In vivo regulation of the β-myosin heavy chain gene in soleus muscle of suspended and weight-bearing rats

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Giger, Julia M., Fadia Haddad, Anqi X. Qin, and Kenneth M. Baldwin. In vivo regulation of the β-myosin heavy chain gene in soleus muscle of suspended and weight-bearing rats. Am J Physiol Cell Physiol 278: C1153–C1161, 2000.—In the weight-bearing hindlimb soleus muscle of the rat, ~90% of muscle fibers express the β-myosin heavy chain (β-MHC) isoform protein. Hindlimb suspension (HS) causes the MHC isoform population to shift from β toward the fast MHC isoforms. Our aim was to establish a model to test the hypothesis that this shift in expression is transcriptionally regulated through specific cis elements of the β-MHC promoter. With the use of a direct gene transfer approach, we determined the activity of different length β-MHC promoter fragments, linked to a firefly luciferase reporter gene, in soleus muscle of control and HS rats. In weight-bearing rats, the relative luciferase activity of the longest β-promoter fragment (~3500 bp) was threefold higher than the shorter promoter constructs, which suggests that an enhancer sequence is present in the upstream promoter region. After 1 wk of HS, the reporter activities of the ~3500, ~914, and ~408-bp promoter constructs were significantly reduced (~40%), compared with the control muscles. However, using the ~215-bp construct, no differences in promoter activity were observed between HS and control muscles, which indicates that the response to HS in the rodent appears to be regulated within the ~408 and ~215 bp of the promoter.

β-myosin heavy chain promoter; direct gene transfer; hindlimb suspension; dual luciferase

SKELETAL MUSCLE FIBERS are generally classified as fast glycolytic, fast oxidative, and slow oxidative, which describe the speed of shortening, energy metabolism, and fatigue resistance of the muscle fibers (11, 25). These classifications of muscle fibers are in part related to the type of myosin heavy chain (MHC) that is expressed (24). Typically, there are four MHC isoforms expressed in adult skeletal muscles: one slow type, designated as type I or β; and three fast types, designated as IIα, IIx, and IIB (24). Each isoform has slightly different biochemical properties that impact the shortening properties of the fiber (1). Different MHC isoform(s) are expressed in the various muscle fiber types depending on the functional characteristics of the muscle type. For example, the weight-bearing hindlimb soleus muscle expresses primarily the β-MHC isoform (15), whereas the nonweight-bearing fast-twitch tibialis anterior (TA) muscle expresses a predominance of the IIB MHC isoform (13).

Despite the specific pattern of isoform expression in certain fiber types, there is a high degree of MHC phenotype plasticity that occurs in response to variations in contractile activity, neural input, and thyroid hormone status (2, 6, 12, 29). In the soleus muscle, for example, the expression of β-MHC is responsive to changes in circulating thyroid hormone levels, manipulations in load, and denervation (3, 12, 15). Specifically, when hyperthyroidism or hypothyroidism is induced in the rat, β-MHC mRNA expression in the soleus is downregulated or upregulated, respectively, relative to the euthyroid state in rats (12). Unloading of the weight-bearing soleus muscle by hindlimb suspension (HS) or microgravity causes a reduction in the expression of β-MHC and a shift in the MHC isoform population toward the faster MHC types (3, 15). Likewise, the suppression of contractile activity through denervation results in decreased levels of β-MHC and increased relative levels of IIX and IIAMHCs (12). This plasticity of the β-MHC phenotype is believed to be regulated in part by transcriptional processes (15, 29). However, the specific transcriptional mechanisms by which exogenous signals, such as unloading, influence the expression of the β-MHC gene are not known.

Putative cis-acting elements within the promoter sequence of the β-MHC gene and corresponding transcription factors have been the subject of numerous studies on the regulation of the β-MHC gene (7, 10, 14, 18, 30, 32). Most studies have focused on the highly conserved proximal region of the promoter that contains, in addition to the ubiquitous basal transcription machinery, specific positive and negative regulatory sites (7, 18, 30, 32). It has been suggested by findings from transgenic (18, 32) and muscle cell culture (30) experiments that three positive elements: βe2 or MCAT-binding site (~285/~269); the CCAC box (~245/~233); and the βe3 element (~210/~188) are essential and sufficient for muscle-specific gene activation (18, 30). In addition to these positive motifs, a repressor element that flanks the βe2 element upstream has been identified, and it appears to play a distinct role in β-MHC expression in skeletal and cardiac muscle cells (7, 9, 10). When this repressor element, βe1 (~330/~300), is deleted or mutated, a significant increase in reporter activity is observed (7, 9). The role, if any, that these positive and negative elements in the gene promoter play in the regulation of β-MHC expression in response...
to the loading state of soleus muscle has not been determined.

The main goal of the present study was to establish a model to examine β-MHC gene regulation in an in vivo setting using the approach of direct gene injection. One aim was to verify that the injected β-MHC promoter-linked reporter construct was expressed in a muscle type-specific manner by comparing reporter activity in soleus and TA muscles. Also reported herein is a deletion analysis to determine which segments of the β-MHC promoter sequence are essential for gene expression in the soleus muscle during normal weight bearing, and which are relevant to the altered β-MHC expression in response to HS. The findings derived from this initial study indicate that a significant distal enhancer sequence is present between −3500 and −2500 bp, which is required for full promoter activity. Conversely, the specific response to unloading appears to be regulated through a more proximal region of the promoter, between −408 and −215 bp.

**METHODS**

Design and Sequence of Experiments

The experiments described herein were conducted in three phases. Phase I was designed to verify the application of the direct gene injection technique in the study of β-MHC regulation, including the muscle type-specific expression of the β-MHC promoter-linked reporter. Phase II focused on deletion analysis of the −3500-bp β-MHC promoter to determine the significant promoter fragments involved in the regulation of β-MHC expression. Phase III focused on characterizing the pattern of promoter activity in the context of weight-bearing and HS rats.

For each phase, female Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing 95–125 g were anesthetized (ketamine acepromazine, 10–20 mg/100 g) during all surgical and gene injection procedures. A skin incision was made to expose the muscle of interest, and 20 µl PBS containing an equimolar [equivalent to 10 µg −3500-bp MHC-firefly luciferase (FLuc)] mixture of two supercoiled DNA plasmids was injected into the muscles using a 29-gauge needle attached to a 0.5-ml insulin syringe. To improve plasmid uptake, the muscles were preinjected with 25% sucrose in 20 µl PBS based on the method of Wolff et al. (33).

All experiments were performed for a duration of 7 days, which was verified in pilot experiments to be within the time-course window of maximum reporter expression (33). After 7 days, muscle tissues were excised after pentobarbital sodium (100 mg/kg) euthanasia, and the samples were quick frozen and stored at −80°C. All animals undergoing HS were prepared as such immediately after plasmid injections. The HS model employed a tail-traction method using a noninvasive casting procedure described by Caiazzo et al. (4). The tail casting included a swivel attachment that was hooked to the top of the cage, allowing the rat to move freely about the cage using only its front legs. All animals in the study were allowed food and water ad libitum, and all procedures were approved by the institutional Animal Care and Use Committee.

**Reporter Plasmid Constructs**

The plasmids −3300 to +34 and −215 to +34 β-MHC-CAT were a kind gift from Dr. P. C. Simpson (University of California, San Francisco, CA), and the −3500 to +462 β-MHC-CAT was kindly provided by Dr. K. Ojamaa (North Shore University Hospital, Manhasset, NY). The 2675-bp IIB MHC promoter-linked pGL3 plasmid was a kind gift from S. Swoap (Williams College, Williamstown, MA) (26). The −3500 and −215 β-MHC sequences were subcloned into a firefly luciferase expression vector (pGL3 basic; Promega) (34). Deletion mutations of the 5' end were derived from this long fragment using mapped sites for restriction endonucleases and subcloned by standard procedures into the reporter plasmid. All β-MHC sequences terminated at +34 from the transcription start site. These experiments were predicated on the assumption that the level of luciferase activity is proportional to the degree of promoter activity.

To correct for variation in gene transfer efficiency in the direct injection technique, a plasmid containing a viral promoter, such as the CMV (cytomegalovirus) promoter, linked to a different reporter gene, is typically coinjected with the test plasmid. However, in our hands, the CMV promoter subcloned into a renilla luciferase expression vector (CMV-RLuc; Promega) proved unsatisfactory as a control vector. Although repeated experiments were performed, CMV-RLuc activity was persistently variable (Fig. 1A) and did not reflect transfection efficiency. For example, CMV-RLuc activity was often significantly lower than the test plasmid (−3500 β-MHC-FLuc) activity. Therefore, we chose instead to use the promoter sequence of another muscle sarcomeric protein, the 2-kb human skeletal α-actin (gift from S. Swoap), linked to a renilla luciferase reporter as the control reporter plasmid. Unlike the MHC, skeletal α-actin is the only actin isoform expressed in all types of adult rodent skeletal muscle, and, therefore, is not fiber type specific. Figure 1B shows that the skeletal α-actin reporter plasmid was effective in the normalization of transfection efficiency because the level of firefly luciferase activity correlated with the level of renilla luciferase activity in control muscles. Although it is expected that as an integral muscle protein, skeletal α-actin expression may be decreased somewhat in response to HS-induced atrophy, we regard it to still be useful as a means for correcting for transfection efficiency. The reduction in β-MHC promoter expression in response to HS-induced atrophy was normalized relative to the response of the actin promoter. This procedure enabled us to compare the activities of the different β-MHC fragments in response to both weight-bearing and unloading states.

**Reporter Expression Assays**

Frozen muscle tissues were homogenized in ice-cold 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 the Promega dual luciferase assay kit, which is designed for sensitive detection of both firefly and renilla luciferase activities in a single extract aliquot. Activities were measured as total light output (as measured by a Monolight 2010C luminometer) per muscle per second and were expressed as relative light units (RLUs). Background levels, based on luciferase activities of noninjected tissue, were subtracted from the activities of test samples. In this assay system, RLU activity was directly proportional to the amount of tissue aliquot analyzed.

**Gel Mobility Shift Assay**

Isolation of skeletal muscle nuclei. Skeletal muscle nuclei were isolated according to the method described previously.
Nuclei isolated from soleus muscles (5–6 muscles were pooled per sample) were stored at −80°C in a nuclei storage buffer consisting of 25% glycerol, 1 mM dithiothreitol, 20 mM Tris (pH 7.8), 1 mM EDTA, 1 mM MgCl2, 0.2 mM phenylmethylsulfonyl fluoride, 2.5 µg/ml aprotinin, and 2.5 µg/ml leupeptin for subsequent nuclear extraction. A 30-µl aliquot was saved for determining the DNA content.

DNA determination. DNA concentration in the total homogenate as well as in the nuclei suspension was determined by fluorometry using a minifluorometer (TK0100; Hoeffer Scientific, San Francisco, CA). Bis-benzimide H-33258 was used as the fluorescent dye (20), and calf thymus DNA was used as a standard.

Nuclear extraction. Nuclei material designated for extraction (equivalent to 300 µg DNA) were pelleted down by centrifugation at 2,000 g for 5 min. The supernatant was discarded, and the nuclei were suspended in 300 µl of nuclei storage buffer (see Isolation of skeletal muscle nuclei contain- ing 0.42 M KCl, and fresh protease inhibitors were added. After 30-min incubation on ice with gentle agitation, the nuclei were pelleted down by centrifugation at 5,000 g for 5 min, and the supernatant was discarded. DNA concentration in the total homogenate as well as in the nuclei suspension was determined by fluorometry using a minifluorometer (TK0100; Hoeffer Scientific, San Francisco, CA). Bis-benzimide H-33258 was used as the fluorescent dye (20), and calf thymus DNA was used as a standard.

Gel mobility shift assay. Gel mobility shift assays were used to examine and quantify binding of nuclear extract protein to the β2 cis-regulatory element of the rat type I MHC gene promoter (30). This approach allows one to detect changes in transacting factor content and/or changes in pattern of shifted bands resulting from binding of one or more factors.

β2 oligonucleotides were purchased from GIBCO, and the wild-type sense strand consisted of the following sequence: 5′-AGTGA GCTGTGGAATGT AAGGGAT-3′. After strand annealing, the double-stranded probe was end labeled with [γ-32P]ATP (6,000 Ci/mmol) using T4 polynucleotide kinase (Promega). For each reaction, 10 µl of nuclear extract were preincubated for 10 min at room temperature with 6 µg poly(dI-dC) homopolymer used as nonspecific competitor, in a binding buffer containing 60 mM KCl, 1 mM DTT, 1 mM EDTA, 1 mM MgCl2, 0.2 mM phenylmethylsulfonyl fluoride, 2.5 µg/ml aprotinin, and 2.5 µg/ml leupeptin for subsequent nuclear extraction. A 30-µl aliquot was saved for determining the DNA content.

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Muscle type-specific expression of the β-MHC promoter. Our initial studies confirm that the method of direct gene injection is a useful approach to examine β-MHC gene regulation in skeletal muscle by demonstrating that the promoter-linked reporter, −3500 β-MHC-Fluc, was expressed in a muscle fiber type-specific pattern similar to that of endogenous β-MHC mRNA expression (15). Figure 2 (top) shows that the level of activity of the −3500 β-MHC-Fluc was high in the control soleus muscle. The fast-twitch muscle TA also expressed the reporter gene, but at a level ~3% of the level observed in soleus, similar to the endogenous β-MHC mRNA expression in TA muscle (13). As an additional control to ensure that the luciferase expression in muscle was due to the specificity of the β-MHC promoter, we injected the basic pGL3 reporter plasmid, which is the same as the test plasmid but does not contain the β-MHC promoter sequence fragment. The activity of basic pGL3 in soleus muscle was just above background levels and measured 0.75% of the activity of the −3500-bp promoter-driven reporter (data not shown).

Comparison of β and IIB MHC promoter activities in fast- and slow-twitch muscles. Muscle fiber type specificity of two MHC isoforms, β and type IIB, was demonstrated using the direct gene transfer technique in soleus and TA muscles (Fig. 2, bottom). When a plasmid consisting of a 2.6-kb fragment of the IIB promoter fused to a renilla luciferase reporter (IIB-RLuc) was simultaneously injected with the −3500 β-MHC-Fluc plasmid into the soleus and TA muscles, a clear pattern of tissue-specific expression was exhibited. In the soleus muscle, the −3500 β-MHC-Fluc activity was significantly higher (140-fold) than that of the IIB-RLuc activity, and this pattern of expression was reversed such that in the TA muscle, the IIB-RLuc activity was much greater (37-fold) than the −3500 β-MHC-Fluc activity. The fact that both MHC isoform promoters were activated in a characteristic pattern in the slow- and fast-twitch muscle suggested that there was no inherent difference in the ability of the two muscle types to express both reporter constructs.

Collectively, these results indicate that the pattern of −3500 β-MHC-Fluc expression parallels that of the endogenous β-MHC gene expression (shown elsewhere [15]), and thereby confirms that the direct gene injection technique is useful for studying β-MHC regulation in rodent skeletal muscles. Moreover, this method permits deletion and site-directed mutation analysis of the β-MHC promoter in the context of an in vivo setting.

Deletion Analysis of β-MHC Promoter Activity in Weight-Bearing Animals

Deletion mutations of the β-MHC promoter were incorporated into the Fluc reporter plasmid to determine the minimal sequence length that is essential for the activation of the β-MHC gene under normal conditions. Four fragments: −2500, −914, −408, and −215, of the −3500-bp β-MHC promoter, were examined in control soleus muscle (Fig. 3). Although all of these deletion fragments were sufficient to activate reporter activity in control soleus muscle, the Fluc activity of the deletions was reduced significantly to only 20–40% of the −3500 β-MHC-Fluc activity. Examination of the deletion fragments revealed no significant differences in reporter activity between −2500, −914, and −408 β-MHC-Fluc constructs. However, the activity of the −215 β-MHC-Fluc decreased 35% relative to the −408 β-MHC-Fluc construct. This decrease in activity was not altogether surprising in light of the fact that previous investigators have reported the existence of...
several positive regulatory elements upstream of −215 (18, 30, 32). In fact, the −215 deletion results in the elimination of two of these elements, the βe2, or MCAT-binding site, and the CCAC box. Overall, the deletion analysis data indicate that the region between −3500 and −2500 bp contains an enhancer sequence that is required to induce full activation of the reporter gene. The proximal region of the promoter also contains an important positive regulatory sequence(s) between −408 and −215.

Activity of the β-MHC Promoter in Response to Hindlimb Unloading

We are interested in understanding the role that weight-bearing activity plays in the transcriptional regulation of the β-MHC gene in soleus muscle. It has been shown previously that when the normal load-bearing stimulus was removed by HS, β-MHC mRNA and protein levels were downregulated in the soleus, and a shift toward the fast MHC isoforms was observed (15). To determine if a “load-responsive” region exists in the β-MHC promoter sequence, deletion mutants of this promoter were injected in the soleus muscles of control and HS-treated rats, and FLuc reporter activities were compared. Significant differences between the control and HS groups in FLuc activity of deleted promoters would infer that a particular fragment is responsive to unloading. Relative to control rats, there was a 46% decrease in −3500 β-MHC-FLuc activity in soleus muscle after 1 wk of HS (Fig. 4). Reporter constructs containing the −914, −408, and −215 deletion mutants were also tested in soleus muscles of control and HS groups. In spite of lower activity levels of −914 β-MHC-FLuc and −408 β-MHC-FLuc of the control group, compared with −3500 β-MHC-FLuc, a significant HS effect was still observed (Fig. 4). The relative degree of reduction of luciferase activity by HS was similar with the three promoters: −3500, −914, and −408; however, there was no difference between the control and HS groups using the −215 β-MHC−FLuc construct. No difference between the two groups suggests that perhaps a load-responsive element was eliminated during the creation of the −215 deletion construct. Therefore, the sequence between −408 to −215 bp appears to be a load-responsive region.

It should be mentioned that the attenuation of reporter expression in response to HS was associated specifically with the β-MHC promoter. The inset graph of Fig. 4 depicts an increase in the type IIB MHC promoter-linked FLuc activity after HS. This result reflects the antithetic plasticity of certain MHC mRNAs in response to unloading (15), and confirms that the decrease of β-MHC is not merely the result of a generalized depression in transcriptional activity by the HS intervention, but is a specific effect due to muscle unloading.

Gel Mobility Shift Assay Comparing Weight Bearing and Unloaded Soleus Muscle Nuclear Extracts

Considering that the relative activity of the −215 β-MHC-FLuc appears to be unresponsive to HS, one may postulate that either or both of the two positive regulatory elements, C-rich and βe2 motifs, that were eliminated by the −215 deletion may be involved in the HS response. As this was an initial examination of β-MHC gene regulation, we chose to focus first on the βe2 element rather than the C-rich box on the basis of two findings. First, other investigators have reported that the enhancer activity of the C-rich motif appears to be more relevant in cardiac myocytes than in skeletal muscle cells, whereas the βe2 appears to be necessary for a robust activation response in both cell types (30). Second, the enhanced interaction between the βe2

Fig. 3. Deletion analysis of β-MHC promoter in soleus muscle of normal control rats. Derived from −3500-bp β-MHC promoter sequence, deletion fragments −2500 bp (n = 8), −914 (n = 7), −408 (n = 14), and −215 (n = 14) were subcloned into FLuc reporter plasmid. Each deletion construct was injected in an equimolar amount relative to parent plasmid construct (−3500 bp; n = 15), and coinjected with α-actin-RLuc control plasmid. Activity is expressed as β-MHC-to-skeletal α-actin ratios. Values are means ± SE. *P < 0.05 vs. −3500 β-MHC activity, as determined by ANOVA. †P < 0.05 −408 vs. −215 (t-test).

Fig. 4. Deletion analysis in soleus muscles of hindlimb suspended (HS) and weight-bearing (NC) rats. Reporter activities of deletion fragments: −914 (n = 12 NC, 14 HS), −408 (n = 14, 18), and −215 (n = 17, 17), and of parent promoter construct, −3500 (n = 15, 22), were compared in normal control (NC) and HS rats. Activity is expressed as β-MHC-to-skeletal α-actin ratios. Inset depicts relative reporter activity of IIB MHC-FLuc in soleus of NC and HS rats. Increase in IIB MHC-FLuc activity reflects shift that occurs in MHC isoform expression in response to unloading, as has been reported previously (15). All values are means ± SE. *P < 0.05 NC vs. HS (t-test).
sequence and nuclear proteins extracted from pressure-overloaded heart tissue suggested that this element plays a regulatory role in a load-induced response (28).

A gel mobility shift assay was performed that examined the specific interactions of the \( \beta e2 \) element with nuclear proteins extracted from the soleus muscle of control and HS groups (Fig. 5). Two retarded bands, labeled A and B, were competed out by an unlabeled \( \beta e2 \) probe, and thus were indicative of specific interactions between nuclear proteins and the \( \beta e2 \) element. As determined by densitometric analysis, there was no difference in the specific binding pattern between the control and HS nuclear extracts. These results suggest that \( \beta e2 \) is probably involved in \( \beta \)-MHC activation to the same degree under both conditions of weight bearing and unloading and does not play a specific role in the response to HS.

**DISCUSSION**

It is well known that skeletal muscle fibers undergo changes in both mass and MHC phenotype as an adaptive response to different contractile activity demands. What is not known is the specific mechanism(s) involved in establishing the MHC phenotype under normal conditions and during an adaptation response. In the present report, we established an in vivo gene injection model to examine the regulation of \( \beta \)-MHC gene expression in normal and unloaded rodent soleus muscle. Examination of \( \beta \)-MHC promoter-linked reporter activity enabled us to ascertain which subregions of the promoter sequence are essential in the regulation of \( \beta \)-MHC gene expression in a physiological context of weight bearing.

Deletion analysis revealed that the upstream sequence between \(-3500\) and \(-2500\) bp was necessary for full activation of the \( \beta \)-MHC promoter in soleus muscle. The presence of an upstream enhancer in the region between \(-3500\) and \(-2500\) bp has been proposed in our recent report on the \( \beta \)-MHC promoter activity in rodent cardiac muscle (34). Deletion constructs of \( \beta \)-MHC promoter were injected into the left ventricle of rats, and the pattern of reporter activity that was observed was similar to that in the soleus (Ref. 34 and Fig. 3). Evidence that an enhancer exists in the upstream region was revealed through a mutation experiment (34). A three base substitution in the prox-
nal region of −408 promoter construct completely eliminated promoter activity in ventricular samples; however, when this same mutation was tested in the −3500 promoter, some activity (10%) was recovered. These data indicate that the sequence(s) in the upstream promoter is a strong enhancer in that it accounted for about 70% of the promoter activity, and it also compensated for the loss of an essential downstream element (34).

The upstream region of the β-MHC promoter has been virtually unexplored as a potential regulatory region. Sequence analysis reveals that there are several sites that are homologous to MCAT elements, basic helix-loop-helix motifs (N-box and E-boxes), and NF-AT (nuclear factor of activated T cells) binding site motifs in this region. These potential upstream regulatory elements have not yet been specifically examined. The NF-AT factor holds particular interest as a potential regulator. Recently, NF-AT was shown to be involved in muscle phenotype regulation, specifically, in the expression of slow isoforms, sTnI and myoglobin (5). NF-AT activation is regulated by calcineurin, a phosphatase enzyme that is dependent on intracellular Ca2+ concentration. The authors assert that the neural activity pattern typical of slow-twitch muscle nerves sustains a level of intracellular Ca2+ that maintains calcineurin activity and ultimately the NF-AT factor, in an activated state. NF-AT, once activated via a dephosphorylation mechanism, translocates to the nucleus and interacts with a binding site in the genes of sTnI and myoglobin. These findings implicate the NF-AT factor as a possible link between neural activity and the expression of slow isoforms in skeletal muscle.

In addition to β-MHC gene regulation in normal soleus, we are also interested in understanding the mechanism that underlies the shift in MHC expression in response to HS. Our research group has previously demonstrated that after HS, there is a significant decrease in the level of β-MHC mRNA and a concomitant increase in IIx and IIb MHC mRNA in soleus muscle (15). Likewise, in the present report, the −3500 β-MHC promoter activity was reduced, and the IIb MHC promoter activity was increased in HS soleus, compared with control. Deletion analysis revealed that even though shorter fragments of the β-MHC promoter generated less reporter activity than the −3500 β-MHC-FLuc, an HS effect was still observed with all fragments except the −215 β-MHC-FLuc construct. Therefore, the HS response appears to be conferred between −408 and −215 bp of the proximal region of the promoter. In fact, this same region appears to harbor positive regulatory element(s) essential to normal β-MHC gene expression, because, in control soleus, there was a significant downregulation of activity when the sequence between −408 and −215 was eliminated (Fig. 3).

Other investigators, using muscle cell culture and transgenic mouse models, have found that the proximal region is sufficient to confer muscle-specific expression (18, 30, 32). As previously mentioned, numerous positive regulatory elements: βe2, βe3, and CCAC, have been identified in skeletal and cardiac cell culture studies (30). The βe2 element (−285 to −269) contains a 12-bp sequence that is identical to the simian virus 40 enhancer element, AP5/GTII (30), and within which the muscle-specific MCAT motif is harbored. Mutation analysis indicates that the interaction of βe2 and transcriptional enhancer factor 1 (TEF-1), the MCAT-binding protein (8), is essential for β-MHC expression in cardiac and skeletal cells (30). The CCAC box (−250 to −230) represents a binding site for the Sp1 transcription factor. Although this is an ubiquitous factor, it has been shown to act cooperatively with the βe2 in the activation of β-MHC gene in cardiomyocytes (30). The βe3 element (−210 to −188) is also a recognition site for TEF-1 (16), and deletion analysis indicates that it is essential for β-MHC gene activation by α-adrenergic stimulation or protein kinase C activation in cardiomyocytes (17). However, in vivo studies of β-MHC gene regulation are inconsistent with the findings from the cell culture experiments. In a transgenic mouse model, β-MHC promoter-linked CAT activity was still maintained when any of the three βe2, CCAC, or βe3 elements were mutated (18). However, when all three elements were mutated simultaneously, there was a significant reduction in CAT activity. Therefore, these results indicate that any of the three elements are dispensable, but all three are needed for full gene activation. Yet, another transgenic study concluded that the simultaneous mutation of all three elements did not abolish the β-MHC gene activation in response to functional overload in the plantaris muscle (31).

It should be mentioned that these disparities between the results from muscle cell cultures and transgenic mice models may stem from the various limitations inherent in these model systems. There is evidence that the pattern of MHC gene expression is dependent on loading state as well as the presence of the nerve (23), and these physiological influences cannot be effectively mimicked in culture. Although these influences would be maintained in a transgenic mouse model, this time- and labor-intensive approach is largely limited to only one species thus far. Moreover, due to the inherent problems in controlling the copy number of transgenes, interpreting data is difficult and deletion analysis is troublesome. For example, the level of reporter activity is dependent on the transgene copy number, which varies in each animal. Therefore, the validity in assuming that different reporter activities are attributable to different deletion fragments could prove problematic. In fact, some animals with a relatively high β-MHC transgene copy number exhibited flawed gene regulation by inappropriately expressing considerable levels of the β-MHC transgene in the adult mouse ventricle (19). Our in vivo gene injection technique has the advantage that it can be performed on rats, the species in which this research group has established experimental models and has extensively characterized MHC isoform expression in different muscle types. Moreover, the genes can be injected in any specific muscle at any time during a given experimental period, and coindexing a control plasmid normalizes the plasmid uptake...
and relative expression of the transferred gene, making the deletion/mutation analyses relatively easier to interpret.

Our results indicate that the HS response is most likely conferred within the −408- and −215-bp fragment. The possibility that the aforementioned positive regulatory elements play a role in this regulation was considered. The CCAC box (−245 to −233) was not specifically examined in this current paper because previous evidence strongly suggests that this element is pertinent only in cardiac myocytes (30). However, this does not preclude its possible relevance in the regulation of β-MHC expression during an unloading response, and its role, if any, should be considered in the future. Our gel mobility shift assay revealed that the βe2 sequence (−285 to −269) did interact with proteins from control and HS soleus nuclear extracts, but there was no difference in the degree of binding between the two groups. Therefore, although the βe2 element may enhance the promoter activity in normal soleus, we concluded that it was not specifically involved in the regulation of the HS response. The influence of the βe3 element (−210 to −188) was considered irrelevant because it is contained within the −215 bp. It is of interest to note that McCarthy et al. (21) reported that the proximal 600-bp sequence of the mouse β-MHC gene promoter was sufficient to direct an HS response in a transgenic mouse model. Moreover, they found that the HS effect remained unchanged despite the simultaneous mutations of the βe2, C-rich, and βe3 elements within both 5600- and 600-bp promoter sequences (21). Therefore, their results imply that the three elements may not be involved at all or may require the interaction with some other element(s) within this subregion of the β-MHC promoter to confer the HS response.

Within the −408- and −215-bp sequence, a putative repressor element termed βe1 (−330/−300) has been identified (7, 9). The repressor activity of βe1 was exposed through deletion analysis experiments using the human β-MHC gene in skeletal and cardiac muscle cell culture and rat heart gene injection models (7, 9). It was found that when the βe1 element was deleted or mutated, a large increase in CAT expression was detected (7, 9). It is conceivable that a repressor signal protein is upregulated in soleus muscle in response to unloading and then binds to the βe1 site in the β-MHC promoter, thus suppressing β-MHC gene expression. In fact, a recent study from McCarthy et al. (22) identified two proteins (50- and 52-kDa) derived from soleus muscle nuclear extracts of suspended rats that show highly enriched specific binding activity with a human βe1 sequence probe (equivalent to −322 to −301 bp of the rat β-MHC βe1 sequence) in electrophoretic mobility shift assays. In light of all of the findings at this point, we would like to test a −299 β-MHC deletion construct, as well as a −408 β-MHC construct harboring a βe1 mutation in our HS model. As both constructs will in effect hinder the signal protein(s) from binding to the βe1 site, we would be able to ascertain whether the βe1 element is essential to confer the HS response.

Gel mobility shift assay experiments comparing binding activity of the βe1 sequence with nuclear extracts from soleus muscle of control and suspended rats are also planned.

In summary, we have established a working model, using the gene injection technique, to examine the regulatory role of specific promoter regions of the β-MHC gene in vivo. Initial results indicated that the upstream sequence of the β-MHC promoter was necessary for full promoter activity. However, the proximal sequence was sufficient to confer promoter activation in soleus muscle tissue of both normal and HS rats. Specifically, our findings indicate that a conceivable HS-responsive site may exist in the −408- to −215-bp subregion.

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REFERENCES


