An E-box within the MHC IIB gene is bound by MyoD and is required for gene expression in fast muscle

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Wheeler, Matthew T., Emily C. Snyder, Melissa N. Patterson, and Steven J. Swoap. An E-box within the MHC IIB gene is bound by MyoD and is required for gene expression in fast muscle. Am. J. Physiol. 276 (Cell Physiol. 45): C1069–C1078, 1999.—The myosin heavy chain (MHC) IIB gene is selectively expressed in skeletal muscles, imparting fast contractile kinetics. Why the MHC IIB gene product is expressed in muscles like the tibialis anterior (TA) and not expressed in muscles like the soleus is currently unclear. It is shown here that the mutation of an E-box within the MHC IIB promoter decreased reporter gene activity in the fast-twitch TA muscle 90-fold as compared with the wild-type promoter. Reporter gene expression within the TA required this E-box for activation of a heterologous construct containing upstream regulatory regions of the MHC IIB promoter linked to the basal 70-kDa heat shock protein TATA promoter. Electrophoretic mobility shift assays demonstrated that mutation of the E-box prevented the binding of both MyoD and myogenin to this element. In cotransfected C2C12 myotubes and Hep G2 cells, MyoD preferentially activated the MHC IIB promoter in an E-box-dependent manner, whereas myogenin activated the MHC IIB promoter to a lesser extent, and in an E-box-independent manner. A time course analysis of hindlimb suspension demonstrated that the unweighted soleus muscle activated expression of MyoD mRNA before the de novo expression of MHC IIB mRNA. These data suggest a possible causative role for MyoD in the observed upregulation of MHC IIB in the unweighted soleus muscle.

somatic gene transfer; transcription; myosin heavy chain; hindlimb suspension

DIFFERENCES IN SKELETAL muscle contractile characteristics are the product of differences in gene expression among muscle fibers (2, 30). Chief among these proteins is the heavy chain protein of myosin (MHC). In mammalian skeletal muscle, nine different MHC isoforms have been identified, four of which are expressed in adult skeletal muscles of the rat (2). These are, in order of increasing contractile velocity, type I, type IIA, type IIX, and type IIB.

Although contractile and metabolic characteristics arise during development, they are not fixed in adult muscle; rather, fiber phenotype changes in response to altered hormonal environments, contractile activity, myotrophic factors, and other influences (2, 30). In particular, the unweighting associated with hindlimb suspension or microgravity induces specific slow muscles of the hindlimb, e.g., soleus and vastus intermedius, to undergo a slow to fast fiber-type conversion, with de novo expression of the MHC IIB isoform mRNA in the soleus muscle (2, 7, 16, 24). Although the course of skeletal muscle adaptations in fiber-type diversity have been well characterized and defined, the regulatory mechanisms that drive fiber-type specific expression of genes are largely unknown. With the use of nuclear runons, transcriptional processes have been shown to regulate expression of some members of the MHC gene family, including the MHC IIB gene, cardiac α-MHC, and type I/β-MHC (1, 4, 25, 28). Furthermore, transcriptional studies of the MHC IIB promoter utilizing somatic gene transfer have determined that only the proximal 295 bp of the MHC IIB promoter are required to drive reporter gene differentially in fast- and slow-twitch muscle (24).

Numerous elements that have been shown to be required for muscle-specific expression of many genes reside within the proximal 295 bp of the MHC IIB promoter. These include AT-rich regions, a CArG region, and two E-boxes, which serve as binding regions for members of the MEF2 family of proteins (8), serum response factor (19), and myogenic regulatory factor (MRF) family of transcription factors, respectively (22). Among the members of the MRF family, including MyoD, myogenin, Myf-5, and MRF-4, two have been shown to selectively accumulate in muscles of different fiber type: MyoD in fast-twitch muscle and myogenin in slow-twitch muscle (10, 11, 29). The pattern of MyoD distribution changes with corresponding changes in the pattern of fast and slow fiber-type distribution after thyroid hormone treatment or cross-reinnervation (11), suggesting a potential regulatory role of MyoD in transition of adult muscle phenotype.

Using somatic gene transfer, we found that the proximal E-box of the MHC IIB promoter was required for high level of reporter gene activity in vivo. Mutations within the E-box that decreased the activity of the MHC IIB promoter also disrupted binding of nuclear proteins to this region in an electrophoretic mobility shift assay. This mutation also decreased the preferential activation of the MHC IIB promoter by MyoD in cell culture. Finally, we found that the de novo expression of the MHC IIB mRNA in the soleus in response to unweighting was preceded by an induction of expression of the MyoD mRNA, with no change detected in myogenin mRNA. These data suggest that MyoD is a good candidate for the regulation of MHC IIB gene expression in response to altered physiological demands placed on adult skeletal muscle.

EXPERIMENTAL PROCEDURES

Generation of deletion constructs. PCR was utilized to generate both deletion and mutation constructs (Fig. 1 and

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For generation of deletion constructs, 5’-primers were designed such that each contained an internal Kpn I site and annealed to the positions as described in Table 1. PCR sewing was employed to generate internal deletion constructs or mutations as described previously (21). The final PCR products were cloned into pGL3basic (Promega). All clones generated by PCR were checked for fidelity by sequencing of both strands. Heterologous promoters were made through ligation of a 264-bp PCR product cloned from either pGL3IIB0.3 or pGL3IIB0.3MUTE upstream of the basal 70-kDa heat shock protein (HSP70) TATA box (23).

Somatic gene transfer via intramuscular DNA injections. All plasmids were purified using Qiagen columns and resuspended in filter-sterilized PBS. DNA concentrations were quantified through spectrophotometry at 260 nm. Each firefly luciferase reporter plasmid was mixed with an equal quantity of pRL-CMV and directly injected into TA muscle (100 µg of each construct) or soleus muscle (50 µg of each construct). Number of successful injections for each construct (n) is listed next to each horizontal bar. After 1 wk, injected muscles were analyzed for firefly and Renilla luciferase activities. Data are presented as means ± SE.

Tables 1 and 2). For generation of deletion constructs, 5’-primers were designed such that each contained an internal Kpn I site and annealed to the positions as described in Table 1. PCR sewing was employed to generate internal deletion constructs or mutations as described previously (21). The final PCR products were cloned into pGL3basic (Promega). All clones generated by PCR were checked for fidelity by sequencing of both strands. Heterologous promoters were made through ligation of a 264-bp PCR product cloned from either pGL3IIB0.3 or pGL3IIB0.3MUTE upstream of the basal 70-kDa heat shock protein (HSP70) TATA box (23).

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An intraperitoneal injection of a cocktail containing (in mg/kg body wt) 25 ketamine, 1 acepromazine, and 5 xylazine was used to anesthetize young Sprague-Dawley rats (under 2 mo) with an average weight of 100 g (range = 70–150 g). Injections into the tibialis anterior (TA) and soleus muscles were performed as described previously (24). One hundred micrograms of each construct were injected directly into the TA, while 50 µg of each construct were injected into the soleus muscle. Animals were killed 7 days after injection.

Protein isolation from muscles. A Polytron homogenizer was used to homogenize each muscle sample in 2 ml of a buffer containing 100 mM Tris base, pH 7.8, 1 mM EDTA, 1 mM dithiothreitol (DTT), 2 µg/ml aprotinin, 5 µg/ml leupeptin, 170 µg/ml phenylmethylsulfonyl fluoride, and 0.7 µg/ml pepstatin. The homogenate was centrifuged at 10,000 g for 15 min at 4°C. The supernatant was then stored at –70°C until further analysis.

Transfection in mammalian cell culture. Approximately 3 × 10⁵ C2C12 myoblasts were seeded into gelatin-coated wells of a six-well plate in growth media (20% fetal bovine serum, 25 µg/ml gentamicin, in DMEM). Transfections were per-
formed the next day using lipofectamine (GIBCO) with 1 µg of a firefly luciferase reporter plasmid, 76 ng pRL-CMV, and with or without 0.5 µg pEMSV-MyoD or pEMSV-myogenin. After 5 h of transfection, growth media were added to each well for incubation overnight. The following morning growth media were replaced with a low serum medium (2% horse serum, 10 µg/ml insulin, 10 µg/ml transferrin, 50 mM HEPES, pH 7.5, and 25 µg/ml gentamicin, in DMEM) to induce differentiation. After 48 h, protein extracts were made from each sample in a solution of 50% formamide, 1 M NaCl, 3 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, and 24 µg C₂C₁₂ nuclear extract protein. Nuclear protein was incubated at room temperature for an additional 30 min. The following morning, growth media were replaced with or without 0.5 µg pEMSV-MyoD or pEMSV-myogenin. A firefly luciferase reporter plasmid, 76 ng pRL-CMV, and 24 µg C₂C₁₂ nuclear extract protein. Nuclear protein was incubated at room temperature for an additional 30 min.

Table 1. Regions deleted in the clones used for somatic gene transfer

<table>
<thead>
<tr>
<th>Clone Name</th>
<th>Region Deleted/Mutated</th>
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<tbody>
<tr>
<td>pGL3IIB0.3</td>
<td>Deletion of region upstream of −295 relative to TSS</td>
</tr>
<tr>
<td>pGL3IIB0.3AAT3</td>
<td>Deletion of region upstream of −195</td>
</tr>
<tr>
<td>pGL3IIB0.3AAT3AAT2</td>
<td>Deletion of region upstream of −169</td>
</tr>
<tr>
<td>pGL3IIB0.3AAT1</td>
<td>Deletion of region upstream of −85 and −137</td>
</tr>
<tr>
<td>pGL3IIB0.3AAT1AAT2</td>
<td>Deletion of region between −196 and −137</td>
</tr>
<tr>
<td>pGL3IIB0.3CArG</td>
<td>Deletion of region between −110 and −87</td>
</tr>
<tr>
<td>pGL3IIB0.3MUTI10</td>
<td>Mutation of region between −121 and −110</td>
</tr>
<tr>
<td>pGL3IIB0.3MUTE</td>
<td>Mutation of region between −67 and −60</td>
</tr>
</tbody>
</table>

TSS, transcription start site.

Table 2. Sequences of electrophoretic mobility shift assay probes and mutations (bold)

<table>
<thead>
<tr>
<th>Reporter/Gel Shift Probes</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>MUTI10 (reporter)</td>
<td>5'-GTG AAT GCC AAT GTC CGA GAT TGC-3'</td>
</tr>
<tr>
<td>MUTE (gel shift and reporter)</td>
<td>5'-GCT AGG ACC GCC TGC GGC-5'</td>
</tr>
<tr>
<td>E-box (gel shift)</td>
<td>5'-GCT AGG ACA GGT GGC TTC-3'</td>
</tr>
<tr>
<td>Consensus MEF-1 (gel shift)</td>
<td>5'-GAT CCC CCC AAG AGC TGC TGG CTG-3'</td>
</tr>
<tr>
<td>NEG-1 (gel shift)</td>
<td>5'-CCT GTG ACC TCT TCC TCC TCT GCA-3'</td>
</tr>
<tr>
<td>AT-1 (gel shift)</td>
<td>5'-TAT TCC TTA TTA TAT CCA TTC AC-3'</td>
</tr>
</tbody>
</table>

(19:1 acrylamide-bis-acrylamide), 25 mM Tris base, 190 mM glycine gel at 4°C for 3 h. The gel was removed from the cast, dried for 1 h, and exposed to XOMAT AR film for 2–4 h with an intensifying screen at −70°C.

Nuclear extract from C₂C₁₂ cells were grown in growth media to confluence and induced to differentiate in low serum for 1 day. To isolate nuclei, the cells were washed twice with cold PBS containing 1 mM EDTA. Fresh cold PBS-EDTA was then added, and the cells were scraped into a conical tube. The nuclei were centrifuged at 10,000 g for 10 min at 4°C and resuspended in buffer A (15 mM HEPES, pH 7.5, 60 mM KCl, 15 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 10% sucrose, 0.1% octylphenoxy polyethoxethanol, 0.15 mM spermine, 0.75 mM spermidine, 14 mM β-mercaptoethanol, 1 µg/ml aprotinin, and 5 µg/ml leupeptin). The suspension was centrifuged for 10 min and then centrifuged for 5 min at 1,000 g at 4°C. The pellet was resuspended in buffer B (75 mM HEPES, pH 7.5, 60 mM KCl, 15 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.15 mM spermine, 0.75 mM spermidine, 0.5 mM DTT, 1 µg/ml aprotinin, and 5 µg/ml leupeptin). The suspension was centrifuged for 40 s at 4°C to pellet the nuclei. The pellet was resuspended in buffer C (20 mM HEPES, pH 7.8, 0.4 M KCl, 2 mM DTT, 20% glycerol, 2 µg/ml aprotinin, 0.7 µg/ml pepstatin, 170 µg/ml phenylmethylsulfonyl fluoride, and 5 µg/ml leupeptin) and incubated on ice for 15 min. The suspension was centrifuged for 1 min at 4°C, and the supernatant containing crude nuclear extract was stored as aliquots at −70°C.

Hindlimb suspension. Adult female Sprague-Dawley rats weighing 110–220 g were divided into the following groups: 1) normal, 2) 1-day suspension, 3) 2-day suspension, 4) 4-day suspension, 5) 7-day suspension, and 6) 14-day suspension. Animals were suspended by their hindlimbs and killed as described previously (24). The TA and soleus muscles were removed from the animals, trimmed free of visible connective tissue, weighed, and quick-frozen in liquid nitrogen. Muscles were stored at −70°C.

RNA isolation and Northern blotting muscle. RNA was isolated as described previously (24). RNA concentration was determined spectrophotometrically. Ten micrograms of RNA from each sample in a solution of 50% formamide, 1× MOPS, and 2.2 M formaldehyde was heated to 65°C for 10 min and loaded into a 1× MOPS and 1% agarose gel and run in 1× MOPS run buffer for −10–14 h at low voltage (−22 V). The RNA was transferred to a Hybond-N membrane (Amersham) using capillary transfer. The membrane was ultraviolet cross-linked and stored in a sealed bag at 4°C until use.

When using oligonucleotide probes, membranes were prehybridized in 6× SSPE (0.9 M NaCl, 31 mM Na₂PO₄, H₂O), 10 mM EDTA), 10× Denhardt’s solution, 0.1% SDS, and 150 µg/ml salmon sperm DNA at 42°C for 2 h. P32 end-labeled oligonucleotides specific for MHC IIβ or 28S were added to 6× SSPE and 0.1% SDS and hybridized to the membrane overnight at 42°C. When random-primer labeled probes for MyoD or myogenin were used, membranes were prehybridized in 50% formamide, 5× SSPE, 5× Denhardt’s solution, 0.1 mg/ml salmon sperm DNA, and 0.1% SDS for 4 h at 42°C. The labeled DNA was added to a freshly made prehybridization solution and hybridized at 42°C overnight. The membranes were washed extensively, with a final wash in 0.1× SSC, 0.1% SDS at 60°C for 30 min. Blots were exposed to XOMAT AR film with an intensifying screen at −70°C. For reprobing, blots were stripped by pouring boiling 0.1× SSC with 0.1% SDS over the blots and allowing them to cool to room temperature.

Statistics. Data for the variables studied are reported as means ± SE. Statistically significant differences were deter-
RESULTS

In vivo transcriptional analysis of the MHC IIB promoter. Numerous deletions or mutations of the firefly luciferase-linked MHC IIB promoter were generated (Fig. 1) for transcriptional analysis using somatic gene transfer into rat adult skeletal muscle. The TA muscle was chosen for injection because of its relative ease of accessibility and its high level of expression of the MHC IIB mRNA and protein. The soleus muscle was chosen for injection because it does not express the MHC IIB mRNA as determined by Northern (24) or ribonuclease protection analysis (16). As a control for variability inherent in the injection technique (24, 31), a constitutively expressed Renilla luciferase reporter vector (pRL-CMV) was coinjected with each experimental MHC IIB promoter construct. Deletion of a distal E-box and the mAT3 element (pGL3IIB0.3 D AT3) resulted in a decrease in firefly luciferase to Renilla luciferase activity in both the fast-twitch TA and the slow-twitch soleus muscle (Fig. 1). Furthermore, deletion of the distal E-box, mAT3, and mAT2 regions (pGL3IIB0.3AT3AT2) further decreased firefly luciferase activity in both muscles (Fig. 1) and in C2C12 myotubes (data not shown). Both of these constructs (pGL3IIB0.3AT3 and pGL3IIB0.3AT3AT2) induced higher firefly activities in the TA as compared with the soleus, suggesting that these elements play a role of non-fiber type-specific transcriptional activation of this gene. The deletion of the mAT1 region, the CArG region, or a 10-bp mutation from −120 to −111 each had minimal effect on reporter gene expression within the TA and appeared to induce reporter gene expression within the soleus muscle. An E-box located within the 295-bp MHC IIB promoter at −66 to −61 bp from the transcription start site was mutated (Fig. 1 and Table 2 for sequence). The pGL3IIB0.3MUTE construct was 90-fold less active in the TA as compared with the parent pGL3IIB0.3. Activity from this mutated promoter was not significantly above background (pGL3basic; Fig. 1) in either the soleus or the TA. The four mutated base pairs were cloned into the larger 2.6-kb MHC IIB promoter (Fig. 1). Activity of the pGL3IIB2.6MUTE construct was significantly depressed in both the TA and the soleus as compared with the parent vector (pGL3IIB2.6). The E-box is not sufficient by itself to drive a high level of expression in the TA in that a construct consisting of only the E-box and TATA box (pGL3IIB0.085) was minimally expressed in both soleus and TA muscles.

HSP70 TATA-based constructs. Because others (6) have shown that the upstream regulatory region of the MHC IIB promoter requires a specific TATA box for full activity, we wished to determine whether the MHC IIB TATA box was required for the E-box-dependent nature of differential expression of this gene. A 264-bp region including the E-box (or mutated E-box) from −31 to −295, excluding the MHC IIB TATA box, was cloned upstream of the HSP70 TATA box (pGL3TATA), a ubiquitously expressed basal promoter (Ref. 23: Fig. 2). This basal promoter was much less active than the MHC IIB promoter in either the soleus or TA muscles (Fig. 2). Reporter gene activity using the heterologous construct (pGL3TATAIIB0.3) was significantly elevated in only the TA muscle, not the soleus muscle (Fig. 2). However, muscles injected with pGL3TATAIIB0.3MUTE, containing the E-box mutation, did not exhibit elevated activities in the TA or soleus muscles. On the
basis of these data, it appears that the activation of the MHC IIB promoter in fast muscle requires the E-box. This activation seems to be mild in the absence of the MHC IIB TATA box because the relative activity of pGL3TATAIIB0.3 was significantly less than activity of the region linked to the endogenous MHC IIB TATA box (see Fig. 1).

EMSA. Because the proximal E-box appeared to be important for transcriptional activity of the MHC IIB gene, we wished to determine 1) whether a nuclear protein could specifically bind this region and 2) the identity of such a protein(s). EMSA were performed utilizing muscle cell culture nuclear extracts and an oligonucleotide probe spanning the proximal E-box (Table 2). Incubation of the double-stranded probe with C2C12 myotube nuclear extract produced two distinct specific interactions, complex A and complex B (Fig. 3). Competition with 100-fold excess of nonradioactive self-probe, or of a consensus MEF-1 site (Santa Cruz), successfully eliminated the slower mobility interaction (complex A, Fig. 3). The MEF-1 site used here contains an E-box from the muscle creatine kinase gene. Only competition with the self-probe eliminated the faster mobility interaction (complex B, Fig. 3). Neither a probe containing the mutated E-box nor a nonspecific probe (Neg1A, kindly provided by Dr. Deborah Weiss) successfully competed away either interaction. Thus both complex A and complex B were determined both to be specific and to require an intact E-box. Incubation with antibodies to MyoD (Novacastra) or myogenin (DAKO) indicated that both MyoD and myogenin were part of complex A but not part of complex B. An electrophoretic mobility shift reaction with the AT1 element of the MHC IIB promoter radioactively labeled demonstrated 1) a specific interaction with factors from the muscle cell nuclear extract and 2) antibodies directed against MyoD or myogenin did not nonspecifically shift protein-DNA interactions.

Cotransfection of cultured C2C12 and Hep G2 cells. As MyoD and myogenin both bound this E-box in vitro, we wished to determine whether the individual myogenic factors preferentially activate the MHC IIB promoter. Furthermore, we wanted to test the hypothesis that the proximal E-box, which is necessary for high levels of activity of the MHC IIB promoter in vivo (Fig. 1) and for protein-DNA interactions in vitro (Fig. 2), is required for activation by members of the MRF family. The MHC IIB0.3 promoter was activated in C2C12 myotubes by cotransfection with both MyoD and myogenin (Fig. 4A), as has been reported previously (14, 26). MyoD induced a sevenfold increase in reporter gene expression above the 300-bp promoter alone, whereas myogenin induced only a threefold increase (Fig. 4A). Mutation of the proximal E-box of the MHC IIB promoter diminished reporter gene activity eightfold in C2C12 cells (Fig. 4A) as seen in vivo (Fig. 1), but contrary to a previous report (6). With this mutation, MyoD and myogenin were still able to activate this promoter two- to threefold above that of the mutated promoter alone. This activation in the absence of an E-box is presumably due to secondary myogenic effects, since the myogenic regulatory factors initiate a cascade of effectors to activate the muscle gene program (22). Thus it appears that MyoD requires an intact E-box for full activation of this promoter

Fig. 3. MHC IIB E-box has direct interactions with myocyte nuclear extract. Two complexes (A and B) are formed with C2C12 nuclear extract and a 20-bp probe that contains proximal E-box of MHC IIB regulatory region. Both are specific, since they are competed away by 100-fold molar excess of nonradioactive self-probe (self) and are not competed with a 100-fold molar excess of a nonspecific element (NEG-1). Both complexes required an intact E-box for specific binding, since addition of 100-fold molar excess of a double-stranded probe containing identical sequences except for a mutated E-box (MUT E) did not compete for binding. When competing with a consensus MEF-1 site, which contains an E-box, only slower migrating complex (A) is competed away. Complex A contains both MyoD and myogenin as antibodies directed against either supershift complex. Complex B does not contain either MyoD or myogenin by supershift analysis. As a control for supershift experiments, AT1 element of MHC IIB regulatory region was used as a probe. It also generated a specific complex with factors from C2C12 nuclear extract, with a slower mobility than labeled E-box probe. Lack of a supershift with this element suggested that protein-DNA interaction did not contain either MyoD or myogenin. Pre, preimmune serum.
Fig. 4. MyoD preferentially activates MHC IIB promoter in both C2C12 myotubes and in Hep G2 cells. A: C2C12 cells. Proximal 300 bp of MHC IIB promoter (pGL3IIB0.3) or this same region with proximal E-box mutated (pGL3IIB0.3MUTE), or β-MHC promoter (pGL3βMHC) were cotransfected with a MyoD or myogenin expression vector into C2C12 myoblasts. These cells were allowed to differentiate into myotubes for 72 h before harvesting. MyoD activated pGL3IIB0.3 7-fold, whereas it only activated mutated promoter 2.5-fold, suggesting that MyoD has both a direct activation of MHC IIB promoter through E-box (myogenesis independent) and an indirect activation via remaining promoter region (myogenesis dependent). Myogenin, however, upregulated both normal and mutated promoters equally (2.7-fold), suggesting that myogenin induced upregulation of MHC IIB promoter through an E-box-independent, myogenesis-dependent mechanism in C2C12 cells. Neither MyoD nor myogenin substantially upregulated β-MHC promoter. Efficiency of transfection was normalized with pRL-CMV vector. Experiments were triplicated, with an n of 2 in each replicate. Data are shown as means ± SE. MRF, myogenic regulatory factor. B: Hep G2 cells. Same experiment was performed in Hep G2 cells, a human liver cell carcinoma, to lessen myogenic effects of cotransfected MyoD and myogenin.

Table 3. Muscle weight-to-body weight ratio of normal and unweighted soleus muscles

<table>
<thead>
<tr>
<th>Group</th>
<th>Muscle Wt/Body Wt, mg/g</th>
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<tbody>
<tr>
<td>Normal</td>
<td>0.448 ± 0.006</td>
</tr>
<tr>
<td>1-Day unweighting</td>
<td>0.444 ± 0.018</td>
</tr>
<tr>
<td>2-Day unweighting</td>
<td>0.437 ± 0.011</td>
</tr>
<tr>
<td>4-Day unweighting</td>
<td>0.433 ± 0.014</td>
</tr>
<tr>
<td>7-Day unweighting</td>
<td>0.346 ± 0.028*</td>
</tr>
<tr>
<td>14-Day unweighting</td>
<td>0.246 ± 0.030*</td>
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</table>

*P < 0.05 vs. normal.
Northern analysis (Fig. 5B), whereas MyoD mRNA was induced after only 1 day of unweighting and remained elevated through the duration of the experiment (Fig. 5, A and B). The levels of MHC IIB mRNA in the unweighted soleus muscles increased to a maximum at 2 wk of hindlimb suspension (Fig. 5B). Both unweighted and control soleus muscles were seen to express myogenin (Fig. 5A). Upon quantification relative to 28S rRNA (Fig. 5B), no significant difference was found between normal and unweighted soleus muscles. The correlation in the time course of MyoD mRNA expression relative to MHC IIB mRNA expression is consistent with the hypothesis that MyoD plays a causative role in the induction of MHC IIB gene expression in the unweighted soleus muscle.

DISCUSSION

Deletion and mutation analysis of the MHC IIB promoter in vivo. In a search to find an element within the MHC IIB promoter that drives differential expression of this promoter among muscles of the hindlimb,
we have identified a critical region that is required for transcriptional activity in adult fast-twitch skeletal muscle. Mutation of the proximal E-box within the MHC IIB promoter completely abolished activity within the context of the 295-bp promoter in adult skeletal muscle and significantly reduced promoter activity within the context of the larger promoter (Fig. 1). This is in contrast to a previous report that showed no effect of a mutation in this general region (26) in cell culture. This mutation consistently was inactive in cell culture and in vivo in our laboratory. The larger 2.6-kilobase-pair (kb) construct contains the originally identified MEF-1 consensus sequence located at approximately −940 (27), which can also bind MyoD. Thus this site at −940 may act in a partially redundant fashion for the mutated E-box in the 2.6-kb promoter, allowing for the small level of activity observed in the TA when the proximal E-box was mutated (pGL3IIB2.6MUTE; Fig. 1). Deletion of the entire sequence of the promoter distal to the E-box at −65 (pGL3IIB0.085) results in very low expression, indicating that the proximal E-box and consensus mTATA box alone are not sufficient for normal activity of the promoter in vivo or in myotube culture. Linking this region to the basal HSP70 promoter demonstrated that the E-box was definitely required for activity in fast-twitch muscle. Thus it appears that the E-box is necessary, but not sufficient, to drive IIB promoter-driven reporter gene expression in fast-twitch muscle.

In addition, we found that three constructs (pGL3IIB0.3AT1, pGL3IIB0.3CArG, and pGL3IIB0.3MUT110) drove higher levels of reporter gene expression than the parent vector (pGL3IIB0.3) in the soleus muscle. These three constructs contain deletions or mutations in the region between −167 and −89 within the MHC IIB promoter. This induction of reporter gene activity from these constructs suggests that the region between −167 and −89 may be involved with active repression of transcriptional activity of the MHC IIB gene in slow-twitch muscle.

Protein-DNA interactions. Because the proximal E-box is obviously required for promoter activity, we wished to identify the nuclear factor(s) that bind this region. Gel mobility shift assays demonstrated conclusively that factors from C2C12 nuclear extracts specifically interact the MHC IIB proximal E-box (Fig. 3). Proteins immunologically related to both MyoD and myogenin bound to this element in vitro. Both of these proteins are well-characterized basic helix-loop-helix proteins that bind E-boxes (22). Both myogenin and MyoD form heterodimers when they bind to E-boxes (22). It is likely that the observed binding complexes to the E-box of the MHC IIB promoter do in fact contain E-proteins as well, although it has not yet been demonstrated. The identification of these E-proteins will be important to further study the binding relationship of the MHC IIB proximal E-box and myogenin or MyoD. It may be the case that differential expression of E-proteins in different muscle types may result in a different heterodimer complex and thus differential activation of the MHC IIB gene.

Interestingly, at least one more factor can bind to this region in vitro (Fig. 3; complex B). The identity of this factor is not known, but binding was E-box dependent because addition of 100× molar excess of the mutated E-box did not disrupt binding. Also, flanking sequences of the proximal E-box in the MHC IIB gene are important in that the consensus MEF-1 site containing an E-box, or the E-box from the MyoD gene (data not shown), did not successfully compete for the faster migrating complex.

MRF and MHC IIB expression in cell culture. Because both MyoD and myogenin bound the critical proximal E-box of the MHC IIB promoter in vitro, we wished to determine whether either of these MRF could specifically activate the MHC IIB promoter in cell culture. Others have previously reported that any of the MRF can activate the MHC IIB promoter in cell culture (26). In fact, there is conflicting evidence about the magnitude of activation by MyoD vs. myogenin of this promoter (14, 26). Hence, we measured the ability of these expression vectors to activate the MHC IIB promoter utilizing transient transfections into C2C12 myotubes and the nonmuscle cell line Hep G2. We found that both myogenin and MyoD upregulated the MHC IIB 295-bp promoter, even in the presence of the mutated proximal E-box (Fig. 4). However, in C2C12 cells, myogenin activated the promoter to the same degree, regardless of the presence of the mutation. This suggests that the induction of MHC IIB expression in cell culture by myogenin is one that is dependent on the activation of the myogenic program, and not a direct effect on the MHC IIB promoter. MyoD, however, was much more effective in activating the MHC IIB promoter in the presence of the wild-type promoter. This suggests that MyoD has two routes of activation of this promoter: 1) an E-box-dependent pathway likely mediates a direct effect of MyoD, and 2) an E-box-independent pathway mediated by the myogenic program, like that of myogenin.

Time course of expression of MHC IIB, MyoD, and myogenin in unweighting. Although the gel mobility shift assays show that the MHC IIB E-box can be bound by both MyoD and myogenin in vitro, the degree to which these two proteins are coexpressed with MHC IIB in hindlimb musculature is vastly different. Previous studies have indicated that MyoD is preferentially expressed in fast adult muscle while myogenin is expressed in slow muscle (20–22), a finding confirmed here (Fig. 5). Unweighting of the soleus muscle has been used as a model of conversion of slow-twitch muscle to fast-twitch muscle (2). Northern blot analysis revealed that MyoD mRNA was significantly upregulated in unweighted soleus muscles (Fig. 5). However, myogenin mRNA levels remained unchanged, in agreement with a study using another model of muscle disuse, immobilization (15).

Other studies have shown that the level of MRF transcripts changes under different environmental conditions, including regeneration, denervation, cross-innervation, muscle hypertrophy, and stimulation of a stretched muscle (11, 12, 18, 29). However, most of
these studies are complicated by the fact that MRF genes are upregulated within satellite cells during proliferation and differentiation of satellite cells in response to these interventions. In the current study, we focused on the physiological model of hindlimb suspension that does not activate satellite cells (5) but does induce changes in muscle fiber type. MyoD mRNA accumulated in the slow soleus muscle after only 1 day of unweighting (Fig. 5). This suggests that MyoD is a primary response gene to unweighting in the soleus. Furthermore, the MHC IIB mRNA was not detectable by Northern analysis in the soleus until 4 days of unweighting (Fig. 5). One previous study also reported that it was 3 days after the initiation of immobilization that the MHC IIB mRNA became detectable (16). Thus MyoD mRNA accumulated in the unweighted soleus before the detection of the MHC IIB mRNA. This temporal relationship between MyoD and MHC IIB suggests that the MyoD gene product is required for expression of the MHC IIB gene in the soleus muscle. Although not measured here, translational or posttranslational control of MyoD and myogenin may play a role in regulating MHC IIB gene expression. The maintenance of a dephosphorylated state is required for MyoD to bind to other E-proteins and form a complex with the E-box as well as to require stabilization by retinoblastoma protein in its dephosphorylated state (20).

Evidence presented herein suggests that MyoD plays an important role in the induction of MHC IIB in the unweighted soleus muscle. However, in myoD−/− mice, IIB fibers are detected in the musculature (11). In one muscle, the extensor hallucis longus, the number of IIB fibers in myoD−/− mice is reduced about threefold. In the other three muscles examined, the proportion of IIB fibers was normal (11). Thus MyoD may play only a limited role in the establishment of IIB fibers. It is unclear what role, if any, MyoD plays in adaptive response to altered physiological environments known to induce fiber type changes (11). Experiments are ongoing to determine whether the temporal relationship between the induction of MyoD and MHC IIB in the unweighted soleus observed herein is causative or merely correlative.

The mechanism of transcriptional activation of MyoD through its association with the E-box has not been characterized. However, a report has recently shown that MyoD aids in the binding of basal transcription factors to the TATA box area and the transcription start site (9). In the context of the MHC IIB promoter, the mutation of the E-box removes the ability of MyoD to bind to the MHC IIB promoter. This in turn prevents MyoD from maintaining the association of TBP or TFIIID with the mTATA box. This suggests that MyoD either plays a role in the maintenance of basal transcriptional activation or plays an active role in the interaction among the basal transcriptional machinery and upstream enhancer elements. Why myogenin cannot play a similar role in slow muscle, where myogenin is expressed but the MHC IIB gene is not, is currently under investigation. The activation and interaction of MyoD on the proximal E-box may thus be a crucial and important step in the pathway for the activation of MHC IIB expression in response to altered physiological demand on the soleus muscle.

This work was supported by National Science Foundation Grant IBN-9723351.

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Received 22 October 1998; accepted in final form 2 February 1999.

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