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TRANSCRIPTIONAL REGULATION OF THE MYOSIN HEAVY CHAIN IIB GENE IN INACTIVE RAT SOLEUS

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Abstract

The myosin heavy chain (MHC) isoform composition of skeletal muscle is dependent, in part, on the functional demands of the muscle. The rat soleus muscle primarily expresses the slow-contracting type I MHC; however, chronic inactivity increases expression of the faster-contracting type II MHC isoforms. The purpose of this study was to identify the type IIb MHC promoter region(s) that regulate de novo transcription during chronic inactivity of the soleus induced by spinal cord isolation (SI; complete mid-thoracic and high sacral spinal cord transections plus deafferentation). Seven days after SI, transcription of IIb MHC was evidenced by increases in IIb pre-mRNA and mRNA. The activity of an ≈2.2-kb IIb promoter-firefly luciferase reporter plasmid increased in SI soleus over control as compared to that of a promoterless plasmid. Deletion analyses indicated that the regions encompassing –2237 to –1431, –1048 to –461, and –192 to –161 basepairs (bp) each contributed to the increase in transcriptional activity. Moreover, deletions or mutations of AT-rich regions in the proximal –192 bp region abolished the increased promoter activity. These results provide important insights related to how proximal IIb MHC promoter elements regulate the increased expression of the IIb MHC gene in response to inactivity of a predominantly slow postural muscle as it undergoes a remodeling of its phenotype and functional characteristics.

Keywords

muscle atrophy; muscle plasticity; gene regulation; gene promoter; spinal cord isolation

Skeletal muscles of adult mammals typically express types I, IIa, IIx, and IIb isoforms of the myosin heavy chain (MHC) contractile proteins, each of which is encoded by a separate MHC gene. The relative composition of MHC isoforms expressed within a given muscle has a strong influence on its contractile properties and, in turn, the MHC profile is largely dependent on the functional history, activation level, and loading state of the muscle.¹⁴ Identifying the mechanisms that regulate the expression of specific adult MHC isoforms in

response to altered activation and loading states is an ongoing topic of investigations aimed at defining the regulatory mechanisms of phenotype plasticity.

In rats, the soleus is a highly active postural muscle in the hindlimb. It predominantly expresses the slow-contracting type I, or beta, MHC gene. However, when the soleus is subjected to chronic neuromuscular inactivity and/or unloading, the muscle atrophies, and transcriptional activity of the type I MHC gene decreases.⁶ In contrast to suppression of type I MHC during soleus muscle atrophy, expression of the fast-contracting type IIx and IIb MHC genes increases.^{5,6,9} In the case of the fastest-contracting IIb MHC iso-form, the increased expression is considerable, because the rat soleus normally does not express the IIb MHC gene based on lack of detection of IIb mRNA⁹ or protein.⁵ Recently, it was demonstrated that the increase in IIb mRNA during atrophy of the soleus was accompanied by an increase in the IIb pre-mRNA, which is the primary product of transcription.⁹ These results suggest that a transcriptional control mechanism is involved in increased IIb gene expression during remodeling of muscle in response to chronic decreases in neuromuscular activity. This increased expression of IIb MHC in the soleus thus provides a model system for investigating how chronic decreases in neuromechanical activity regulate the expression of muscle-specific genes that alter muscle phenotype and functional characteristics. A greater understanding of these regulatory mechanisms has the potential to foster the development of pharmacological and/or neuromechanical rehabilitation therapies aimed at the maintenance or recovery of neuromuscular function following traumatic injury to the nervous system.

Regulatory region(s) within the promoter of the IIb MHC gene that control its transcription have been investigated in vitro using different muscle cell lines. Initial reports by Whalen's group^{8,13} focused on the ~2600 basepairs (bp) upstream promoter and identified regions that were important for controlling transcription in C₂C₁₂ myoblasts. These studies identified two well-conserved muscle AT-rich regions located -192 to -147 bp proximal to the transcription start site (TSS) that cooperatively bound the transcription factors OCT-1 and myocyte enhancer factor-2 (MEF-2) to control the activity of IIb promoter-reporter plasmids. Only a few details are known about this promoter region with regard to the in vivo control of IIb transcription in mammals. Using in vivo plasmid DNA gene transfer, Huey et al.⁶ reported that 7 days of chronic inactivity of the rat soleus muscle increased the transcriptional activity of a IIb promoter fragment that encompasses the proximal 1400 bp upstream of the TSS. Swoap¹² reported that the -295 to -63 bp upstream promoter contained regions important for the expression of IIb in normal rat tibialis anterior (TA) muscle as well as its induction in the soleus muscle after hindlimb unloading. Subsequently, Swoap's group¹⁵ reported that mutation of an E-box in position -66 to -61 decreased promoter activity in normal rat TA and also prevented binding of the myogenic regulatory transcription factors MyoD and myogenin in gel mobility shift assays. In addition, more recent reports have indicated that the mAT2 region (MEF-2/OCT-1 binding site) and a CArG-like box residing in the -120 to -80 bp promoter region are binding sites for the serum response factor (SRF) and act as enhancers of IIb transcriptional activity in the predominantly fast TA muscle of adult mice.¹ Collectively, these prior data implicate the highly conserved proximal 300 bp of the IIb MHC promoter in its regulation in skeletal muscle in vivo.

The purpose of the present study was to identify the *cis*-regulatory regions within the upstream promoter that induce MHC IIb promoter activity in the rat soleus during chronic neuromuscular inactivity. The IIb pre-mRNA expression was analyzed to monitor the transcriptional activity of the endogenous IIb gene in the same muscles subjected to injection with IIb promoter constructs. Spinal cord isolation (SI) was used as the model of neuromuscular inactivity/unloading to induce muscle atrophy and slow-to-fast remodeling of

the MHC phenotype of the soleus.⁶ We hypothesized that SI would induce the IIB MHC promoter activity in the inactive soleus, and that deletions and/or mutations of the AT-rich regions in the proximal promoter would attenuate the IIB promoter activity.

MATERIALS AND METHODS

Experimental Model

This study followed the National Institutes of Health (NIH) Animal Care Guidelines and was approved by the University of California, Los Angeles, Animal Care and Use Committee. Female Sprague–Dawley rats (≈ 135 – 140 g) were anesthetized, and a skin incision was made to expose the soleus. Twenty μ l of phosphate-buffered saline (PBS) containing plasmid DNA mixtures were injected into the soleus using a 29G needle attached to a 0.3-ml insulin syringe. Plasmid mixtures consisted of a mouse type IIB MHC test promoter (2 pmoles/injection) and a human skeletal α -actin reference promoter construct (HSA2000-pRL; 1 pmole/injection). Following plasmid injection the rats either underwent spinal cord isolation surgery (SI; $n = 7$ – 8) or were maintained as normal ambulatory controls (Con; $n = 8$). For the SI procedure the spinal cord was transected at both a mid-thoracic and high sacral level, and bilateral dorsal rhizotomy was performed between the two transection sites as described previously.^{3,7,11} The unique feature of the SI model is that the surgical treatment renders the motor neuron pools in the isolated region of the spinal cord virtually inactive, while maintaining neuromuscular connectivity with the leg musculature.¹¹ Thus, under these electrically silent conditions the electromyographic activity of the soleus after 7 days of SI is reduced to $<1\%$ of normal, and the muscles fail to contract and generate force.¹¹

After 7 days the animals were euthanized by decapitation and the soleus was dissected, weighed, quickly frozen, and stored at -80°C until subsequent analysis. The 7-day timepoint was selected because: (1) prior studies by Huey et al.⁵ reported significant phenotype shifts in MHC mRNA expression after 7 days of SI, including the induction of IIB mRNA expression; (2) prior studies by Giger et al.² had determined that the optimal level of promoter activity occurred 7 days after plasmid injection; and (3) the SI soleus muscle was virtually electrically silent during this 7-day period.¹¹

Plasmid Constructs

The mouse IIB promoter from -2554 to $+13$ bp relative to the transcription start site (TSS) was linked to a firefly luciferase (FLuc) reporter gene in the pGL3 basic vector (a kind gift from Dr. Steven Swoap, Williams College, Williamstown, Massachusetts). The IIB MHC promoter sequence was initially described by Takeda et al.¹³ and is found in GenBank Accession No. M92099. Deletion fragments of the 5' end were produced from this long fragment using high-fidelity polymerase chain reaction (PCR) to generate -2237 bp, -1431 bp, -1048 bp, -461 bp, -224 bp, -192 bp, and -160 bp IIB promoter fragments. The forward primer was designed to contain an XhoI recognition site in addition to the target IIB promoter sequence, while the reverse primer was designed to contain a HindIII site in addition to 19 bp sequences corresponding to bases from -6 to $+13$ bp relative to TSS. Both XhoI and HindIII do not cut the IIB promoter but are part of the multicloning site of pGL3 basic, thus facilitating cloning of a shorter fragment in the reporter vector upstream of the FLuc reporter gene. PCR used the original -2554 to $+13$ bp pGL3 plasmid template (100 ng/reaction) and pfu polymerase (Stratagene, La Jolla, California), a high-fidelity DNA polymerase, to amplify products for 25 cycles. PCR products were separated by DNA gel electrophoresis, isolated by gel extraction, and after an overnight digestion with XhoI and HindIII they were ligated into the pGL3 basic plasmid that was predigested with the same enzyme pair. Additional PCR primers were used to create a $-1.4 \Delta(-181/-161)$ construct

which had an internal deletion of the region between -181 and -161 bp within the -1431 bp construct. This deleted region contained a muscle AT-rich region known as mAT2.¹³ Cloning of the -1.4 Δ (-181/-161) promoter fragment introduced an XhoI site (CTCGAG) sequence between the flanking regions. Screening of the region with this XhoI insertion using MatInspector (Genomatix Software, Munich, Germany), however, did not reveal the creation of any new putative transcription factor binding sites. An overlap extension technique using high-fidelity PCR was utilized for site directed mutagenesis.⁴ Internal deletions and mutations were directed at the mAT1 and mAT2 sites within the -1431 bp IIB promoter (Fig. 1).

All of the prepared IIB MHC promoter fragments including the shorter deletions, the internal deletion, the mutations, and the entire fragment were sequenced using an ABI Big Dye Kit and 3700 sequencer (UCI DNA core facility). Sequencing results confirmed the accuracy of the PCR-generated products and the presence of the mutation or deletion as designed.

Each of the IIB promoter constructs and a promoterless (basic) pGL3 plasmid were individually tested in 11 separate 7-day experiments. The reference promoter (also a kind gift from Dr. Steven Swoap) consisted of the human skeletal α -actin gene extending from -2000 to +250 bp relative to TSS and linked to the renilla luciferase (RLuc) reporter gene in the pRL vector (Promega, Madison, Wisconsin). The α -actin reference promoter activity (RLuc) was used to correct for the inherent variability of plasmid uptake by expressing the IIB test promoter activity (FLuc) relative to the α -actin promoter activity. The activity of each tested IIB promoter fragment was expressed as SI relative to Con and normalized to the activity of a promoterless pGL3 under the same conditions. Using the α -actin as a reference promoter may not be considered as the most ideal choice since its activity decreases with the SI manipulation. However, the decrease in α -actin promoter was accounted for by normalizing the ratio data to that of the promoterless vector (pGL3 basic) coinjected with the same amount of α -actin promoter construct. In our hands we found that a promoterless construct is associated with a low basal expression of the reporter. This expression appears to be dependent on the amount of plasmid taken up by the tissue (data not shown) and not on the manipulation, since a promoterless construct has no regulatory elements to control the expression of the reporter gene. In addition, although the α -actin pRL activity was different across experiments due to differences in plasmid uptake, we found that the magnitude of the decrease in the α -actin promoter activity in response to SI was very consistent across these experiments, ranging from a 74%–94% decrease with no significant difference among individual experiments ($P > 0.05$). Therefore, we feel that normalizing the IIB promoter/ α -actin promoter ratio to the promoterless/ α -actin promoter ratio reflects IIB promoter activity without the interference of the α -actin decrease.

Reporter Expression Assays

Each soleus was homogenized in 1 ml of ice-cold passive lysis buffer (Promega) supplemented with protease inhibitors (AESBF, aprotinin, and leupeptin) and centrifuged at 10,000g 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 FLuc and RLuc activities within a single extract aliquot. Five μ l of supernatant was used in the assay, and light output was integrated over 10 s using a luminometer (Analytical Luminescence) and expressed as relative light units (RLUs).

MHC IIB Pre-mRNA and mRNA Analyses

Soleus muscles from one of the promoter experiments were used for RNA analyses. Total RNA was extracted from 250 μ l of muscle homogenate using TRI-reagent LS according to the supplied procedure (Molecular Research Center, Cincinnati, Ohio). The RNA pellet was

suspended in nuclease-free water and treated with DNase I according to the supplier's recommendation (Invitrogen, La Jolla, California) to remove any genomic DNA contamination. After DNase I treatment, RNA concentration was determined by UV absorbance at 260 nm. The obtained RNA had an OD₂₆₀ to OD₂₈₀ ratio of ≈ 2.0 , consistent with a pure RNA preparation. In addition, RNA integrity was examined by agarose gel electrophoresis using 0.5 μg of total RNA and ethidium bromide staining. High-quality, nondegraded RNA was confirmed by the existence of sharp 28S and 18S ribosomal RNA bands visible on the gel. Only high-quality RNA was utilized for subsequent analyses ($n = 8$ Con, and $n = 14$ SI).

One μg of RNA was reverse-transcribed in 20 μl total volume using Superscript II (Invitrogen) and a primer mix consisting of oligo-dT (100 ng/ μl) and random decamers (200 ng/ μl) according to the supplier's recommendation (Invitrogen). PCR amplification to detect MHC IIb pre-mRNA used 1 μl of cDNA amplified for 30 cycles to produce a 448-bp product using the following primers: a forward primer based on intron 39 sequence, TG TAGTCATCATAGCATCCTCCAT, and a reverse primer corresponding to the 3' untranslated region in exon 40 of the IIb MHC mRNA of this sequence: GATTTCTTCTGTACCTTTCAACA. PCR reactions to determine IIb mRNA utilized 0.1 μl cDNA amplified for 25 cycles to produce a 539-bp product using the following primers: a forward primer common to all MHC isoforms, AGAAG-GAGCAGGACACCAGC, and a reverse primer specific for the IIb MHC isoform, GTGTGATTT CTTCTGTACC. Amplification was performed using a Stratagene Robocycler. All samples were run in duplicate and PCR products were separated on 2% agarose gels and stained with ethidium bromide. Pictures were taken in UV light conditions using a digital camera, and band intensity on the digitized image was determined by volume integration using Image Quant 5.0 software (Molecular Dynamics, Sunnyvale, California).

Statistical Analyses

The left and right soleus of each rat within the SI and Con groups were included in the comparisons. Two-factor analysis of variance (ANOVA) was used to compare the dependent variables (muscle wet weight; promoter activity) among the different IIb promoter constructs and treatments (SI vs. Con). Fisher's post-hoc tests were used to compare between the tested promoters. Independent *t*-tests were used to compare the Con and SI groups for the IIb pre-mRNA and mRNA. Pearson's correlation coefficient was computed between the IIb pre-mRNA and mRNA. $P \leq 0.05$ was considered significant. StatView and GraphPad Prism software were used for the statistical analyses. Data are shown as mean $\pm$ SEM unless indicated otherwise.

RESULTS

Muscle Weight

After 7 days of SI, soleus wet weight among all 11 experiments decreased similarly by a mean of $60 \pm 1.0\%$ (Con = 68.7 ± 1.3 mg; SI = 27.3 ± 0.8 mg; $P < 0.05$).

IIb MHC Transcription

The Con soleus expressed a very low level of the type IIb MHC gene transcripts, whereas 7 days of inactivity induced marked transcription of the gene (Fig. 2). The IIb pre-mRNA and mRNA levels were higher ($P < 0.0001$) in SI than Con soleus; in addition, the pre-mRNA and mRNA values were significantly correlated ($r = 0.79$; $P < 0.0001$).

Selection of the –2237 bp I Ib Construct as the Reference I Ib Promoter

The –2237 bp upstream promoter served as the reference I Ib promoter. While the –2554 bp was the longest promoter region studied, we used blast analyses to compare the I Ib MHC promoter to mouse genomic sequences in GenBank database (NCBI website) and found that the region between –2554 and –2237 bp corresponds to a genomic repeat of the Line1 type that is not specific to the I Ib MHC promoter. Moreover, further analysis revealed that the 5'-most region between –2554 and –2237 bp is not part of the I Ib promoter sequence for the mouse C57BL/6J strain, but it is in the SV129 substrain, a source of genomic DNA commonly used for cloning and was initially used to clone the –2554 I Ib fragment.¹² The reasons for discrepancies in the distal I Ib promoter regions among mouse strains is not entirely clear at this stage. It may be due to genotype variation affecting repeat insertion: its functional significance on I Ib MHC expression remains to be determined. Curiously, the –2554 to +13 I Ib promoter had a 3–5-fold stronger activity than the –2237 to +13 bp construct when tested in rat fast muscles such as the plantaris and medial gastrocnemius (data not shown), which may indicate that the repetitive sequence (–2554 to –2337) has some activating influence on the I Ib promoter activity in muscles that normally express I Ib MHC.

I Ib MHC Promoter Is Activated by SI

The activity of the I Ib promoter constructs is shown in Figure 3. The greatest activity occurred for the –1048 bp promoter, which had high activity in both Con and SI muscles. The deletion of the region between –1048 and –461 bp resulted in a decrease ($P < 0.0001$) in activity in Con and SI muscles. The activity of the promoter fragment was similar to the basic promoterless plasmid after deletion to –160 bp.

The promoter activity induced by SI was the greatest for the –2237 bp promoter fragment, which exhibited an ≈ 16 -fold increase ($P < 0.0001$) as compared to the SI response of the promoterless (basic) plasmid (Fig. 3, inset). The promoter activities induced by SI were similar for the –1431, –1048, and –461 bp constructs and about 5–7-fold above the promoterless plasmid ($P \leq 0.016$). Deletion of the –461 to –224 bp regions reduced ($P = 0.02$) the promoter activity induced by SI to ≈ 3 -fold above the promoterless plasmid. Upon further deletions the promoter activity induced by SI was statistically similar, albeit lower, for the –192 bp construct, and the –160 bp construct showed no response above the promoterless pGL3 plasmid (Fig. 3, inset).

mAT1/mAT2 Mutation Blunts I Ib Promoter Activation in SI Soleus

Deletion of the region from –181 to –161 bp (encompassing the mAT2 region; Fig. 1) reduced ($P = 0.0008$) the promoter activity as compared to the –1431 bp construct (Fig. 4) and abolished the SI-induced increase in the promoter activity to a level similar to the promoterless control (Fig. 4, inset). Mutation of the mAT2 region within the –1431 bp fragment only modestly blunted the promoter activity; however, the SI-induced increase in promoter activity was decreased ($P = 0.002$; Fig. 4, inset). Mutation of the mAT1 site blunted the promoter activity to a level similar to that of the deletion from –181 to –161 bp and the promoterless control, and decreased to response to SI (Fig. 4 and inset).

DISCUSSION

Seven days of SI induced a marked 60% atrophy of the soleus that was essentially identical to that reported in prior SI experiments.⁶ Atrophy of the soleus was accompanied by a significant increase of the I Ib MHC gene transcription in the soleus of SI rats as evidenced by increases in the I Ib pre-mRNA and mRNA. These results are consistent with prior reports and provide confirmation of a transcriptional control mechanism underlying the phenotype

shift from slow to fast MHC isoforms, consisting of a decreased expression of type I and IIa MHC and concomitant increased expression of IIx and IIb MHC.^{5,6,9} This study demonstrates that the exogenous IIb promoter-reporter activity in skeletal muscle simulates the transcription of the endogenous gene as evidenced by the increases in promoter activity paralleling those of the pre-mRNA and mRNA observed in this study and others.⁹ In contrast, the use of an exogenous IIx promoter construct was not successful in gene transfer experiments in vivo, as its activity did not parallel that of the endogenous gene.¹⁰ Thus, studying the regulation of the IIb MHC promoter in the soleus muscle in response to SI represents a practical model system for investigating the regulatory region(s) that are critical for activating fast MHC gene transcription in skeletal muscle undergoing atrophy and phenotype transition.

Given the increased IIb transcription in the SI soleus, we were interested in localizing the region(s) of the IIb promoter that regulate transcriptional activity by using a series of deletion constructs that were tested by plasmid injection. The greatest activity in both Con and SI muscles occurred for the -1048 bp promoter fragment. The greater response of the -1048 bp fragment than the longer fragments could be indicative of the existence of a negative response element in the region between -1431 and -1048 bp. The deletion of the region between -1048 and -461 bp resulted in a decrease in activity in Con and SI muscles. The activity of the promoter fragment was similar to the basic promoterless plasmid after deletion to -160 bp indicating that control elements responsible for a minimal level of activity reside between the -192 and -160 bp region (Fig. 3).

The -2237 bp promoter had the greatest SI-induced response, whereas the responses of the -1431, -1048, and -461 bp constructs were similar and about 20%–40% of the longest -2237 bp promoter fragment (Fig. 3, inset). The deletions to -224, -192 and -160 bp further reduced the SI response to similar levels, with the -160 bp response entirely absent, i.e., equivalent to the promoterless plasmid (Fig. 3, inset). The data in Figure 3 suggest that regulatory elements within the -192 to -160 bp region are necessary for IIb promoter transcriptional activation in vivo. Therefore, we focused on identifying the role of regulatory elements with this proximal region (-192 to -160) in the context of a longer IIb promoter.

The region between -192 and -147 bp contains two muscle AT-rich regions (mAT1 and mAT2; Fig. 1) capable of binding the transcription factors Oct-1 and MEF-2.⁸ These AT-rich regions are highly conserved among mouse, rat, and human MHC IIb genes. Blast analyses (NCBI website) revealed a 100% homology for the mAT1 site and two single base differences in humans for the mAT2 site. Such highly conserved promoter regions are suggestive of regulatory function and could indicate the presence of *cis* regulatory binding sites that control gene expression. To explore how the mAT2 region controls transcription of the IIb gene during SI, clones of the -1431 bp fragment were designed to contain deletions or mutations within this AT-rich region. Deletion between -181 to -161 (includes mAT2 region) within a -1431 bp construct reduced the promoter activity as compared to the full-length -1431 bp construct (Fig. 4), and also decreased the SI-induced promoter activity (Fig. 4, inset). Mutation of the mAT2 region within the -1431 bp fragment only modestly blunted the promoter activity; however, the SI-induced increase in promoter activity was decreased similarly to the mAT2 deletion (Fig. 4, inset). Because the -181 to -161 bp deletion also removed bases directly adjacent to the mAT1 site (Fig. 1), it was conceivable that transcription factor binding within the mAT1 site was affected by this deletion that included the mAT2 site. Indeed, the mutation of the mAT1 site blunted the promoter activity to a level similar to that of the deletion from -181 to -161 bp and the promoterless control. Thus, deletions and/or mutations of these mAT-rich regions within the -1431 bp promoter all resulted in a lowered and comparable response to SI (Fig. 4 and inset). These results are

consistent with cooperative binding of transcription factors within the mAT1 and mAT2 sites to induce I Ib promoter activity in the soleus of SI rats.

The interaction between the mAT1 and mAT2 regions and the specific transcription factors interacting with these two sites to control induction of I Ib promoter activity in the soleus of SI rats is not entirely clear. Cooperative binding between mAT1 and mAT2 to initiate transcription was reported by Lakich et al.⁸ in C₂C₁₂ myoblasts and C2 myotubes. These authors reported that MEF-2 and Oct-1 transcription factors can bind to these AT-rich regions to influence transcription, but they preferentially bind to mAT1 and mAT2, respectively. Moreover, their mutation analysis indicated that distinct binding sites exist for Oct-1 and MEF-2 within the mAT2 site, and that binding by either factor is essential for MEF-2 to cooperatively bind mAT1 and initiate promoter activity.⁸ Although promoter activity in our in vivo study was evident in the Con and SI soleus for the mAT2 mutation, in which bases were changed within both the Oct-1 and MEF-2 binding domains, the relative SI response was similar to a promoterless plasmid (Figs. 1, 4 inset). Thus, it appears that both the mAT1 and mAT2 sites contain positive response elements for inducing I Ib expression in the SI soleus.

A recent report by Allen et al.¹ further highlights the importance of the mAT2 region for controlling the expression of the three fast MHC iso-forms, i.e., I Ia, I Ix, and I Ib. These authors demonstrated by mutagenesis that as few as 2–4 nucleotide base substitutions in the otherwise well-conserved mAT2 region significantly decreased activity of the I Ia and I Ix promoters in C₂C₁₂ myoblasts, with the I Ib native mAT2 sequence being the most active MHC promoter. In their study, electrophoretic mobility shift assays (EMSA) confirmed that Oct-1 and MEF-2 binding was impaired by substitutions present in the I Ia and I Ix mAT2 regions.¹ In our studies we performed EMSAs (data not shown), but the results were inconclusive and we were unable to determine whether these or additional transcription factors bound to the AT-rich regions. Determining the specific transcription factors that regulate these AT-rich and other promoter regions that control the increased I Ib MHC expression in the soleus during decreased neuromuscular activity in vivo requires further investigation.

One previous study by Wheeler et al.¹⁵ investigated regions of the proximal I Ib promoter for controlling in vivo I Ib expression in fast and slow muscles. In their study they reported that mutations or deletions within the –167 and –89 bp promoter region increased I Ib activity in the soleus of control rats and suggested that negative elements within this region might repress I Ib transcription in slow-twitch muscles. The regions deleted by Wheeler et al.¹⁵ are directly downstream of mAT2 and encompass mAT1 as well as a CA-rich region known as a CA_nG box. In our study we found no differences in basal soleus promoter activity between the intact –1431 promoter fragment and the –1431 mAT1 mutation or the –181 to –161 deletion (Fig. 4), perhaps indicating that the relatively larger deletions created by Wheeler et al.¹⁵ eliminated the binding of multiple and/or cooperative transcription factors that acted as repressors of I Ib expression in the soleus of control rats. There was some indication that our mAT2 mutation, which was a few bases upstream of the Wheeler et al. deletions, increased basal promoter expression. The combined effects of the slightly higher Con and lower SI activities in the mAT2 mutation, however, resulted in a diminished SI response (Fig. 4, inset). Moreover, both the mAT1 and mAT2 promoter regions were important for inactivity-induced expression, as both the deletion and mutations decreased the response to SI (Fig. 4 and inset).

Combined, the results of the present series of experiments provide new insights into the regulation of the fast I Ib MHC gene in vivo. The data show that injection of plasmid constructs that contain the isolated I Ib promoter linked to a reporter gene is a valid tool to

dissect the transcriptional regulation of the IIf gene in rat skeletal muscle. The IIf promoter-reporter construct activity was induced in response to chronic neuromuscular inactivity induced by SI. It is concordant with the endogenous gene response as evidenced by increased pre-mRNA and mRNA in the soleus of SI rats. Further, we have identified regions within the proximal –2237 bp upstream region of the IIf MHC gene that regulate IIf promoter activity in the soleus of Con and SI rats. Deletion analysis provided some indication of a repressor element(s) in the region between –1431 and –1048 bp (Fig. 3). The three regions encompassing –2237 to –1431, –461 to –224 bp, and –192 to –160 bp were involved in regulating the total SI-induced responses of the –2237 bp promoter fragment (Fig. 3, inset). Internal deletions and mutations further indicate that two AT-rich regions in the proximal –192 bp are critical for eliciting IIf MHC transcription in the soleus of SI rats. While prior in vitro studies have reported that the transcription factors MEF-2 and Oct-1 cooperatively bind to these AT-rich regions,^{8,1} the specific transcription factors that induce IIf transcription in the soleus in response to chronic neuromuscular inactivity (SI) remain undefined. Highly active postural muscles like the soleus exhibit considerable plasticity in terms of phenotype shifts in response to decreased neuromuscular activity/loading such as that caused by traumatic injuries to the nervous system or other conditions of reduce physical activity. Defining the regulatory region(s) that control the expression of muscle-specific genes and influence the muscle contractile and metabolic phenotype in response to the chronic state of neuromuscular activity/loading has important implications for developing effective strategies for optimizing neuromuscular function.

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Abbreviations

Con	control
EMSA	electrophoretic mobility shift assay
FLuc	firefly luciferase
MEF-2	myocyte enhancer factor-2
MHC	myosin heavy chain
RLuc	renilla luciferase
SI	spinal cord isolation
SRF	serum response factor
TA	tibialis anterior
TSS	transcription start site

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-224

mAT3 -188 mAT2 -167 -160 mAT 1

CTATCAGATGCTTATAAAGAACCTAGATCATCCCCTGTCAAATTTATTTATAGATGACAAGAAATATTTCTAATT

-137 -103 CARG -94

ATATCCATTACAGCCACAGTCAGTGAATATTGTGTAAAGAGATTGCCAAAATGGTTTTGCCAAGTAGGTCCGC

-74

AGCTAGGACAGCTGAGGTGGCTGCTGTGTTTGCAGAACAGCCTCTATAAAAGTGAAGCTGGGGTGCCTCTGTCC

+1 TGTCTTCCTCAA +13

mAT1 mutation: TATTTCTAcTTA_{ggc}CCATTCAC

mAT2 mutation: TGTC_{cggc}TATTT_{cgcga}GAC

deleted region -181 to -161: TTATTTATAGATGACAAGAAA

FIGURE 1.

Design of the internal deletion and mutations for mAT1 and mAT2 regions within the -1431 bp promoter. Sequence shown above begins at -224 bp relative to the TSS as designated by +1. Bold bases were targeted for mutation; TATAAAA is the TATA box.

FIGURE 2.

Pre-mRNA and mRNA content of soleus muscles in Con ($n = 8$) and SI ($n = 14$) rats. Values are means and SEM. *Significant increase from Con ($P < 0.0001$). Inset shows the relationship between MHC IIb mRNA (y-axis) and pre-mRNA (x-axis) expression in soleus muscles of Con and SI rats ($r = 0.79$; $P < 0.0001$). Line was generated by regression analyses.

FIGURE 3.

Activity of the MHC IIb promoter constructs expressed as a fold-change from the activity of a basic promoterless plasmid. Values are means and SEM ($n = 9-16$ muscles/group/construct). Significant main effects exist between Con versus SI and among the promoter constructs (ANOVA, $P < 0.0001$). Significant interaction between main effects ($P < 0.0001$). †Significantly different from all other constructs ($P \leq 0.0009$). *Significantly different from -2237, -1431, and -1048 bp constructs ($P \leq 0.0008$). @Significantly different from -224 ($P = 0.044$). Inset depicts the response to SI of the test promoter divided by the response to SI of the basic promoterless plasmid. †Significantly different from all other constructs ($P < 0.0001$). *Significantly different from -160 and basic ($P < 0.02$). @Significantly different from -224 and -192 ($P < 0.02$).

FIGURE 4.

Activity of -1431 bp MHC IIb promoter constructs that contain internal deletions or mutations expressed as a fold-change from the activity of a basic promoterless plasmid. Values are means and SEM ($n = 9-16$ muscles/group/construct). Significant main effects for Con versus SI and among the promoter constructs (ANOVA; $P < 0.0001$). Significant interaction between main effects ($P < 0.0001$). *Significantly different from -1431 and -1431 mAT2 mutation constructs ($P \leq 0.0002$). Inset depicts the response to SI of the test promoter divided by the response to SI of the basic promoterless plasmid. *Significantly different from all other constructs ($P < 0.002$).