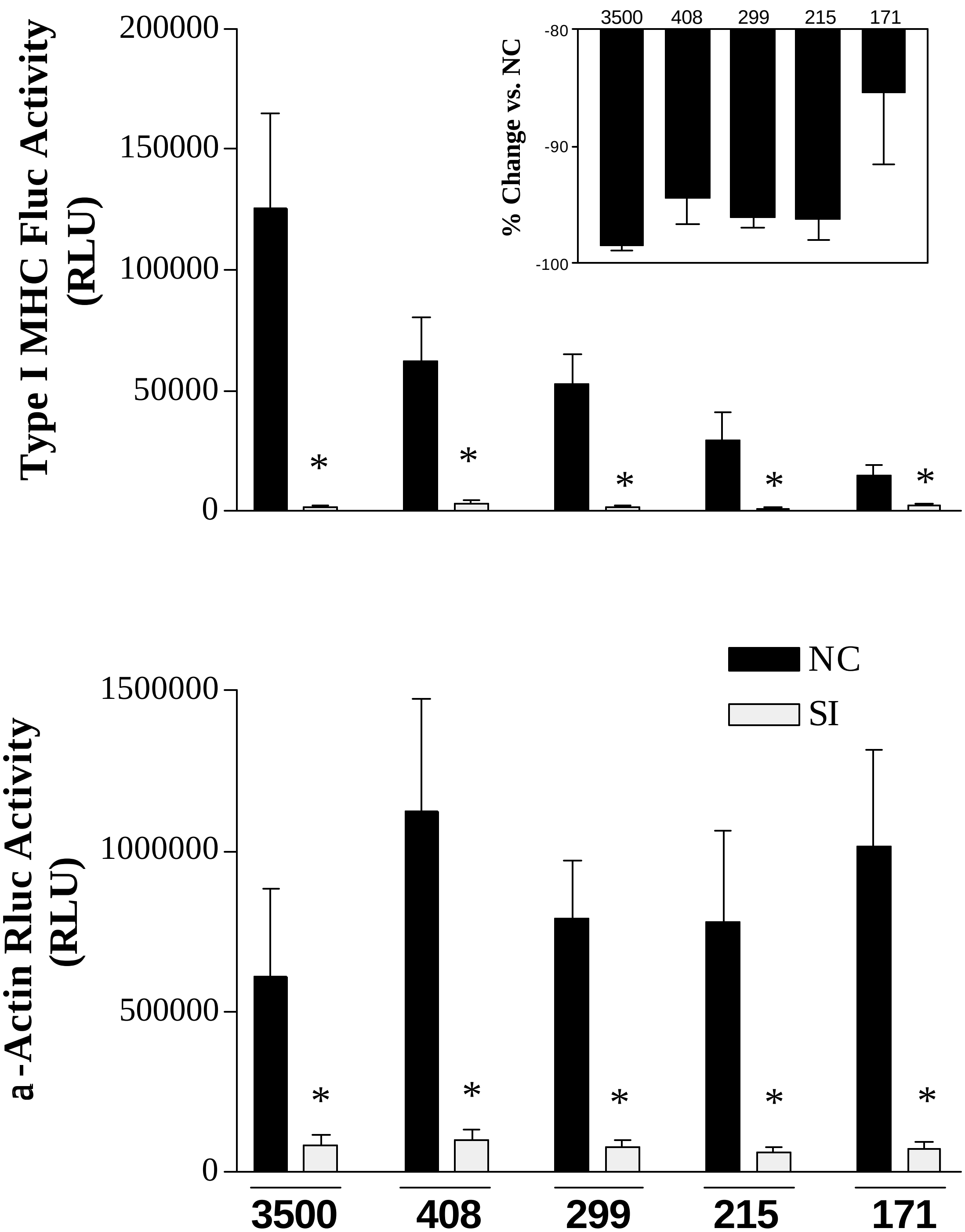

Type I MHC Fluc Activity (RLU)
200000
150000
100000
50000
0
% Change vs. NC
3500
408
299
215
171
-80
-90
-100
*
NC
SI
a -Actin Rluc Activity (RLU)
1500000
1000000
500000
0
3500
408
299
215
171

Figure 3

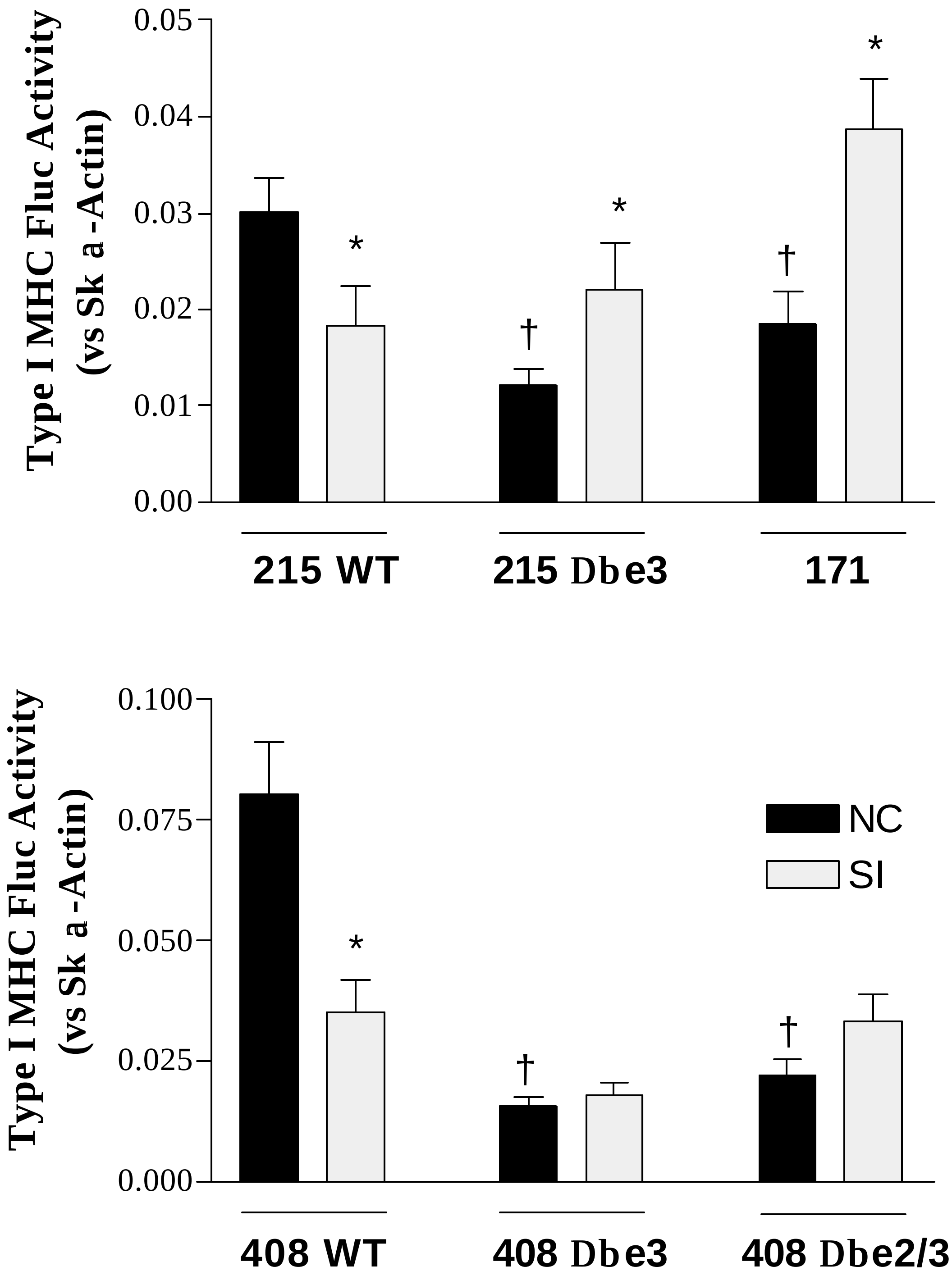

Type I MHC Fluc Activity (vs Sk a -Actin)
0.05
0.04
0.03
0.02
0.01
0.00
*
*
*
†
†
215 WT
215 Dbe3
171
Type I MHC Fluc Activity (vs Sk a -Actin)
0.100
0.075
0.050
0.025
0.000
*
†
†
NC
SI
408 WT
408 Dbe3
408 Dbe2/3

Figure 4

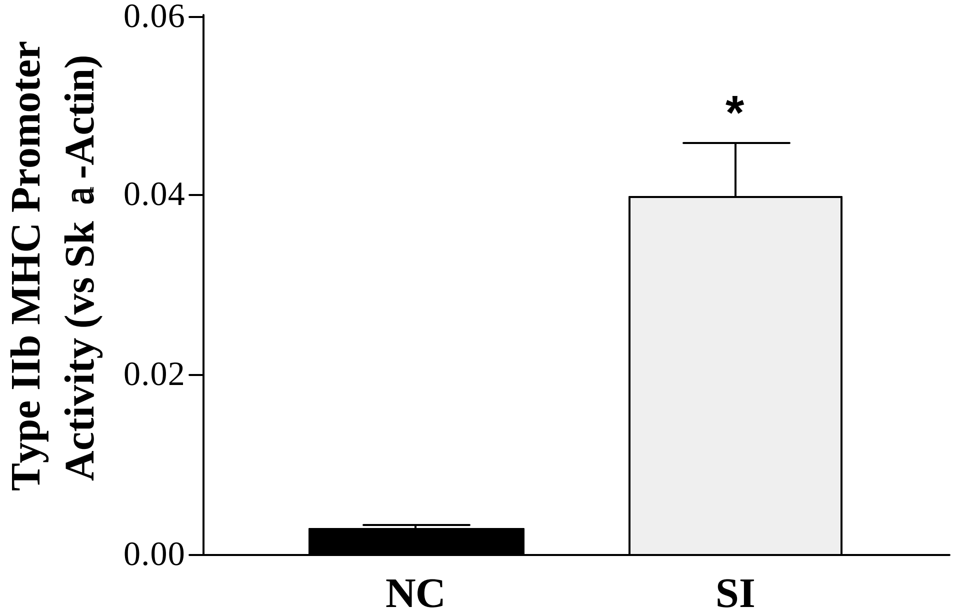
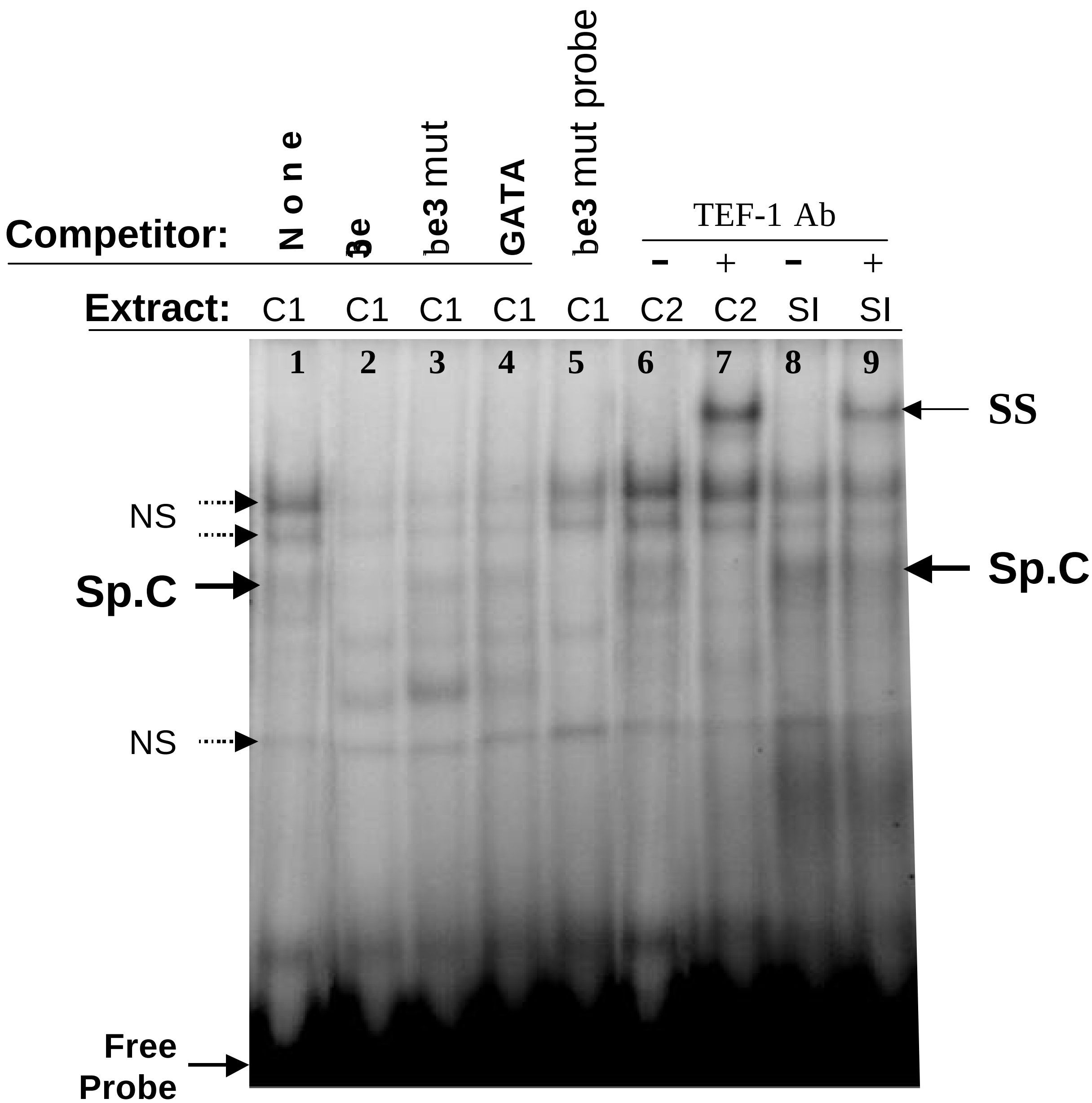

Type IIb MHC Promoter Activity (vs Sk a -Actin)
0.06
0.04
0.02
0.00
*
NC
SI

Figure 5


Competitor:
None
3e
be3 mut
GATA
be3 mut probe
TEF-1 Ab
-
+
-
+
Extract:
C1
C1
C1
C1
C1
C2
C2
SI
SI
1
2
3
4
5
6
7
8
9
SS
NS
Sp.C
NS
Free Probe
Sp.C