Transcriptional regulation of the type I myosin heavy chain gene in denervated rat soleus

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Huey, K. A., F. Haddad, A. X. Qin, and K. M. Baldwin. Transcriptional regulation of the type I myosin heavy chain gene in denervated rat soleus. Am J Physiol Cell Physiol 284:C738–C748, 2003. First published November 20, 2002; 10.1152/ajpcell.00389.2002.—Denervation (DEN) of rat soleus is associated with a decreased expression of slow type I myosin heavy chain (MHC) and an increased expression of the faster MHC isoforms. The molecular mechanisms behind these shifts remain unclear. We first investigated endogenous transcriptional activity of the type I MHC gene in normal and denervated soleus muscles via pre-mRNA analysis. Our results suggest that the type I MHC gene is regulated via transcriptional processes in the denervated soleus. Deletion and mutational analysis of the rat type I MHC promoter was then used to identify cis elements or regions of the promoter involved in this response. DEN significantly decreased in vivo activity of the −3,500, −2,500, −914, −408, −299, and −215 bp type I MHC promoters, relative to the α-skeletal actin promoter. In contrast, normalized −171 promoter activity was unchanged. Mutation of the β3 element (−214/−190) in the −215 promoter and deletion of this element (−171 promoter) blunted type I downregulation with DEN. In contrast, β3 mutation in the −408 promoters was not effective in attenuating the DEN response, suggesting the existence of additional DEN-responsive sites between −408 and −215. Western blotting and gel mobility supershift assays demonstrated decreased expression and DNA binding of transcription enhancer factor 1 (TEF-1) with DEN, suggesting that this decrease may contribute to type I MHC downregulation in denervated muscle.

slow muscle; transcriptional regulation; pre-mRNA; β3 DNA regulatory element; denervation

The importance of neural activation and loading state in the determination and maintenance of myosin heavy chain (MHC) expression in rodent skeletal muscle has been clearly demonstrated in models such as cross-reinnervation, electrical stimulation, spinal cord isolation (SI), and denervation (DEN); for reviews, see Refs. 37 and 43). When neuromuscular activation is absent or decreased as occurs with spinal cord transection (44), SI (20), or DEN (19), there is a rapid loss of muscle mass associated with a specific reduction in the relative expression of slow type I myosin heavy chain (MHC) at the expense of the faster MHC isoforms in slow muscles such as the soleus. Although similarities exist in the phenotypic shift in a muscle deprived of either neural activation and/or loading, the molecular mechanisms leading to these transformations may be different and specific to the imposed perturbation. For example, SI is unique in that there is virtually no residual activation or loading of the hindlimb muscles, but the motoneuron-muscle connections remain intact (35, 38). Thus, although both SI and DEN muscles are inactive, the models differ significantly due to the presence (SI) or absence (DEN) of an intact motoneuron-muscle connection and the associated neurotrophic influences. Consequently, the affected muscle is exposed to different conditions that potentially lead to significant divergence in the gene expression regulatory pathways between the two models. For example, several studies have shown that neurotrophic factors may influence gene expression independent of electrical activity (6, 9, 18, 25, 29, 50). Therefore, even though both DEN and SI decrease type I MHC, the molecular mechanisms mediating the shifts with DEN are likely unique in the absence of motoneuron-muscle connections.

Previous studies have demonstrated significant decreases in type I MHC expression at both the mRNA and protein levels in the rat soleus in response to DEN (19, 21, 31). However, no studies to date have investigated whether these changes are initiated at the transcriptional or posttranscriptional level. Gene expression can be regulated at several cellular levels, i.e., transcriptional, posttranscriptional/pretranslational, translational, and posttranslational levels. One may gain understanding as to the site of regulation by examining the relationship among expression levels of protein, mRNA, and pre-mRNA. A previous study reported that DEN induces a time-dependent decrease in type I MHC expression at both the mRNA and protein level (19), suggesting that this downregulation is largely pretranslational. Furthermore, this pretranslational control could be mediated via transcriptional or posttranscriptional processes. Assessment of the transcriptional activity of endogenous genes is commonly determined with nuclear run-on assays. However, because of the difficulties in obtaining skeletal muscle nuclei, and perhaps the inconvenience of the run-on

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assay (using large amounts of radioisotopes), very few studies have reported nuclear run-on results for muscle-specific gene transcription (12, 17). Alternatively, analysis of pre-mRNA expression can be used to assess a gene’s transcriptional activity (5, 33, 34). To our knowledge, this approach has not been applied to study MHC gene transcription. In this study, we have utilized this approach to investigate transcriptional regulation of the type I MHC gene in denervated rat soleus. We found that type I MHC pre-mRNA expression decreased in the denervated soleus, suggesting transcriptional regulation of the gene in this model. Therefore, we used in vivo gene transfer and promoter-reporter system analyses to characterize promoter function and delineate cis-regulatory elements involved in this response.

Slow type I MHC expression has been studied extensively in both cardiac and skeletal muscle, and the gene has been isolated from several mammalian species, including human (27, 39), rat (28), mouse (13, 48), and rabbit (8). Transgenic mice have been utilized to demonstrate that type I MHC promoter activity in muscles is fiber-type specific. Type I MHC reporter activity was detected primarily in cardiac ventricles and slow skeletal muscles, whereas activity was not detected in pure fast muscles (tibialis anterior) or non-muscle tissue (liver) (36). Furthermore, in vivo rat studies using direct muscle gene injection demonstrated 25-fold greater activity of the type I MHC promoter in the slow soleus compared with the fast tibialis anterior (TA) (11). Transient transfection assays in cardiac and muscle cell culture (4, 7, 22, 23, 40, 45) have identified several highly conserved positive and negative cis regulatory elements within the most proximal region of the type I MHC promoter [within 350 base pairs (bp) of the transcription start site]. For example, at least three positive elements that are necessary for muscle-specific expression have been identified: βe2 (−285/−269), CACC Box (−245/−233), and βe3 (−210/−188) (45). A negative element, βe1 (−330/−300) has also been identified (4). Furthermore, the upstream distal region (−3,500/−2,500) was found to be required for maximal promoter activity in both cardiac and slow skeletal muscles (11, 53).

Studies have implicated the βe1 repressor region in type I MHC downregulation in response to skeletal muscle unloading (11, 30). All three positive elements have been implicated in the adaptation to skeletal muscle overloading (46, 49), and the βe3 element has been implicated in the overload-induced type I MHC upregulation in the rat plantaris (10). Recently, we reported that the βe3 element is involved in the type I MHC downregulation associated with SI (20). It remains to be determined whether type I MHC promoter activity is also downregulated in response to DEN and the proximal regulatory elements potentially involved in this response. In particular, the role of βe3 is of interest on the basis of its role in type I MHC downregulation in a different model of muscle inactivity, SI (20).

βe3 element-mediated regulation of type I MHC gene expression has been shown to involve transcription enhancer factor 1 (TEF-1) binding to this element (22, 23, 41, 47). In the SI model, the involvement of the βe3 element in type I MHC downregulation was associated with decreased TEF-1 expression and DNA binding. The present study also supports the finding that type I MHC gene downregulation in inactive muscles involves changes in the expression and binding of TEF-1 to the βe3 element. In contrast, stretch-overload-induced muscle hypertrophy was associated with a significant increase in TEF-1 binding to the α-actin promoter (2). Whereas increased expression and DNA binding of TEF-1 may be associated with muscle hypertrophy, a reduction in TEF-1 may contribute to muscle atrophy. One potential model of muscle gene regulation is that TEF-1 could upregulate muscle contractile genes in response to muscle overload (2) while downregulating these genes in response to chronic inactivity.

Consequently, through deletion and mutational analysis of the type I MHC promoter, this is the first study to establish the functional significance of regulatory elements in the type I MHC promoter in a denervated muscle. Understanding the molecular mechanisms underlying MHC phenotype shifts in response to DEN has clinical importance not only for peripheral nerve injury but also aging, which is also associated with selective muscle fiber denervation. This research can ultimately help identify ways to maintain muscle phenotype in a denervated muscle that will eventually be reinnervated.

The purpose of the present study, therefore, was to test the hypothesis that downregulation of type I MHC expression in the denervated rat soleus is regulated at the transcriptional level, using pre-mRNA expression as a marker of gene transcriptional activity. Our goal was to characterize type I MHC gene promoter activity in the denervated soleus muscle and to identify cis-acting regulatory sequences necessary for its downregulation in response to DEN. We report that 1 wk of DEN was associated with a significant decrease in type I MHC endogenous gene transcription. Promoter analyses demonstrated that important cis DNA regulatory elements mediating this response are contained within 215 bp upstream of the transcription start site. Within this fragment, the βe3 element is likely to be involved in the type I downregulation, because both deletion and mutation of this element reduced the responsiveness of the type I MHC promoter to DEN. Furthermore, βe3 regulation likely involves decreased TEF-1 expression and binding to this element.

MATERIALS AND METHODS

Experimental design and animal procedures. These experiments were conducted over a period of −1 yr and consisted of repeating the following experimental design. Ten adult female Sprague-Dawley rats (96 ± 1.0 g) were used for each tested promoter construct. Rats were anesthetized and underwent surgery for intramuscular plasmid injection into both left and right soleus muscles as described previously.
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 −3.5 kb 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). Approximately 15 min after plasmid injection and while anaesthetized, the rats underwent unilateral hindlimb muscle DEN by removal of ~2 mm of the sciatic nerve in the midthigh region. The contralateral leg served as a control based on our previous results showing that soleus MHC composition was similar between a normal control and the contralateral muscle when studied 4 wk after DEN (13).

To test whether subsequent DEN after plasmid injection affected type I MHC gene promoter responsiveness, we performed a pilot experiment in which DEN was performed 72 h after plasmid injection. Plasmid mixes (10 μg 3,500 type I MHC pGL3 + 3.29 μg HSA pRL) were injected in both left and right soleus muscles of eight adult rats. Three days post plasmid injection, the left leg was harvested. The reporter was euthanized 4 days post denervation, equivalent to 7 days post plasmid injection. Reporter activity was analyzed, and results show that at 4 days post-DEN, both type I MHC and α-actin promoter activities were significantly reduced in the denervated muscle. The percentages of decrease in promoter activity 4 and 7 days post-DEN, respectively, were 86 and 92% for type I MHC and 77 vs. 78% for type I/actin ratio. These results show that plasmid injection and subsequent DEN does not interfere with reporter expression and data interpretation.

Additional rats (n = 24) were used to obtain soleus for muscle nuclei extraction for use in the DNA gel mobility shift assays. All experiments were 7 days in duration. After 7 days, the animals were euthanized and the soleus muscle was quickly removed, frozen between blocks of dry ice, and stored at −80°C until subsequent analysis. This study was approved by the Animal Use Committee at the University of California, Irvine, 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 (Fluc) reporter gene in the pGL3 vector (Promega). A series of deletions of the type I MHC promoter were tested in this study as follows: −3,500, −2,500, −914, −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 this laboratory (10, 20, 52). The −408 and −215 type I MHC promoter constructs with β3 mutation, the −408 β2/β3 mutant, and −408 β2/β3/GATA mutant were also tested in the normal control (NC) and DEN soleus. Mutations of the target promoter sequence consisted of changing ACC to CGG in the β3 element (22, 45), and GTG to TGT in the β2 element (45), GATAT to ACTCG in the GATA element, and CCCAACC to CCCAAAG in the C-rich region and were designed to disrupt specific transcription factor binding sites (see Ref. 52 for details on mutagenesis).

In addition to the various type I MHC promoter constructs, a type IIb MHC-pGL3 promoter-reporter construct was also tested in NC and DEN soleus to examine the promoter response of a gene that is upregulated in response to DEN. This experiment would also rule out the possibility of global transcriptional downregulation with DEN. This IIb MHC construct consisted of a mouse IIb MHC promoter fragment extending from −1,400 bp to +13 bp relative to the TSS and linked to a Fluc reporter gene (pGL3 basic, Promega). The IIb and human skeletal α-actin promoter fragments were the kind gift of Dr. Steve Swoap, Williams College, Williams-town, MA, and their activity in rat muscles follows the same specificity as the expression of the endogenous gene (42). Plasmids were amplified in *Escherichia 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 ultraviolet absorbance at 260 nm using the conversion factor of 50 μg/ml per optical density 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.

**Rationale for the use of skeletal α-actin as a reference promoter.** To correct for variation in gene transfer efficiency in the direct injection technique, a reference promoter linked to a different reporter gene is coinjected with the test plasmid. Typically, a plasmid containing a viral promoter, such as the CMV (cyto megalovirus) or SV40 (simian virus) promoter, is selected as the reference vector because the viral promoters are ubiquitous, ubiquitously expressed in the surrounding nonmuscle tissue, which compromises its function in the normalization of plasmid uptake in muscle cells. Previously, we have shown (11) that the level of Fluc activity (type I MHC-Fluc) correlated well to the level of Rluc (α-actin-Rluc) activity in control soleus muscles, demonstrating the effectiveness of α-actin reference plasmid as a control for transfection efficiency. On the basis of reporter activity generated in this study, a strong relationship between Fluc and Rluc activity is observed in control muscles (Fig. 1A).

Ideally, the reference promoter activity should not change in response to experimental manipulation. However, as a sarcomeric muscle protein, we expect α-actin to be downregulated in response to the atrophic stimuli of DEN. However, because the sciatic nerve DEN procedure is the same across all experiments, the degree of atrophy is also expected to be equivalent across experiments. This is demonstrated by the similar decrease in muscle mass 7 days post-DEN (see RESULTS). Therefore, although it is likely that the skeletal α-actin promoter activity may be decreased with global transcription changes after DEN, these changes are not fiber type specific. Furthermore, the degree of downregulation should remain relatively constant across all experiments. Indeed, in the present study, the average decrease in α-actin activity was not significantly different among the various experiments, with an average decrease of 65 ± 3% relative to...
control (Fig. 1/H9251 B). The consistency of the α-skeletal actin-Rluc as a reference promoter. A relationship between firefly luciferase activity (Fluc), driven by the test promoter, and renilla luciferase activity (Rluc), driven by the actin promoter in normal control (NC) soleus. Analysis is shown for 2 separate experiments, the 3,500 and the 299 promoter fragments. Correlation was significant for both studies, demonstrating that although variability in the test promoter expression was high due to variable plasmid transfer efficiency, the expression is predictable based on renilla expression when driven by the actin promoter. Thus normalizing to actin promoter activity can account for variable gene transfer. B: % change in Rluc activity in the denervated soleus muscle relative to control in the various experiments. Lanes 1–7 are from the deletion analysis reported in Fig. 3. Lane 8 is from the −215 βε3 mutation reported in Fig. 4. Lanes 9–12 are from the −408 mutants reported in Fig. 5. No significant difference was detected among the means of the different groups. Den, Denervation; MHC, myosin heavy chain.

As an additional control, plasmid uptake can be determined by directly measuring the amount of plasmid DNA in the tissue using PCR (12, 17). Two sets of experiments were sampled using this method to assess whether plasmid uptake was different between NC and DEN soleus: the 3,500 type I MHC pGL3 and the 408 type I MHC pGL3. In these two experiments, tissue plasmid content was analyzed for eight NC and eight DEN muscles in each experiment. No significant difference was detected in plasmid content between NC and DEN muscles for either test construct, although the DEN muscles tended to have a slightly higher plasmid content. Relative densitometric values in NC and DEN soleus, respectively, were 44 ± 9 and 65 ± 13 for the 3,500 type I MHC construct and 109 ± 24 and 120 ± 12 for the 408 type I MHC construct. Thus the decreased reporter activity in denervated muscles is not the result of a reduced plasmid uptake but rather decreased reporter gene expression. Theoretically, reporter activity can be normalized to plasmid uptake to compare promoter activity between control and experimental muscles. However, DEN (or any experimental manipulation) may alter gene expression both transcriptionally and posttranscriptionally (e.g., RNA stability and translation and degradation rates). Therefore, normalizing Fluc activity to plasmid DNA would not accurately represent transcriptional activity of the test promoter because it would not reflect potential changes in posttranscriptional activity. Therefore, dividing the type I MHC promoter-driven firefly activity by α-actin-driven Fluc activity is a superior method of normalization because it factors out posttranscriptional processes occurring in the atrophying muscle. Thus any change in the type I MHC-Fluc/α-actin-Rluc reporter activity in response to DEN results from a specific decrease in type I MHC isoform promoter activity. On the basis of this rationale, type I MHC promoter activity is reported as the ratio of Fluc activity to Rluc activity, the latter being driven by the actin reference promoter.

Reporter gene assays. For the luciferase assay, each soleus was homogenized in 1 ml of ice-cold passive lysis buffer (Promega) supplemented with protease inhibitors (0.2 mM AEBSF, 5 μg/ml aprotinin, and 5 μg/ml leupeptin), and 200 μl were transferred into a tube containing 600 μl of TRI reagent LS (Molecular Research Center), mixed immediately, and stored at −80°C for subsequent RNA extraction. The remaining homogenate was centrifuged at 10,000 g for 10 min at 4°C. The supernatant was reserved for the luciferase assay 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. Reporter activity was assayed using 5 μl of supernatant, and light output was integrated over 10 s using a Monolight 2010-C luminometer (Analytical Luminescence Laboratories). Background activity levels, based on measurements in noninjected tissue for both lucifers, were determined and subtracted from the activities measured in the tissues injected with the test plasmid.

Type I MHC mRNA and pre-mRNA analyses. For the first two experiments, total RNA was extracted from 200 μl of the luciferase homogenate using TRI reagent LS according to the manufacturer’s protocol. After extraction, the RNA pellet was suspended in nuclease-free water and immediately treated with DNase according to supplier’s (Promega) recommendation to remove any trace of genomic DNA contamination. At the end of DNase treatment, the RNA samples were re-extracted with TRI-reagent LS, and the RNA pellet was suspended in nuclease-free water. RNA concentration was determined by ultraviolet absorption at 260 nm. RNA samples were stored at −80°C for future analysis with reverse transcription-polymerase chain reaction (RT-PCR).

Semiquantitative RT-PCR was used to analyze the expression of type I MHC mRNA and pre-mRNA in normal control and denervated soleus RNA samples. One microgram of total RNA was reverse transcribed using Superscript II reverse transcriptase according to the supplier’s recommendations (Invitrogen), but with substitution of oligo dT primers with the type I MHC mRNA antisense primer, which is complementary to the unique 3′-untranslated end of type I MHC mRNA (primer 2 in Table 1). The PCR for amplification of type I MHC mRNA were carried out in 25-μl reactions containing 1× PCR μl, 1.5 mM MgCl2, 0.2 mM dNTP, 0.75 unit Biologe DNA polymerase (Bioline, Randolph, MA), 1 μl of cDNA template diluted 40-fold, and 15 pmoles of each of primers 1 and 2 (Table 1). Annealing was set at 56°C, and amplification was carried out for 24 cycles, using a 1-min extension period for each cycle, which resulted in a 546-bp PCR product. PCR to amplify type I MHC pre-mRNA were
similar to the above except cDNA dilutions were fivefold, primers 3 and 4 were used, and the number of cycles was 30. This resulted in a 186-bp product. Note that primers 1 and 2 also have the potential to amplify pre-mRNA, but this does not occur because the pre-mRNA product would be \(>2.5\) kb (extension time of 1 min is not sufficient for full amplification) and its abundance is much less than the mRNA. Primer 3 is derived from the last intron of the type I MHC gene (Haddad and Baldwin, unpublished work) and, thus, can target only pre-mRNA. The number of cycles and the PCR conditions for each target mRNA was optimized so that the amplified signal was still on the linear portion of a semilog plot of the yield expressed as a function of the number of cycles. As a check for genomic DNA contamination, PCR reactions using an equal amount of nonreverse transcribed RNA produced no bands, thus validating the effectiveness of DNase treatment. PCR products were separated on a 2% agarose gel by electrophoresis, stained with ethidium bromide, and quantified using laser scanning densitometry, as reported previously (51). It should be noted that for this type of RT-PCR, unlike RT reactions using oligo(dT) or random primers, the template cDNA used for these analyses is specific for the type I MHC gene, including both the pre-mRNA and mRNA species. This specificity was necessary to obtain a clean pre-mRNA signal. The restrictive RT conditions set by the antisense gene specific primer makes it impossible to target an internal control such as 18S or other constitutive genes for normalization. Without an internal standard, meaningful results depend on accurate RNA concentrations and pipetting. To minimize errors, RNA concentration was measured twice for each sample (average value was used) and each RT product was amplified by PCR in duplicate. All duplicates were within 5% of each other, and if the difference was >10% between duplicates, the sample was repeated. Furthermore, accuracy of the results was verified on a few samples by amplifying serial dilutions of the cDNA. The results were consistently linear and the comparisons between NC and DEN muscles were similar for all dilutions.

**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 DEN soleus muscles \((n = 6\) group, each “n” consisted of a pool of four muscles for control and six muscles for DEN, \(-200\) mg/pool) and stored at \(-80^\circ\)C. Double-stranded oligonucleotides consisting of 24 bp from the type I MHC gene promoter spanned the \(\beta 3\) regulatory elements (10, 20, 30). After strand annealing, double-stranded probes were end-labeled with \(^{32}\)P using T4 polynucleotide kinase (Promega).

Binding reaction conditions, supershift assays using TEF-1 antibody (BD Transduction Laboratories), gel conditions, and image analysis were as described in details previously (20).

**Western blotting.** Approximately 10 \(\mu\)g of soleus nuclear proteins were subjected to SDS-PAGE according to standard protocol (26) and electrophoretically transferred to a PVDF membrane (Immobilon-P) using 10% methanol, 25 mM Tris, and 193 mM glycine, pH 8.3. The membrane was reacted overnight with a TEF-1 monoclonal antibody (BD Bioscience) at 1:500, and after washing and reaction with the secondary antibody, the signal was detected using enhanced chemiluminescence (ECL Plus Kit, Amersham) according to the manufacturer’s protocols. Signal intensity was determined by laser scanning densitometry (Molecular Dynamics/ImageQuant). Antibody reaction resulted in a specific \(-53\)-kDa band matching the band obtained from a positive control A431 cell lysate (provided by BD Bioscience) and a band from HeLa nuclear extract (Santa Cruz). A duplicate membrane blot of the same samples was reacted with preimmune mouse serum and a secondary antibody under similar conditions as the TEF-1 blot, and signal was used as negative control to insure the specificity of TEF-1 antibody reaction.

**Statistical analysis.** Statistical analysis was performed with GraphPad Prism 3.0 statistical software. Values are mean \(\pm\) SE. Differences between mean type I MHC promoter activity in NC and DEN soleus was determined by a paired, two-tailed \(t\)-test. \(P < 0.05\) was accepted as the level of statistical significance. Differences among mean type I MHC promoter activity of the deletion constructs in either NC or DEN soleus were determined by a one-way ANOVA with Newman-Keuls post hoc comparisons.

**RESULTS**

**Body and muscle weights.** The average initial body weight at the time of unilateral denervation and gene transfer was \(96 \pm 0.7\) g and increased to \(133 \pm 0.8\) g after 7 days. Mean soleus weight was \(58 \pm 0.7\) mg and \(34 \pm 0.8\) mg for the NC and DEN soleus, respectively. In all conducted experiments, whether to measure promoter activity or to obtain muscle nuclei, 7 days of DEN were associated with similar muscle atrophy. The average muscle loss in the denervated soleus ranged from 35 to 46% (overall average was 42%, \(P < 0.01\)).

**Endogenous type I MHC gene mRNA and pre-mRNA expression.** Seven days of DEN was associated with a 36% reduction in pre-mRNA (\(P < 0.05\)) and a 56% decrease in mRNA expression (\(P < 0.05\)) (Fig. 2). Although the mature mRNA changes appear more pronounced than the pre-mRNA, these changes are not significantly different (\(P = 0.2\)). These results demonstrate that transcriptional processes primarily regulate the reduction in type I MHC mRNA expression in denervated muscles.

**Deletion analysis of the type I MHC promoter.** In the normal control soleus, the longest promoter fragment (\(-3,500\) type I MHC) demonstrated the greatest activity, confirming the existence of positive regulatory elements in the distal upstream region of the promoter (Fig. 3). In addition to the \(-3,500\) type I MHC promoter, six shorter promoter fragments were tested for their responsiveness to DEN. All of the deletion fragments tested (\(-2,500, -914, -408, -299, -215, \) and \(-171\)) were active in NC soleus, but to a lesser degree than the \(-3,500\) promoter fragment. In response to DEN, \(-3,500\) type I MHC promoter activity was reduced by 77% relative to control. The responsiveness to
DEN was similar in all deletion fragments down to −215 (Fig. 3), with the decrease ranging from 59 to 76% relative to activity in normal control muscle (Fig. 3, inset). In contrast, the −171 promoter activity was not significantly changed with DEN (Fig. 3). This lack of responsiveness to DEN of the −171 compared with the −215 promoter suggests that regulatory elements essential for type I MHC downregulation with DEN are likely located between 215 and 171 bp of the type I promoter. One putative cis DNA regulatory element in this region is the $\beta_3$ element ($-214/-190$) (45). Thus, to assess the role of the $\beta_3$ element in type I MHC downregulation with DEN, experiments were performed that involved mutation of this element in two promoter fragments, the −408 and −215 type I MHC promoters.

**Mutational analysis of the type I promoter.** Mutation of the $\beta_3$ element in the −215 type I promoter significantly decreased activity in the NC soleus, confirming the role of the $\beta_3$ element as a positive regulatory element in normal expression of the type I MHC gene in slow skeletal muscle (Fig. 4). Mutation of this element blunted the promoter downregulation in response to DEN (Fig. 4). Thus decreased responsiveness to DEN when the $\beta_3$ element is mutated suggests that this element may confer responsiveness of the type I MHC promoter to DEN.
Similar to the results on the −215 promoter, mutation of the same β3 element in the −408 type I promoter also significantly decreased activity in the NC soleus, confirming its role as a positive element (Fig. 5). Despite its reduced level in the normal soleus, the activity of the −408 β3 mutant was significantly decreased in response to DEN, suggesting that additional upstream elements were able to restore type I MHC promoter responsiveness to DEN (Fig. 5). To test which additional elements may be involved in rescuing type I MHC promoter responsiveness to DEN, we simultaneously mutated several regulatory elements along with the β3 element. Simultaneous mutations of the β3 and β2 (−285/−269) elements reduced promoter activity in NC soleus by ~75% compared with the WT and ~50% compared with the β3 mutant, demonstrating that both β3 and β2 are positive regulatory elements for type I MHC expression in normal rat soleus (Fig. 5). However, the −408 β3/β2 mutant’s responsiveness to DEN was similar to that of the WT 408 promoter (Fig. 5). In addition, a 408 promoter fragment with a triple mutation (β2, β3, and GATA-like sequences) was still responsive to DEN compared with the WT (Fig. 5). It was not until a C-rich mutation was incorporated to create a quadruple mutant that the responsiveness to DEN was attenuated compared with the WT (Fig. 5, inset).

Activity of the type IIB MHC promoter. Denervation of slow muscle is associated with an increase in fast MHC isoform expression (19, 21, 31). A type IIB MHC-pGL3 promoter-reporter construct was injected into NC and DEN soleus to examine the promoter response of a gene that is upregulated in response to DEN. Our results show that the activity of a −1,400/+13 type IIB MHC promoter construct was significantly increased in the soleus of DEN rats compared with NC (Fig. 6). This upregulation of the type IIB promoter demonstrates that DEN does not cause a global decrease in the transfected promoter activities. Furthermore, it shows that the IIB promoter response to DEN is in the same direction as the endogenous IIB MHC gene.

Gel mobility shift assays. The findings that mutation of the β3 element in the −215 promoter fragment and that deletion of this element (−171 fragment) decreased type I responsiveness to DEN suggest that this element is involved in the response to DEN. Thus gel mobility shift assays were used to examine interactions of the β3 element with nuclear proteins extracted from NC and DEN soleus (Fig. 7A). Although DNA-protein interactions were resolved into several complexes on the gel, only one complex, based on competition with different DNA probes, was specific to the β3 probe (labeled Sp. C in Fig. 7A). This specific band (Sp. C) was competed out by 100-fold molar excess of unlabeled β3 probe. In contrast, an unlabeled β3 mutant probe and an unrelated sequence (GATA) were not able to effectively compete out binding to the radiolabeled β3 probe when incubated at 100-fold molar excess. Densitometric analysis determined that there was a significant 3.6-fold increase in binding with the DEN nuclear extracts compared with NC. Further, the increase was not due to differences in nuclear protein concentration between the groups, because binding to probes containing other cis DNA elements (GATA or
NFAT) was not different between the two 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. 7A). Whereas DEN significantly increased nuclear protein binding to the β3 element 3.6-fold, there was 50% less protein supershifted by the TEF-1 antibody in DEN samples (450,000 ± 120,000 vs. 219,000 ± 50,000 densitometric units for NC and DEN, respectively). This decrease in the supershifted bands with TEF-1 in the DEN nuclear extract is due to a decreased abundance of TEF-1, as determined by Western blot analysis using TEF-1-specific antibody (Fig. 7B). Nuclear extract from denervated soleus produced a TEF-1 signal intensity that on average was 30% of the NC signal, n = 5/group (Fig. 7B).

**DISCUSSION**

Motor innervation and muscle activation are critical factors influencing muscle gene expression. Previous denervation studies at both the mRNA and protein levels have demonstrated that the muscle shifts its MHC phenotype when the motoneuron-muscle connection is severed (19, 21, 31). Similar to other models of muscle inactivity, the MHC remodeling that occurs with DEN is characterized by decreased expression of slow type I MHC and increased expression of the faster MHC isoforms (19, 21, 31). The findings reported herein provide the first evidence that the previously reported decrease in type I MHC protein and mRNA in denervated slow muscles is mediated largely via decreased transcriptional activity of the endogenous type I MHC gene based on pre-mRNA and mRNA analyses of type I MHC gene expression. Furthermore, the findings reported herein demonstrate that the activity of an exogenous type I MHC promoter fragment, when studied via reporter gene assays 7 days after direct gene transfer in vivo, is also downregulated in denervated soleus similar to the endogenous gene. This downregulation in reporter gene activity was observed for both the type I MHC and α-skeletal actin promoters; however, reporter activity increased severalfold when driven by a fast type Ib MHC promoter fragment (Fig. 6). This latter observation validates the specificity of the system and negates the possibility of a generalized decrease in reporter activity in the denervated muscle. A systematic functional analysis of the type I MHC promoter via serial deletions of the 5' flanking region demonstrates that promoter responsiveness to DEN is contained within 215 bp upstream of the transcriptional start site (Fig. 3). Mutation analysis of important cis-regulatory elements partially implicates the β3 DNA regulatory element (45) in the response to DEN (Figs. 4 and 5).

Likely, both the neurotrophic effects and activation-associated factors present with an intact motoneuron-muscle connection are essential for maintenance of
slow type I MHC expression in a slow rat hindlimb muscle. We recently reported that SI is associated with type I MHC promoter downregulation in the rat soleus (20). SI is unique in providing a baseline for complete inactivity while maintaining potential activity-independent neural influences. In contrast, the motoneuron-muscle connection is severed with DEN, thus eliminating both activity-dependent and -independent neural influences on the muscle. Despite possible differences between these two models of inactivity, the βe3 element was involved in type I MHC downregulation in both models. Although the βe3 element is likely a critical activity/load response element, there are some subtle differences in type I MHC transcriptional regulation when the motoneuron-muscle connection is maintained. With SI, the βe3 element was necessary and sufficient for transcriptional downregulation of the type I MHC gene promoter (20). Specifically, mutation of this element in two different promoter fragment lengths (−215 and −408) prevented type I MHC downregulation. In contrast, although mutation of the βe3 element in the −215 fragment blunted type I MHC downregulation in response to DEN, mutation of this element in the −408 fragment did not prevent type I MHC downregulation. The differing −408 promoter response between DEN and SI with the βe3 mutation suggests that in SI muscles the βe3 element was the primary mechanism whereby type I MHC promoter activity was blunted. In contrast, severing the motoneuron-muscle connection with DEN increased potential pathways for the muscle to downregulate type I MHC gene expression. Despite the mutation of four important DNA regulatory elements, the −408 type I MHC promoter fragment was still responsive to DEN (Fig. 5). This responsiveness of the triple mutant (βe2, βe3, and GATA) was similar to the −408 WT but was slightly attenuated (P = 0.06 ANOVA) when the C-rich mutation was added to the previous three elements (Fig. 5, inset).

These results point to the complex regulation of the type I MHC promoter in denervated muscle. The βe3 element is important for conferring responsiveness to DEN in the −215 promoter; however, this same element’s regulatory capacity is overridden by other influences in the longer 408 promoter fragment. It is puzzling that simultaneous mutations of the βe3 with other known upstream regulatory elements in the −408 fragment did not abolish the responsiveness to DEN observed with −215 βe3 mutant. This suggests that other untested elements located between 408 and 215 bp upstream of TSS may play a role in type I MHC downregulation in the absence of neural activation and trophic factors. These elements could involve the β1 repressor element (4) or other unknown elements.

The present study demonstrates that decreased type I MHC expression with skeletal muscle inactivity, independent of motoneuron-muscle connectivity, is transcriptionally regulated and involves the βe3 regulatory element. Studies in cardiac myocytes have characterized the enhancer core of the βe3 element, also referred to as the myocyte-specific CAT (M-CAT) cis element (22, 24). We have demonstrated that βe3 is a positive element in the normal weight-bearing soleus muscle, as evidenced by the significant decrease in −215 and −408 type I promoter activity when the βe3 element is mutated (Figs. 4 and 5). 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 (22, 23, 41, 47), αMHC (14), and skeletal α-actin (24). For example, TEF-1 overexpression and activation of a −215 type I MHC promoter in cardiac myocytes required an intact M-CAT element (41).

Studies in both cardiac and skeletal muscle suggest TEF-1 involvement in muscle contractile gene regulation. Recently, a TEF-1-related protein, RTERF-1, was implicated in cardiac myocyte hypertrophy (47). Skeletal muscle hypertrophy is also associated with significantly increased TEF-1 binding to α-actin promoter (2). Although increased expression and DNA binding of TEF-1 may be associated with muscle hypertrophy, a reduction in TEF-1 expression and binding may contribute to muscle atrophy. Our supershift assays demonstrate that TEF-1 is part of the nuclear protein complex bound to the β3 element (Fig. 7). A TEF-1 antibody supershifted protein complexes binding to the β3 oligonucleotide in nuclear extracts from both NC and DEN soleus. Although total nuclear protein binding to the β3 oligonucleotide increased almost fourfold with DEN, protein supershifted by the TEF-1 antibody was only 50% of that observed in NC soleus. These results, taken together with our previous finding in the soleus of SI rats (20), demonstrate that two different models of skeletal muscle inactivity decrease TEF-1 binding to the type I MHC promoter. This decreased binding to the β3 probe in the GMSA resulted from decreased expression of TEF-1 protein as demonstrated by Western blot analysis (Fig. 7B). Consequently, one mechanism by which muscle inactivity could downregulate type I MHC is via decreased TEF-1 expression, thus decreasing activation of the β3 element of the type I MHC promoter. This possibility is supported by the significant reduction in promoter responsiveness when βe3 element was mutated or deleted in the shorter promoter fragment. Potentially, TEF-1 could upregulate muscle contractile genes in response to muscle overload (2) while downregulating these genes in response to chronic inactivity.

Because DEN significantly increased nuclear protein binding to the β3 oligonucleotide while decreasing the amount of TEF-1 binding, the residual binding was likely composed of other yet to be determined transcription factors that increase in response to DEN. Another possibility is that DEN causes changes in the availability of promoter-specific cofactors. For example, TEF-1 and a transcription factor designated as Max associate to exert a positive cooperative effect for gene regulation (14). Consequently, residual binding to the βe3 element may include factors such as Max; however, without TEF-1, gene activation is blunted.
In NC soleus, IIb MHC mRNA and protein expression are below detection limits. After 7 days of DEN, we detected de novo expression of type IIb MHC mRNA up to 6 ± 2% of the total MHC mRNA pool as determined by RT-PCR (unreported data). Testing the IIb MHC promoter in the denervated soleus showed that its activity increases several fold, mimicking the regulation of the endogenous gene (Fig. 6). This observation further validates gene injection as an approach to study regulation of promoter activity. However, although short-term inactivity and/or unloading is sufficient to activate IIb MHC promoter activity and significantly increase endogenous mRNA, such inactivity is unable to induce expression of IIb protein. For example, in hindlimb-unloaded rats, IIb mRNA was detected with no changes at the protein level (16). Similarly, 60 days of SI, 30 days of DEN (15, 19), or 14 days of tetrodotoxin paralysis (31) did not induce de novo IIb protein expression. Taken together, these observations suggest that in a slow muscle there is a marked uncoupling between transcriptional and translational events in the upregulation of IIb MHC. However, more prolonged inactivity (e.g., 90 days of SI) or decreased activity coupled with other factors such as thyroid hormone status has induced IIb protein expression in the soleus. For example, hindlimb unloading coupled with triiodothyronine treatment increased both IIb mRNA and protein in the rat soleus (3, 16).

In summary, using a novel approach, we demonstrated that type I MHC downregulation in the denervated soleus involves transcriptional processes. Using the direct gene transfer approach, we have characterized important regulatory elements in the type I MHC gene promoter that mediate downregulation in response to DEN. Deletion and mutation analyses suggest involvement of the β3 DNA regulatory element in the type I MHC downregulation; however, other potential mechanisms exist with the addition of upstream regulatory elements. Supershift assays demonstrated that one mechanism contributing to type I MHC downregulation with DEN is decreased TEF-1 DNA binding, potentially leading to a decreased activation of the β3 positive regulatory element on the type I MHC promoter (Fig. 7). Mutation of the β3 element mutation in the −215 promoter fragment rendered it unresponsive to DEN (Fig. 4). Furthermore, the −171 promoter, which lacks the β3 element, was also unresponsive to DEN (Fig. 4). However, strong involvement of the β3 element was dependent on promoter length as the addition of upstream regulatory elements (−408 promoter) altered the role of β3. Consequently, future studies to further delineate the role of β3 in conferring this responsiveness could ligate a single or double 24bp-β3 element upstream of the 171-bp promoter or the 215 β3 mutant promoter to test whether responsiveness to DEN is restored. Furthermore, utilization of different models of reduced muscle activity will be useful in establishing the distinct roles of neurotrophic factors, neural activation, and loading on muscle contractile gene expression.

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