Effect of unloading on type I myosin heavy chain gene regulation in rat soleus muscle

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Giger, Julia M., Fadia Haddad, Anqi X. Qin, Ming Zeng, and Kenneth M. Baldwin. Effect of unloading on type I myosin heavy chain gene regulation in rat soleus muscle. J Appl Physiol 98: 1185–1194, 2005. First published December 10, 2004; doi:10.1152/japplphysiol.01099.2004.—Slow-twitch soleus, a weight-bearing hindlimb muscle, predominantly expresses the type I myosin heavy chain (MHC) isoform. However, under unloading conditions, a transition in MHC expression occurs from slow type I toward the fast-type isoforms. Transcriptional processes are believed to be involved in this adaptation. To test the hypothesis that the downregulation of MHC1 in soleus muscle following unloading is controlled through cis element(s) in the proximal region of the promoter, the MHC1 promoter was injected into soleus muscles of control rats and those subjected to 7 days of hindlimb suspension. Mutation analyses of six putative regulatory elements within the −408-bp region demonstrated that three elements, an A/T-rich, the proximal muscle-type CAT (β3e), and an E-box (−63 bp), play an important role in the basal level of MHC1 gene activity in the control soleus and function as unloading-responsive elements. Gel mobility shift assays revealed a diminished level of complex formation of the β3e and E-box probes with nuclear extract from hindlimb suspension soleus compared with control soleus. Western blots showed that the relative concentrations of the transcriptional enhancer factor 1 and myogenin factors bind the β3e and E-box elements, respectively, in the control soleus. Western blots showed that the relative concentrations of the transcriptional enhancer factor 1 and myogenin factors were significantly attenuated in the unloaded soleus compared with the control muscle. We conclude that the downregulation of MHC1 in response to unloading is due, in part, to a significant decrease in the concentration of these transcription factors available for binding the positive regulatory elements.

spaceflight or hindlimb suspension (HS) models. This transition also occurs in inactivity models like hindlimb denervation or spinal isolation (SI). The exact cellular mechanisms that regulate this transition are unknown, but transcriptional processes are believed to be involved in this adaptation (16, 24, 25, 28–30, 32, 34).

The possible transcriptional mechanism can be examined at the level of the gene promoter using direct gene transfer in hindlimb skeletal muscles. It has been shown previously that the proximal promoter (−408 bp) of the type I MHC gene is important for reporter expression in normal slow muscle. This proximal promoter contains positive regulatory sites, including two muscle-type CAT (MCAT) binding sites termed β2 (−285/−269) and β3 (−210/−188), and an A/T-rich site (−269/−258) (21, 33). A repressor site, β1 (−311/−301), has also been identified within this proximal region (6, 8).

Models that manipulate load or activity stimuli of hindlimb muscles indicate that the elements identified above are relevant in the regulation of the type I MHC gene. For example, we have recently shown that the most proximal MCAT site, β3e, is required for type I MHC promoter activation in response to the model of functional overload (10). In inactivity models such as denervation and SI, the type I MHC promoter shows drastic decreases in activity levels in soleus muscle. Mutation analysis has demonstrated a significant role of the β3e site in conferring this response in SI soleus (16). Similarly, in the denervated soleus, a β3e mutation in a short −215-bp promoter fragment abolished the response to denervation, but promoter activity was recovered when the mutation was present in the longer −408 fragment. Only until the simultaneous mutation of four elements, β2e, β3e, CACC site, and A/T-rich in the −408 promoter, was the response to denervation clearly abolished (15). These data reveal that a slightly different regulatory mechanism is at work in these two models of inactivity.

Regulatory factors affecting MHC gene expression may differ in the different models because the manipulations that are imposed may stimulate different signaling pathways. The manipulation of denervation involves the severing of the sciatic nerve, which removes neural factors as well as makes the muscle inactive (see Ref. 15). The SI model also eliminates the neuromuscular activity and loading, but the motoneuron-muscle connection remains intact so that potential activity-independent neurotrophic factors are maintained. On the other hand, the suspension model leaves the hindlimb nerves intact so that the muscles can still contract and the hindlimbs can move about, but the load stimuli alone is reduced. We are

IN THE ADULT RODENT SKELETAL muscle, the myosin heavy chain (MHC) exists as several isoforms, consisting of slow type I and fast types IIa, IIx, and IIb. These isoforms have different biochemical properties that are directly related to the speed of muscle contraction and are generally expressed in a fiber-type-specific manner and depend on the functional characteristics of the muscle type. A unique feature of muscle is its ability to adapt to different functional demands through altered expression of contractile components. The expression of the MHC isoforms is sensitive to variations in load, motor nerve input, and thyroid hormone (12–14). The slow-twitch soleus muscle, which is a weight-bearing hindlimb muscle, predominantly expresses the type I MHC isoform. Yet a transition in MHC isoform expression occurs from slow type I toward the fast-type isoforms following unloading conditions, such as in
interested in determining whether or not the regulatory factors and promoter elements that cause the downregulation of type I MHC in response to unloading by HS are the same as those previously identified in other models of type I MHC down-regulation, such as denervation and SI. In response to unloading, the downregulation of the gene may involve a reduction in normal positive transacting factors or an increase in suppressor-type factors.

We have previously examined the type I MHC promoter in control and unloaded soleus muscle (9). The ~3,500-, ~914-, and ~408-bp promoter fragments exhibited an ~40% decrease in reporter activity in response to unloading by HS, but the smaller ~215-bp deletion did not respond to HS, indicating that the response to unloading is conferred within the proximal promoter (9). Evidence exists that certain elements in the proximal promoter may play a role in conferring the response to unloading. Further examination, using GMSAs and Western blot analyses, demonstrated that MCAT elements β2 and β3 showed a decreased level of binding when incubated with soleus extracts of suspended rats.

The experiments described herein test the hypothesis that the downregulation of type I MHC in soleus muscle following HS is controlled through the cis element(s), especially β3, in the proximal region of the gene promoter. In the present study, we comprehensively examined the effects of single and multiple mutations of six cis elements on the activity of the proximal promoter to more precisely determine which cis elements and potential binding factors are relevant in regulating the response to HS. Determining the regulatory mechanism(s) involved in MHC gene expression in response to pathological conditions should enhance our understanding of the cellular signaling factors or pathways that impact muscle phenotype and function and may also shed light on possible restorative interventions.

The findings herein suggest that three elements, A/T-rich, β3, and E-box, are important in the basal activity of the promoter in normal soleus, as well as confer a response to unloading. Further examination, using GMSAs and Western blot analyses, demonstrated that the amount of transcriptional enhancer factor (TEF)-1 and myogenin proteins, which bind the β3 and E-box elements, respectively, are significantly reduced in the soleus extracts of suspended rats, thus generating a profile that resembles that of the fast-twitch plantaris muscle.

METHODS

REPORTER PLASMID CONSTRUCTS

Plasmid construction was as described previously (9). The ~3,500 and all shorter promoter fragments and specific mutations were subcloned by standard procedures into the reporter plasmid, a firefly luciferase expression vector (pGL3 basic, Promega). All type I MHC sequences terminated at +34 from the transcription start site. A 2-kb promoter sequence of the human skeletal α-actin (a gift from S. Swoap, Williams College, Williamstown, MA), linked to a renilla luciferase (Rluc) reporter was used as the reference vector (skeletal α-actin-Rluc) (9). Mutation constructs consisting of base substitutions in the ~408 type I MHC promoter were designed to disrupt binding to the targeted site in the promoter. Custom primers (Invitrogen, Carlsbad, CA) were used for mutagenesis (QuickChange Site-Directed Mutagenesis Kit, Stratagene) using the ~408 wild-type sequence as a template. (See Table 1 for mutation sequences.) The initial report by Thompson et al. (33) that identified pertinent cis elements in the proximal promoter was referred to, in part, as a guide for targeting specific cis elements in the mutation studies. String-based search query using TESS database (URL: http://www.cbil.upenn.edu/teSS) at default settings was also used to identify transcription factors’ binding sites. This type of in silico analysis screens the test sequence, highlights possible matches of the sequence, and gives the percent identity to known consensus sequences. Highlighted matches of interest were then compared with published consensus sequences.

JUSTIFICATION FOR THE USE OF SKELETAL α-ACTIN AS A REFERENCE PROMOTER

To correct for gene transfer efficiency, we chose to use the reporter sequence of the 2-kb human skeletal α-actin, linked to a Rluc reporter to be co-injected as the reference vector. As we have reported previously (9), the activities of the typically selected viral promoters, cytomegalovirus-Rluc and Simian virus-40-Rluc (Promega), were persistently variable, whereas the α-actin-Rluc plasmid correlated well to test plasmid. Ideally, the reference promoter activity should not change in response to the experimental manipulation. However, as a sarcromeric muscle protein, we expect α-actin to be downregulated in response to the stimulus of HS. The activity of α-actin promoter decreased in response to HS, with an average decrease of 54% below control (coefficient of variation = 39%). We feel that dividing the type I MHC promoter-driven firefly activity by α-actin-driven renilla activity is a better conceived method of normalization because it factors out the generalized decrease in transcriptional activity due to atrophic stimuli. Thus any decrease in the type I MHC-Fluc/α-actin-Rluc reporter activity in response to HS is a result of the specific decrease in type I MHC isoform promoter activity.

ANIMAL PROCEDURES

The experiments described herein were conducted for a period of >2 yr. To examine ~15 different promoter constructs in vivo, ~350 female rats (95–110 g) were randomly assigned to normal control (NC) and HS groups (n = 10–15 animals/group). Rats were anesthetized (ketamine-acetromazine-xylazine, 50:1:4 mg/kg) for aseptic surgical and injection procedures. A skin incision was made to expose the soleus muscle. Twenty microliters of PBS containing a mixture of two supercoiled DNA plasmids (1.8 pmol of type I MHC-Fluc test plasmid and 1.8 pmol of control skeletal α-actin-Rluc reporter plasmid) were injected into the muscle using a 29-gauge needle attached to a 0.5-ml insulin syringe. All animals undergoing HS were prepared as such immediately following plasmid injections. The HS model

<table>
<thead>
<tr>
<th>Table 1. Mutation sequences of MHC1 promoter</th>
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<tbody>
<tr>
<td>AP1 Wt 5’ GGGTACGAGGGAGAAGGTCAGGCTAGGCCCCCTTGC 3’</td>
</tr>
<tr>
<td>AP1 mut 5’ GGGTACGAGGGAGAAGGTCAGGCTAGGGCTTGC 3’</td>
</tr>
<tr>
<td>β1e Wt 5’ CTGGTGGACTGGGTG 3’</td>
</tr>
<tr>
<td>β1 mut 5’ CTGGTGGACTGGGTG 3’</td>
</tr>
<tr>
<td>β2 Wt 5’ CGGGCCTTTCAC 3’</td>
</tr>
<tr>
<td>β2 mut 5’ CGGGCCTTTCAC 3’</td>
</tr>
<tr>
<td>A/T-rich Wt 5’ AATGTAAGGGATATTTTTGCTTCAC 3’</td>
</tr>
<tr>
<td>A/T-rich mut 5’ AATGTAAGGGATATTTTTGCTTCAC 3’</td>
</tr>
<tr>
<td>β3 Wt 5’ CATGGCAGCAGGCCCTCTTGTC 3’</td>
</tr>
<tr>
<td>β3 mut 5’ CATGGCAGCAGGCCCTCTTGTC 3’</td>
</tr>
<tr>
<td>E-box Wt 5’ TGGGACTGGGTG 3’</td>
</tr>
<tr>
<td>E-box mut 5’ TGGGACTGGGTG 3’</td>
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</table>

Custom primers sequences (Invitrogen, Carlsbad, CA) used for mutagenesis (QuikChange Site-Directed Mutagenesis Kit, Stratagene) using the –408 wild-type (Wt) sequence as a template are depicted in the 5’ to 3’ direction. The base pairs to be mutated (mut) are underlined in wild-type sequences; the base pair mutations are lowercase, bold, and underlined in mutant sequences. MHC, myosin heavy chain.
employed a tail traction method using a noninvasive casting procedure described by Caiozzo et al. (4). The tail casting included a swivel attachment that was hooked to the top of the cage and allowed the rat to move freely about the cage using only its front legs. Seven days after plasmid injection, soleus muscle tissues were excised following pentobarbital sodium (100 mg/kg) euthanasia and were quick frozen and stored at −80°C until analyzed for reporter activity. Despite the likelihood that only a very small fraction of muscle fibers are transfected, we process the whole soleus muscle to ensure that we harvest all of those fibers that were transfected. Soleus muscles from additional control and 7-day HS rats were harvested for RNA analysis (n = 6 rats/group) and nuclear protein extraction (6–10 muscles pooled/sample). Nuclear extract was prepared from 7-day denervated (Den) soleus muscles (6 muscles pooled/sample). Adult female Sprague-Dawley rats (145 ± 10 g) underwent unilateral hindlimb muscle denervation. Following anesthesia, a skin incision was made in the midthigh region, the sciatic nerve was isolated, and a 2-mm section was removed. After 7 days, the denervated soleus muscles were harvested.

All animals in the study were provided with food and water ad libitum, and all procedures were approved by the Institutional Animal Care and Use Committee.

**Reporter Expression Assay**

Frozen muscle tissues were homogenized in ice-cold passive lysis buffer from Promega, using a glass homogenizer. The homogenate was centrifuged at 10,000 g for 10 min at 4°C. The supernatant was reserved for the luciferase activity assay using Promega’s Dual Luciferase Assay kit, which is designed for sensitive detection of both Fluc and Rluc activities in a single-extract aliquot. Activities were measured as total light output (as measured by a Monolight 2010-C luminometer) per muscle per second and were expressed as relative light units. Background levels, based on luciferase activities of noninjected tissue, were subtracted from the activities of test samples. These experiments were predicated on the assumption that the level of luciferase activity is proportional to the degree of promoter activity.

**Nuclear Extraction of Skeletal Muscle Tissue**

Nuclear protein was extracted from skeletal muscle according to the method described by Blough et al. (3). Briefly, frozen muscle tissue (200–300 mg) was homogenized in 35 ml of buffer 1 (10 mM HEPES, pH 7.5, 10 mM MgCl₂, 5 mM KCl, 0.1 mM EDTA, pH 8.0, 0.1% Triton X-100, 0.2 mM PMSF, 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin, 1 mM DTT). Homogenates were centrifuged for 5 min at 3,000 g at 4°C. The pellets were resuspended in 500–1,000 μl of buffer 2 (20 mM HEPES, pH 7.5, 500 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, pH 8.0, 25% glycerol, 0.2 mM PMSF, 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin, 0.5 mM DTT). Suspension was incubated on ice with intermittent mixing for 30 min and centrifuged for 5 min at 4,000 g at 4°C. The supernatant was transferred to an Amicon 2 ml Centric filter unit (YM-10) (Millipore, Bedford, MA). An equal volume of binding buffer (20 mM HEPES, pH 7.9, 40 mM KCl, 2 mM MgCl₂, 10% glycerol, 0.2 mM PMSF, 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin, 0.5 mM DTT) was added to the filter unit and centrifuged for 30 min at 4,500 g at 4°C. Another 500–1,000 μl were added and centrifuged again for 3-min repeat centrifugation until concentrate achieved the desired volume (1 ml). Protein concentration of nuclear extract was determined using the Bio-Rad protein assay with BSA as a standard. Nuclear extract samples were stored at −80°C.

**GMSA**

GMSAs were used to examine binding of nuclear extract protein to the cis regulatory elements of the rat type I MHC gene promoter. All oligonucleotide sequence probes were purchased from Invitrogen Life Technologies (Carlsbad, CA) and are depicted in Table 2. After strand annealing, the double-stranded probe was end-labeled with [γ-32P]ATP (6,000 Ci/mmol) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). For each binding reaction, 20 μg of a control soleus nuclear extract were preincubated for 10 min at room temperature with 225-ng poly(dI-dC) homopolymer, which was used as nonspecific competitor, in a binding buffer containing 40 mM KCl, 1 mM DTT, 1 mM EDTA, 1 mM MgCl₂, 7.5% glycerol, 0.05% BSA, and 20 mM HEPES, pH 7.9, in a total volume of 20 μl. For competition studies, the preincubation was carried out in the presence of 150× molar excess of either cold self or unrelated type I MHC promoter oligonucleotide (nonspecific). At the end of the preincubation, 100,000 counts/min of labeled wild-type or mutated probes were added. DNA-protein complexes are deemed specific to the probe when complexes [specific complex (SC)] are not formed when nuclear extract samples reacted with radioactive mutant probes or when radioactive wild-type probe is challenged with cold self; in contrast, the SC is unaltered when challenged with cold unrelated sequence. Antibodies to potential nuclear proteins were added to the binding reactions to identify the possible transcription factor(s) comprising the specific DNA-protein complex. In theory, the antibody may bind the factor resulting in the disruption of the DNA-protein complex, causing a depletion of the SC on the gel, and/or it may interact with the DNA-protein complex, causing a retardation of the band’s migration, i.e., a “supershifted” upper band. Therefore, in some cases, 2 μl of antibodies were added: TEF-1 (BD Biosciences), myocyte enhancer factor (MEF)-2C (Cell Signaling), or myogenin (Santa Cruz). Antibodies used but not shown are Sp1, upstream stimulatory factor-1 (Santa Cruz), and Yin Yang 1 (Active Motif). Reactions were then incubated for 30 min at room temperature. At the end of the reaction, 2 μl of loading buffer (20% glycerol, 0.2% bromophenol blue, and 0.2% xylene cyanol) were added, and the reaction mixtures were loaded on a 6% polyacrylamide gel, which was preelectrophoresed at 20 mA/gel for 2 h. Electrophoresis was carried out in 0.5× Tris-borate-EDTA buffer at constant current (30 mA) at room temperature for 2 h. Following electrophoresis, the gels were dried, and the bands were visualized by phosphorimaging (Molecular Dynamics, Sunnyvale, CA).

**MHC RNA Analyses**

**Total RNA isolation.** Total RNA was extracted from preweighed frozen muscle samples using the Tri-Reagent (Molecular Research Center, Cincinnati, OH), according to the supplied protocol by the manufacturer, which is based on the method described by Chomczynski and Sacchi (5). Extracted RNA was precipitated from the aqueous phase with isopropanol, and, after washing with ethanol, it was dried and suspended in a small volume of nuclease-free water. All of the RNA samples used for these analyses were treated with DNase (Promega) to remove any trace of genomic DNA contamination. Following DNase treatment, the RNA samples were reextracted with Tri-Reagent Liquid Sample kit (Molecular Research Center), and the RNA pellet was suspended in nuclease-free water. The RNA concentration was determined by UV absorption using the conversion factor of 40 μg/ml per 1 unit 260 nm optical density, and the samples were stored at −80°C for subsequent analysis with the RT-PCR.

<table>
<thead>
<tr>
<th>Table 2. Gel mobility shift assay sequences</th>
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<tr>
<td>β3c Wt</td>
</tr>
<tr>
<td>β3c mut</td>
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<tr>
<td>E-box Wt</td>
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<td>E-box mut</td>
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</tbody>
</table>

Custom primers sequences (Invitrogen, Carlsbad, CA) used for gel mobility shift assay double-stranded probes are depicted in the 5’ to 3’ direction. The base pairs to be mutated are underlined in wild-type sequences; the base pair mutations are lowercase, bold, and underlined in mutant sequences.
Table 3. Type I MHC-specific RT-PCR primers sequence from the 5’ to the 3’ direction

<table>
<thead>
<tr>
<th>RT primer</th>
<th>Primer 1</th>
<th>Primer 2</th>
<th>Primer 3</th>
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<tbody>
<tr>
<td></td>
<td>GGCTTCAGGGCTTCAACGAGC</td>
<td>AAGGAGGAGGACAGGACG</td>
<td>AAGGCTCTCACAGGATCCT</td>
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<tr>
<td>Primen 1</td>
<td>AAGAAGGAGGACAGGACGAGC</td>
<td>GCCCTGTCCAGGATCCT</td>
<td>GGATCCCTCAGGATCCT</td>
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<tr>
<td>Primen 2</td>
<td>AAGGCTCTCACAGGATCCT</td>
<td>GGATCCCTCAGGATCCT</td>
<td>GGATCCCTCAGGATCCT</td>
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<tr>
<td>Primen 3</td>
<td>GGATCCCTCAGGATCCT</td>
<td>GGATCCCTCAGGATCCT</td>
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The RT primer and primer 2 are complementary to sequences from the 3’ untranslated end of type I MHC mRNA (GenBank accession no. NM_017240). Primer 1 is the common MHC primer used previously (1); primer 3 sequence is from the last intron of the type I (β) MHC gene (GenBank accession no. AY191185.1).

Analyses of type I MHC pre-mRNA. Analysis of pre-mRNA expression can be used to assess a gene’s transcriptional activity as an alternative to the nuclear run-on assay (7, 27). To ascertain transcriptional regulation of the type I MHC gene in NC and HS soleus, the expression of MHC pre-mRNA and mRNA was analyzed using a selective RT-PCR approach. The RT primer was designed to specifically target the type I MHC RNA transcripts (see Table 3 for all primer sequences). Thus, in this approach, only type 1 mRNA and pre-mRNA are reverse transcribed and amplified. For each sample, 1 μg of total RNA was reverse transcribed using Superscript II (Invitrogen) and type I MHC mRNA-specific antisense primer (RT primer in Table 3) in a 10-μl reaction volume. This specific RT reaction was followed by specific amplification of both the mRNA and pre-mRNA. The PCR for amplification of type I MHC mRNA used primers 1 and 2, and a 1-μl cDNA template diluted 40-fold (equivalent to 2.5 μg total RNA) was used. This reaction was carried out for 24 cycles, which resulted in a 546-bp PCR product. PCR to amplify type I MHC pre-mRNA was similar to the above, except that the cDNA dilutions were only fivefold (equivalent to 20 ng total RNA). The primers used were primers 3 and 4, and the number of cycles was raised to 30. This resulted in a 186-bp product. Primer 3 is derived from the last intron of the type I MHC gene. Thus this primer can target only pre-mRNA. The number of cycles and the PCR conditions for each target mRNA were 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 were carried out using an equal amount of non-reverse-transcribed RNA. These reactions turned out negative, thus validating the effectiveness of DNase treatment. PCR products were separated on a 2% agarose gel by electrophoresis, and they were stained with ethidium bromide. The signal quantification was conducted by laser scanning densitometry, as reported previously (36).

Note that, in this procedure, it is not possible to normalize the signal to a reference RNA standard because the RT reaction was selective to type I MHC RNA/pre-mRNA. Accuracy and precision are important for such a determination. To keep the processing variability to a minimum, all of the samples (control and experimental) were processed at the same time, under identical conditions, and used the same premixed reagents to ensure homogeneity. Also, all of the samples were run in duplicates.

Western Blots

Equal amounts of soleus nuclear protein (10 μg) were loaded on a 12.5% acrylamide gel, according to standard protocol (22), and electrophoretically transferred to a polyvinylidene difluoride membrane (Immobolin-P) using 10% methanol, 25 mM Tris, and 193 mM glycine, pH 8.3. The membrane was reacted overnight with a TEF-1 (BD Bioscience) or myogenin (Santa Cruz) anti-mouse monoclonal antibody at 1:500 dilution, and, after washing and reacting with the secondary antibody, the signal was detected using enhanced chemiluminescence (ECL Plus Kit, Amersham), according to the manufacturer’s directions. Signal intensity was determined by laser scanning densitometry (Molecular Dynamics/ImageQuant). Antibody reaction resulted in a specific band matching the band obtained from the positive controls. A431 cell lysate was used as a positive control for TEF-1 (provided by BD Bioscience), and protein isolated from differentiated C2C12 cells was used as a positive control for myogenin and contrasted to that of nonmuscle cell extract, A431, as a negative control.

Statistical Analysis

Statistical analysis was performed by using the Graphpad Prism 4.0 statistical software package. Values are means ± SE. Differences between the means of two experimental groups were assessed by an unpaired, two-tailed t-test. P < 0.05 was taken as the level of statistical significance.

RESULTS

Seven-day Hindlimb Unloading Was Associated With Muscle Atrophy

Seven days of HS resulted in a significant decrease in body weights, and this decrease ranged from 9 to 12% for all of the experiments used in plasmid injection (15 experiments HS vs. NC, n = 10–15 rats/group), with an average of 11% decrease. A more dramatic decrease of 45.6% was measured in the soleus muscle wet weight of suspended rats (32.5 ± 0.7 mg) compared with controls (39.8 ± 0.8 mg), as averaged across all animals used for the study. The normalized soleus weight-to-body weight ratio was 36.3% lower in suspended rats. All of these differences were highly significant (P < 0.0001, n = 150 NC; n = 200 HS), and the decreases were similar among all of the experiments using the different plasmid constructs.

Unloading Caused a Downregulation of Endogenous Type I MHC Expression

Type I MHC is the predominant (~90%) isoform expressed in normal rat soleus muscle (12, 13). Total RNA was extracted from soleus muscles of control (NC) and HS rats, and the
levels of MHC1 mature mRNA and pre-mRNA were compared (Fig. 1). Although there was no significant difference in the mature mRNA levels after 1 wk of HS, there was a significant 41% decrease in the amount of pre-mRNA. This indicates that expression changes due to unloading of soleus muscle are regulated at the transcriptional level.

**Rat Type I MHC Promoter Analysis**

Deletion analysis demonstrated that reporter activity of the −3,500-bp type I MHC promoter, were injected into soleus muscles [−3,500- , −914-, −408-, and −215-bp data have been published previously (9)]. Shaded bars, NC group; open bars, HS group. Promoter activity values represent relative firefly luciferase (Luc) activity determined by the ratio of type I MHC promoter-driven firefly luciferase light units divided by skeletal α-actin-driven renilla luciferase light units (type I MHC-Fluc/sk.α-actin-Rluc). AP, activator protein. Values are means ± SE; n ≥ 8 soleus muscles per each construct per group tested. *P < 0.05, t-test, NC vs. HS.

**Fig. 2. Deletion analysis of type I MHC promoter in soleus muscles of NC and HS rats.** Left: type I MHC promoters with the marked putative regulatory cis elements. Right: bar graph derived from the parent promoter, −3,500 bp (top to bottom), deletion constructs −914, −408, −323, −299, and −215 bp of the type I MHC promoter, were injected into soleus muscles [−3,500-, −914-, −408-, and −215-bp data have been published previously (9)]. Shaded bars, NC group; open bars, HS group. Promoter activity values represent relative firefly luciferase (Luc) activity determined by the ratio of type I MHC promoter-driven firefly luciferase light units divided by skeletal α-actin-driven renilla luciferase light units (type I MHC-Fluc/sk.α-actin-Rluc). AP, activator protein. Values are means ± SE; n ≥ 8 soleus muscles per each construct per group tested. *P < 0.05, t-test, NC vs. HS.

in response to 7 days of HS (9), a reduction that is similar in the same degree as that of the endogenous pre-mRNA. However, the −323-, −299-, and −215-bp promotors showed no difference between HS and NC groups (Fig. 2). Considering the fact that the deletion construct −323, which removed only 85 bp from the 5′ end of −408 fragment, abolished the HS response, we investigated whether this small region contained an important response element(s) (Fig. 3). A 36-bp sequence between −383 and −344 contains several putative sites, mainly including putative binding motifs for activator proteins (AP) 1 and 4.

**Fig. 3. Some distal mutations have no effect on unloading response of reporter activity.** Graph depicts comparison of reporter activity, from top to bottom, a wild-type −408 type I MHC promoter as depicted in Fig. 2, and 408-AP1mut, 408-βe1mut, and 408-βe2mut, mutation promotors in soleus muscles of NC and HS rats. X, Mutated elements. Shaded bars, NC group; open bars, HS group. Activity values represent relative firefly luciferase activity determined by the ratio type I MHC-Fluc/sk.α-actin-Rluc. Values are means ± SE; n ≥ 8 soleus muscles per each construct per group tested. *P <0.05, t-test, NC vs. HS.
A 5-bp mutation, which disrupts the core motif designated AP (−367/−360), was tested in a −408 promoter in NC and HS soleus. Although the AP mutation had significantly less reporter activity than the wild type in NC and HS rats, it still showed HS responsiveness, indicating that this sequence may be a positive regulator of the gene promoter but probably is not a key HS response element.

Other reporter plasmids consisting of the −408 promoter with mutations of putative regulatory elements were individually tested in NC and HS soleus. The promoter activity of the 408-βe2 mutation was similar to that of the wild-type promoter in both NC and HS groups (Fig. 3). The mutation of the “repressor” βe1 caused a 100% increase in activity compared with wild type; however, the βe1 mutation was still responsive to unloading. Mutations of A/T-rich (−269/−258), an MCAT site βe3 (−210/−188), and an E-box (−62/−57) individually tested in the −408 fragment each resulted in a significant decrease in activity of NC soleus, confirming that these proximal elements are positive elements (Fig. 4). The E-box mutation had the most impact on the activity in control muscle, which suggests that it is an important regulatory element. More significantly, the individual mutation of these three elements abolished the response to suspension, indicating that each one may be an unloading-responsive element (Fig. 4).

The significance of the A/T-rich, βe3, and E-box elements in conferring the unloading response was further examined with the creation of double and triple mutants (see Fig. 4). The activity of the double mutation 408-mβe3-A/T-rich was of a similar pattern compared with the single A/T-rich mutant but with the same level of activity in the NC group as that of the βe3 single mutation. The 408-mβe3-E-box double mutant caused a drastic reduction in reporter activity of both control and suspended rats. The triple mutant 408-mβe3-A/T-rich-E-box did not differ much from the 408-mβe3-E-box double mutation, indicating that the additional mutation of A/T-rich had less impact on activity compared with the other two elements. It is apparent that the βe3 and E-box elements contribute most significantly to the regulation of the type I MHC promoter activity, both under control conditions and in response to the unloading stimulus. It should be mentioned that, although the activities of some mutants appear to be increased in response to HS, the increase actually reflects a larger relative decrease in α-actin reporter activity compared with the test promoter. The activities are reported as a ratio, i.e., relative to α-actin, so, in response to HS, the raw values of the mutant −408 promoter activities (numerator) are not actually higher in response to HS but either are unaffected by HS or are only somewhat decreased, yet the α-actin promoter (denominator) is consistently decreased, so the final ratio is higher than for control.

**GMSA**

Mutation analyses of the proximal promoter (Fig. 4) indicated that the βe3 and E-box elements contribute most significantly to the regulation of type I MHC as positive regulatory sites. Therefore, the downregulation in expression in response to unloading may be due to diminished interaction of transcription factors with these *cis* elements, possibly because of lower concentration of transacting factors brought about by the atrophyng process. GMSA was performed as a qualitative assessment to determine whether there was a different banding pattern and/or changes in the intensity of SC(s) between samples of a control and unloaded soleus. Radioactive probes

![Diagram](http://jap.physiology.org/)

**Fig. 4.** The mutation of three elements abolishes the unloading response of reporter activity. Graph depicts comparison of reporter activity, from top to bottom, a wild-type −408 type I MHC promoter as depicted in Fig. 2, and 408-A/T-richmut, 408-βe3mut, and 408-E-boxmut; double mutants 408-A/T-rich-βe3mut and 408-βe3-E-boxmut; and the triple mutant 408-A/T-rich-βe3-E-box in soleus muscles of NC and HS rats. X, Mutated elements. Shaded bars, NC group; open bars, HS group. Activity values represent relative firefly luciferase activity determined by the ratio type I MHC-Fluc/sk.α-actin-Rluc. Values are means ± SE; *n = 8 soleus muscles per each construct per group tested. * *P < 0.05, t-test, NC vs. HS.
corresponding to the βe3 and E-box element sequences (Table 2) were incubated with the nuclear proteins extracted from one control and one unloaded soleus muscle sample, and several DNA-protein complexes were detected (Fig. 5). Specificity of these complexes was determined based on comparison with the pattern obtained using either radiolabeled mutant probe (Fig. 5A, lane 1, or 5B, lane 2) or when the binding with the radiolabeled wild-type probe was challenged with competitors consisting of self, mutant, or unrelated oligonucleotide sequences. The SC1 in the βe3 GMSA (Fig. 5A) was less intense when using the unloaded soleus extract (lane 9) compared with the control sample (lane 2). Compared with lane 2, the addition of an antibody to the MCAT binding protein, TEF-1 (lane 5), disrupted the SC1 complex and resulted in the concomitant appearance of a supershifted upper band labeled as SS. The supershift indicates that the protein(s) comprising the specific protein-DNA complex is antigenically related to TEF-1. A less intense supershifted band also appeared when the TEF-1 antibody was incubated with the unloaded soleus sample (lane 10). The addition of myogenin (lane 7) or MEF-2 antibodies (lane 8) showed no such immunodepletion of the complex or supershift.

Two SCs, SC2 and SC3, were identified using the E-box probe (Fig. 5B). Compared with the control soleus extract (lane 1), the SC2 and especially SC3 were less intense using the unloaded soleus extract (lane 8). Although no supershifted band appeared, the intensity of complex SC2 was partially immunodepleted when the myogenin antibody was added to the control sample (lane 7) compared with lane 1. The intensity of SC2 was only slightly diminished with the TEF-1 antibody (lane 6). Interestingly, the antibodies had little effect when incubated with the unloaded soleus sample (lanes 9–10). Thus the E-box is likely involved in regulation of MHC1 expression in normal muscle and in response to unloading, and myogenin probably binds this site as a positive regulator. In both of the GMSA assays, the intensity of specific bands was attenuated in unloaded samples compared with controls, which indicates that there is either a lower concentration of protein factor available in the unloaded sample or the binding affinity has been altered in unloaded soleus.

Western Blots

Western blot analyses were performed to determine whether the difference between intensity of specific bands of unloaded and control soleus samples and the supershifted bands was due to a decrease in the concentration of transcription factors in the unloaded muscle. Using nuclear protein extracts, the relative concentrations of TEF-1 and myogenin in control, unloaded, and denervated soleus muscles were assessed (Fig. 6). The denervated soleus sample was included to compare HS with another model of soleus atrophy and phenotype shift. Compared with controls, the density of TEF was lower in the unloaded soleus, whereas the density of myogenin was increased in the unloaded soleus. 

Fig. 5. Representative gel mobility shift assays were used to assess the interaction of radiolabeled oligonucleotide probes with 20 μg of nuclear protein from soleus muscle sample. Using rat type I MHC βe3 and E-box radiolabeled oligonucleotides as probes (see Table 2), several specific (SC) and nonspecific binding complexes were resolved. A: βe3 probe binding was examined with extract from a control soleus muscle NC (lanes 1–5, 7–8) and a 7-day unloaded soleus sample HS (lanes 9–10). Binding specificity was analyzed through competition assays by preincubating extracts with 150-fold molar excess of unlabeled oligonucleotides: βe3 (self, lane 3) and nonself (unrelated, lane 4). Only the complex SC1 denotes specific high-affinity complex because it is disrupted by competition with self but not competed by an unrelated (Eboxwt) oligonucleotide and is not detected when incubated with the radiolabeled mutant βe3 probe (lane 1). The addition of a transcriptional enhancer factor (TEF)-1 antibody disrupts the specific band pattern and causes a supershift (SS) (lanes 5 and 10) but has no effect when incubated with only the probe and no extract (lane 6). No disruption is observed when control soleus extracts are incubated with a myogenin antibody (Myog Ab, lane 7) or myocyte enhancer factor-2 (MEF2) Ab (lane 8). B: E-box probe (wtEbox) binding was examined with extract from a control soleus muscle (lanes 1–7) and a 7-day unloaded soleus sample (lanes 8–10). Lane 2, the radiolabeled mutant E-box probe (mEbox); lane 3, unlabeled E-box (self); lane 4, nonlabeled mutant Ebox; lane 5, nonlabeled unrelated (βe3wt); lanes 6 and 10, TEF antibody; lanes 7 and 9, myogenin antibody. Specific high-affinity complexes are arrows SC2 and SC3.
should be noted that significant changes in type I mature
level of type I MHC pre-mRNA after only 7 days of HS. It
differences between NC, HS, and Den groups determined from the above
kDa (15); myogenin is
muscle cells. Molecular weight marker is not shown. The TEF-1 band is
reaction of the positive control sample derived from differentiated C2C12
soleus shifts toward the fast-twitch muscle phenotype.

A sample of control fast-twitch plantaris muscle was in-
cluded to compare the level of these muscle-specific factors in
a typical fast-twitch muscle expressing predominantly type IIb
and Ix MHC against normal and manipulated slow-twitch
soleus. It is not surprising that, compared with the control
soleus, the plantaris muscle contains undetectable levels of
myogenin, as it has been shown to be preferentially expressed
in slow-type muscles (17). The protein expression profile of
TEF-1 and myogenin of unloaded soleus resembles that of the
normal plantaris muscle, supporting the notion that unloaded
soleus shifts toward the fast-twitch muscle phenotype.

**DISCUSSION**

In the present study, we detected significant changes in the
level of type I MHC pre-mRNA after only 7 days of HS. It
should be noted that significant changes in type I mature
mRNA and protein levels have been shown after a longer
experimental period of HS (2 wk) (13). However, we used a
7-day experimental period because we reasoned that tran-
scriptional regulation should precede mRNA and protein changes.
As the precursor for mature mRNA, the pre-mRNA species
signifies the initial step in gene transcription, and, therefore,
with the short experimental time course, we detect relatively
larger differences of pre-mRNA than mature mRNA. The
differences in the level of pre-mRNA in response to HS
validated our reasoning and indicated that a decrease in type I
MHC in HS soleus is regulated at the level of transcription.
This report aimed to determine through promoter analysis which
 cis elements and potential transcription factors are
involved in the load-responsive regulation of type I MHC isoform gene.

Comparisons of promoter cis elements involved in the type
I MHC activity in response to different models show striking
similarities and yet also some key differences. The proximal
MCAT motif, the \( \beta e_3 \) element, is a crucial element in main-
taining promoter activity in the normal, control soleus (this
study and Refs. 15, 16), and it plays a role in the response to
unloading (Fig. 4). The \( \beta e_3 \) element has been shown previ-
ously by our group to be required for upregulation of MHC1 in
the plantaris overload model (10). Moreover, Huey et al. (16)
have shown that the MHC1 promoter containing a \( \beta e_3 \) muta-
tion abolished the response to SI in rat soleus. Although the
\( \beta e_3 \) motif is a key element in conferring the response to
denervation, the additional simultaneous mutations of \( \beta e_3, \)
\( A/T\)-rich, \( \beta e_2 \), and a CCAC in the ~408 promoter were
required to abolish the denervation response (15). The current
analyses examining MHC promoter activity in response to
unloading by HS are similar to the SI and overload models in
that the \( \beta e_3 \) mutation alone can abolish the response compared
with the control state. Yet, in contrast to these other models,
the mutations of the \( A/T\)-rich or the E-box elements also
abolished the response. In fact, mutation analysis indicated that
these three elements are vital to maintain the steady-state level of
MHC1 gene activation in the control soleus.

The significance of the proximal E-box in the regulation of
MHC1 has not previously been examined in vivo, yet the
current report found the E-box to be a very potent cis element
in conferring the expression of MHC1 isoform in adult soleus
muscle. The E-box has been implicated in the regulation of
other muscle-specific genes. It is a significant part of the
conserved positive element motif complexes, FIRE and SURE,
which are involved in troponin I slow-gene regulation (26).
Also, an E-box has been identified as a “hemodynamic load”
element in the \( \alpha M\)-MHC promoter in contracting cardiomyocytes
(37). The mutation of an E-box motif in the MHC2b
promoter caused significant decreases in reporter activity in
soleus and tibialis anterior muscles, and this E-box motif was
proposed to play a role in the response to HS (35).

It should also be noted that the regulation of the MHC1 gene
is more complicated than described in the present study, given
the fact that the ~408 truncations, ~323, ~299, and
~215-bp promoters, were able to abolish the response to HS.
Yet the three elements, \( A/T\)-rich, \( \beta e_3 \), and E-box, that we
identified via mutation analysis as the “unloading” response
elements are within the proximal ~299-bp promoter. It is
likely that some interaction occurs between these very prox-
imal elements and those yet unidentified elements upstream.
We, therefore, examined all mutants in the intact −408 promoter to be confident that the three elements, A/T-rich, βe3, and E-box, in the proximal promoter do indeed impact the basal level of MHC1 gene activity in the weight-bearing control soleus as well as the downregulation of MHC1 gene transcription in response to hindlimb unloading.

It is interesting that our mutation analysis showed that the mutation of any one of the three elements, A/T-rich, and especially βe3 and E-box, was sufficient to abolish the unloading response. One may speculate that a single transcription factor or factor complex must bind all three elements simultaneously to elicit the unloading response, such that the mutation of any one of the identified elements would disrupt the regulation. Moreover, it would appear that every element must be intact because the correct sequence of one element did not rescue the mutation of the other. To pursue the possibility that a single transcription factor or factor complex binds all three elements, GMSAs using sequences of the A/T-rich (not shown), βe3, or E-box elements were performed to qualitatively determine the binding properties of the elements to nuclear protein factors. Possible involvement of the transcription factors, TEF-1 and myogenin, were examined in GMSAs.

The TEF-1 family has been reported to transactivate the type I MHC gene and other muscle-specific genes through binding of the MCAT site (11, 20, 31). Tsika et al. (34) have reported that a nominal TEF-1 antibody supershifted specific binding of the MCAT site (11, 20, 31). Tsika et al. (34) have reported that the myogenin antibody did not react with the SCs of the unloaded soleus extract suggests that myogenin may not be binding to the probe, because of a depletion of the protein or a disrupted binding capacity. Western blot analysis revealed that the relative concentration of the myogenin factor was attenuated in the unloaded soleus compared with the control and denervated muscle. The regulatory pathway involving the E-box site and myogenin appears to differ between the models of unloading and denervation.

We conclude that the downregulation of MHC1 in response to unloading is due, in part, to a significant decrease in the concentration of these transcription factors available for binding the positive regulatory elements. Thus “less” binding at these positive cis elements causes a deactivation of gene transcription. The transcription factor levels in the unloaded soleus resembled those observed in a normal fast muscle, e.g., the plantaris. This similarity, in part, supports the hypothesis that the phenotype transition from slow toward fast MHC that occurs in response to unloading of the weight-bearing slow muscle is conferred through transcriptional processes.

GRANTS

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REFERENCES

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