SKELETAL MUSCLE FIBERS are heterogeneous with respect to contractile properties. Skeletal muscle fiber diversity is attributable to differential expression of isoforms of contractile proteins, including myosin isoforms and several thin filament proteins. Differential expression of myosin isoforms can dictate contractile properties such as maximal velocity of shortening and force-velocity characteristics (6). Myosin molecules are hexameric; they consist of two myosin heavy chain (MHC) proteins and two pairs of myosin light chain proteins. Differentiated mammalian muscle fibers have been shown to be capable of expressing at least four isoforms of MHC: slow type I, intermediate type IIA, fast type IIX, and fast type IIB (reviewed in Refs. 20 and 25). Distinct genes encode each of these MHC isoforms.

Muscle fibers that express different MHC isoforms are adapted for different motor tasks. Motor units used in short-duration, high-power-output activity, such as sprinting and power lifting, express primarily the IIB and IIX MHC isoforms (25). Fast motor units used for endurance activity, i.e., the deep red regions of most muscles, abundantly express the IIA MHC isoform (25).

Fibers that express the type I MHC are abundant in muscle groups adapted for sustained periods of tonic contractile activity, such as anti-gravity function or joint stabilization. Expression of this isoform predominates in the soleus and vastus intermedius muscles, two slow-twitch muscles of the hindlimb.

There is an extensive body of evidence characterizing the adaptive changes in MHC phenotype in response to various types of mechanical and hormonal environments (reviewed in Refs. 20 and 25). Of critical importance to the current study, muscle unweighting, induced either by hindlimb suspension (12) or spaceflight (14), causes a slow-to-fast-type fiber transition whereby the MHC IIB is expressed de novo in the soleus muscle. Transcriptional processes have been shown to regulate in part expression of some members of the MHC gene family, including the MHC IIB gene (10), cardiac α-MHC (2, 27, 33), and cardiac β-MHC (also the type I MHC in skeletal muscle; see Refs. 2, 27, and 33). However, the underlying mechanisms, i.e., signaling pathways and transcription factors, that determine diversity and adaptability among skeletal muscle fibers remain to be elucidated. The lack of a cell culture system that can accurately mimic physiological phenomena such as hindlimb unweighting has impeded our understanding of some of the molecular mechanisms that dictate maintenance and transition of muscle fiber type. The purpose of this study was to delineate the critical cis-acting region(s) within the MHC IIB promoter that drive fiber-type-specific expression of this gene in vivo. To accomplish this goal, somatic gene transfer was used. Numerous groups have shown that plasmid DNA can be injected directly into muscle for adequate expression of reporter genes (1, 5, 7–9, 17, 19, 23, 35, 36, 38). In the current study, the murine IIB promoter was linked to a firefly luciferase reporter gene. It is presented here that only the proximal 295 base pairs (bp) of the MHC IIB promoter are required for both 1) muscle fiber-type-specific expression of reporter gene activity and 2) appropriate upregulation of reporter gene activity within the unweighted soleus muscle.

MATERIALS AND METHODS

Cloning and generation of deletion constructs for the murine IIB promoter. Polymerase chain reaction (PCR) primers were designed to anneal to the sequenced region of the MHC IIB promoter (30). Using high-fidelity long PCR (Boehringer Mannheim, Indianapolis, IN) from mouse genomic DNA, the PCR product generated was cloned upstream of the firefly luciferase gene (pGL3basic, Promega, Madison, WI) and termed pGL3IIB2.6. This construct contains 2,560 bp of sequence upstream of the transcription start site (TSS) and 13 bp downstream of the TSS. Four deletion constructs were made by using pGL3IIB2.6 as a template. pGL3IIB0.3 and

Swoap, Steven J. In vivo analysis of the myosin heavy chain IIB promoter region. Am. J. Physiol. 274 (Cell Physiol. 43): C681–C687, 1998.—The myosin heavy chain (MHC) IIB gene is preferentially expressed in fast-twitch muscles of the hindlimb, such as the tibialis anterior (TA). The molecular mechanism(s) for this preferential expression are unknown. The goals of the current study were 1) to determine whether the cloned region of the MHC IIB promoter contains the necessary cis-acting element(s) to drive fiber-type-specific expression of this gene in vivo, 2) to determine which region within the promoter is responsible for fiber-type-specific expression, and 3) to determine whether transcription off of the cloned region of the MHC IIB promoter accurately mimics endogenous gene expression in a muscle undergoing a fiber-type transition. To accomplish these goals, a 2.6-kilobase fragment of the promoter-enhancer region of the MHC IIB gene was cloned upstream of the firefly luciferase reporter gene and coinjected with pRL-cytomegalovirus (CMV) (CMV promoter driving the renilla luciferase reporter) into the TA and the slow soleus muscle. Firefly luciferase activity relative to renilla luciferase activity within the TA was 35-fold greater than within the soleus. Deletional analysis demonstrated that only the proximal 295 base pairs (pGL3IIB0.3) were required to maintain this muscle-fiber-type specificity. Reporter gene expression of pGL3IIB0.3 construct was significantly upregulated twofold in unweighted soleus muscles compared with normal soleus muscles. Thus the region within the proximal 295 base pairs of the MHC IIB gene contains at least one element that can drive fiber-type-specific expression of a reporter gene.

Skeletal muscle fibers are heterogeneous with respect to contractile properties. The myosin heavy chain IIB promoter region. Am. J. Physiol. 274 (Cell Physiol. 43): C681–C687, 1998.—The myosin heavy chain (MHC) IIB gene is preferentially expressed in fast-twitch muscles of the hindlimb, such as the tibialis anterior (TA). The molecular mechanism(s) for this preferential expression are unknown. The goals of the current study were 1) to determine whether the cloned region of the MHC IIB promoter contains the necessary cis-acting element(s) to drive fiber-type-specific expression of this gene in vivo, 2) to determine which region within the promoter is responsible for fiber-type-specific expression, and 3) to determine whether transcription off of the cloned region of the MHC IIB promoter accurately mimics endogenous gene expression in a muscle undergoing a fiber-type transition. To accomplish these goals, a 2.6-kilobase fragment of the promoter-enhancer region of the MHC IIB gene was cloned upstream of the firefly luciferase reporter gene and coinjected with pRL-cytomegalovirus (CMV) (CMV promoter driving the renilla luciferase reporter) into the TA and the slow soleus muscle. Firefly luciferase activity relative to renilla luciferase activity within the TA was 35-fold greater than within the soleus. Deletional analysis demonstrated that only the proximal 295 base pairs (pGL3IIB0.3) were required to maintain this muscle-fiber-type specificity. Reporter gene expression of pGL3IIB0.3 construct was significantly upregulated twofold in unweighted soleus muscles compared with normal soleus muscles. Thus the region within the proximal 295 base pairs of the MHC IIB gene contains at least one element that can drive fiber-type-specific expression of a reporter gene.

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designed to anneal from position 

constructed was generated using PCR. The 5 bp, was recircularized with blunt ligation. The pGL3IIB1.3 upstream of the TSS. The product, missing the internal 880 Eco vector), and either 

respectively, of sequence upstream of the TSS. To generate 

pGL3IIB0.3 or Pvu II (pGL3IIB0.6). The DNA was recircularized with blunt ligation after the ends were polished with Klenow. pGL3IIB10.88 was generated by restriction of pGL3IIB2.6 with Pvu II, which cleaves the promoter region at positions –63 and –943 upstream of the TSS. The product, missing the internal 880 bp, was recircularized with blunt ligation. The pGL3IIB1.3 construct was generated using PCR. The 5 ′-primer was designed to anneal from position –1282 to position –1259 upstream of the TSS. The PCR product was cloned upstream of the firefly luciferase reporter gene in the Xho I site of the multiple cloning region. Preparation of DNA for injections. Plasmid DNA for injection was prepared from large-scale growth of bacteria harboring the plasmid of interest. The plasmid DNA was purified using Qiagen columns, resuspended in sterile phosphate-buffered saline, quantitated using 260-nm OD spectrophotometric measurements, and stored at a concentration of 4 mg/ml.

DNA injections. Plasmid DNA for the experimental construct (pGL3IIB2.6 or derivative) was combined with pRL-CMV plasmid DNA. pRL-CMV is a CMV-driven renilla luciferase reporter gene (Promega). The final concentration of each construct was 2 mg/ml. The DNA solution was pipetted into an insulin-syringe fitted with a 28-gauge needle. Animals were anesthetized using a cocktail [(in mg/kg body wt) 25 ketamine, 1 acepromazine, and 5 xylazine], and the hindlimbs were shaved. Fifty microliters of the DNA solution (100 µg of each construct) was directly injected into tibialis anterior (TA) muscle. The insertion point of the needle was ~1 cm on the proximal side of the ankle joint. The injection tract ran parallel with the muscle fibers. To inject the soleus muscle, an incision was made on the lateral aspect of the hindlimb. The distal one third of the soleus was isolated for visualization. Twenty-five microliters of the DNA solution (50 µg of each construct) were injected into the soleus muscle. The hindlimb was stitched using 5–0 suture, and the animal was returned to its cage.

Determination of reporter gene activity. One week after the injections, the animals were killed, and the injected muscles were quickly removed, trimmed of all connective tissue, weighed, and frozen in liquid nitrogen. Muscles were homogenized in 2 ml of a buffer containing 100 mM tris(hydroxymethyl)amino-nethane, pH 7.8, 1 mM EDTA, 1 mM dithiothreitol, 2 µg/ml aprotinin, 5 µg/ml leupeptin, 170 µg/ml phenylmethanesulfonyl fluoride, and 0.7 µg/ml pepstatin. The homogenate was centrifuged at 4°C for 15 min at 10,000 g. The supernatant was stored at –80°C until analysis. The protein extracts were brought to room temperature, and 20 µl of these extracts were used to perform the luciferase assays. To measure reporter gene activity, the dual-luciferase reporter assay (Promega) was used. This assay measures and distinguishes luciferase activity from the firefly luciferase protein and the renilla luciferase protein. Light from the chemical reactions was integrated over 10 s using a Turner Designs luminometer. All activities reported were standardized to a reading of 50% on the luminometer.

Immunoprecipitation and Western blot analysis of firefly luciferase. Firefly luciferase was immunoprecipitated from muscle extracts using a commercially available polyclonal antibody directed against firefly luciferase (Promega) and protein G Sepharose (Boehringer Mannheim) using the manufacturer’s recommendations. After immunoprecipitation us-

ing 5 µl of the antibody, the eluted sample was separated on a 10% sodium dodecyl sulfate gel, transferred to a membrane (Bio-Rad, Richmond, CA), and incubated with the luciferase antibody. A chemiluminescence kit (New England Biolabs, Boston, MA) was used for detection of firefly luciferase on the membrane. As a control, 1 ng of purified firefly luciferase (Promega) was also loaded on the gel.

Hindlimb suspension. Sprague-Dawley rats were assigned to one of two groups: 1) normal control (n = 11) and 2) hindlimb suspension (n = 11). For hindlimb suspension, the basic protocol of Diffee et al. (12) was used. Five animals in each group were suspended for 3 wk. At that time, the animals were killed and the soleus muscles were removed, weighed, quickly frozen in liquid nitrogen, and stored at –80°C until analysis. The other six animals in each group were suspended for 2 wk. At that time, the soleus muscles from both normal and hindlimb suspension animals were injected with 50 µg of both pGL3IIB0.3 and pRL-CMV. Care was taken to prevent any weight bearing in the hindlimb suspension animals during preparation and recovery from the injection surgery as well as at the time of death. After one additional week of suspension of the hindlimb suspension group, the animals were killed and the soleus muscles were removed, weighed, and stored as above. Luciferase assays were performed on muscle extracts as described in Determination of reporter gene activity.

MHC mRNA analysis. To extract total RNA, muscles were homogenized in 4 M guanidinium thiocyanate, 20 mM sodium acetate, and 100 mM β-mercaptoethanol. Homogenates were extracted in phenol-chloroform and precipitated with isopropanol. RNA pellets were incubated in 4 M LiCl for 30 min on ice and resuspended in formamide. Electrophoresis and transfer of RNA were performed as described previously (26). Blots were probed with an oligo probe specific for the IIB MHC mRNA (28), stripped, and subsequently probed with an oligo probe specific for 28S rRNA.

Statistics. Data for the variables studied are reported as means ± SE. Statistically significant differences were determined using standard t-tests (hindlimb suspension) or Tukey’s test (deletional analysis) after an analysis of variance. The 0.05 level of confidence was accepted for statistical significance.

RESULTS

Verification of the use of a second normalizing gene construct. One inherent property of injections of plasmid DNA into rat muscle is the variability in uptake of that DNA between muscles and the resultant variability in reporter gene activity (35, 38). A crucial first experiment was performed to determine whether the variability in DNA uptake and reporter gene expression could be normalized using a second construct that is constitutively active. Figure 1 shows that when two promoters, CMV-driven firefly luciferase (pGL3-CMV) and CMV-driven renilla luciferase (pRL-CMV) were co-injected into the rat TA muscle, the relative expression of the reporter genes was the same across a 10-fold difference in activity. Firefly luciferase activity strongly correlated with renilla luciferase activity (Fig. 1).

Deletion analysis of the MHC IIB promoter in the TA muscle. Using the known sequence, the promoter of the MHC IIB gene was amplified with PCR and cloned upstream of the firefly luciferase gene (pGL3IIB2.6). This construct contains 2,560 bp upstream of the TSS of
the MHC IIB gene. Four separate deletion mutants of the MHC IIB promoter linked to the firefly luciferase were made (Fig. 2). These contain 1,282 bp upstream of the TSS (pGL3IIB1.3), 295 bp upstream of the TSS (pGL3IIB0.3), 63 bp upstream of the TSS (pGL3IIB0.06), and an internal deletion of 880 bp within the context of pGL3IIB2.6 (pGL3IIBΔ0.88; between −63 and −943). These constructs were co-injected with pRL-CMV into the TA muscle, a fast-twitch dorsi-flexor muscle that expresses the IIB gene.

One week after injections, the activities of firefly and renilla luciferases within muscle homogenates were determined (Table 1). Renilla luciferase expression derived from the pRL-CMV construct was relatively constant between the different sets of injections. When injecting 2.6 kb of the MHC IIB promoter linked to a firefly luciferase reporter gene (pGL3IIB2.6) into the rat TA, firefly luciferase activity was ~1/20 the activity of the strong CMV-driven renilla luciferase (Table 1). When pGL3IIB1.3 or pGL3IIB0.3 was injected into the TA, similar levels of firefly luciferase activity were observed. However, when pGL3IIB0.06 was injected, activity of the firefly luciferase gene significantly dropped 30-fold (Table 1). Similar results were obtained when 880 bases of the promoter (pGL3IIBΔ0.88) were deleted within the context of the 2.6-kb promoter. The decrease in activities from these two promoters was likely not a result of lack of uptake of DNA by the muscle; both sets of injections showed renilla luciferase activity equivalent to that of the other sets of injections (Table 1). Firefly luciferase activity in muscles injected with either pGL3IIB0.06 and pGL3IIBΔ0.88 was slightly above background, as determined in pGL3basic controls (Table 1). This suggests that a crucial region for high level of expression in the rat TA of the MHC IIB gene lies between bases −295 and −63.

Slow- vs. fast-twitch muscle expression of the IIB MHC promoter. To determine if any or all of these MHC IIB promoter constructs contain the necessary cis-acting element(s) required for muscle fiber-type-specific expression of the reporter gene, each construct was injected into the soleus muscle, a slow-twitch muscle. See Fig. 2 for schematic of myosin heavy chain (MHC) IIB promoter-reporter gene constructs tested. By polymerase chain reaction (PCR), 2.6 kb of murine MHC IIB promoter was cloned and inserted to drive firefly luciferase gene expression. Number shown in each construct name is the approximate size in kilobase pairs of IIB promoter. Included within pGL3IIB0.3 construct are numerous putative transcription factor binding sites previously identified (29, 30). See DISCUSSION for details.

Table 1. Firefly and renilla luciferase activities within tibialis anterior muscle

<table>
<thead>
<tr>
<th>Injected Constructs</th>
<th>Firefly (RLU/10 s)</th>
<th>Renilla (RLU/10 s)</th>
<th>Firefly/Renilla</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRL-CMV + pGL3CMV (25)</td>
<td>15,773 ± 3,827</td>
<td>10,982 ± 2,718</td>
<td>1.495 ± 0.155</td>
</tr>
<tr>
<td>pRL-CMV + pGL3IIB2.6 (20)</td>
<td>496.8 ± 233.3</td>
<td>12,823 ± 5,352</td>
<td>0.062 ± 0.009</td>
</tr>
<tr>
<td>pRL-CMV + pGL3IIB1.3 (9)</td>
<td>410.0 ± 256.6</td>
<td>14,399 ± 9,412</td>
<td>0.045 ± 0.011</td>
</tr>
<tr>
<td>pRL-CMV + pGL3IIB0.3 (18)</td>
<td>307.4 ± 172.9</td>
<td>13,977 ± 8,240</td>
<td>0.071 ± 0.012</td>
</tr>
<tr>
<td>pRL-CMV + pGL3IIB0.06 (8)</td>
<td>14.0 ± 7.4</td>
<td>34,203 ± 17,962</td>
<td>0.002 ± 0.001*</td>
</tr>
<tr>
<td>pRL-CMV + pGL3IIBΔ0.88 (4)</td>
<td>1.5 ± 0.1</td>
<td>18,841 ± 2,221</td>
<td>0.000 ± 0.000*</td>
</tr>
<tr>
<td>pRL-CMV + pGL3basic (7)</td>
<td>0.5 ± 0.5</td>
<td>4,726 ± 1,899</td>
<td>0.000 ± 0.000*</td>
</tr>
<tr>
<td>pRL-CMV (5)</td>
<td>1.3 ± 0.3</td>
<td>9,200 ± 4,017</td>
<td>0.001 ± 0.001*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n (in parentheses) is no. of experiments with construct. Tibialis anterior muscle of rats was injected with 100 µg of above constructs. pRL-CMV, cytomegalovirus (CMV) promoter-driven renilla luciferase gene expression. pGL3-CMV, CMV promoter-driven firefly luciferase gene expression. pGL3IIB, IIB MHC promoter-driven firefly luciferase gene expression. See Fig. 2 for schematic of these constructs. One week after injections, muscles were homogenized and tested for firefly and renilla luciferase activities. There was a significant decrease in ratio of firefly to renilla luciferase activity with pGL3IIB0.06 and pGL3IIBΔ0.88 compared with pGL3IIB2.6. *P < 0.05 vs. pGL3IIB2.6.
muscle of the hindlimb that does not normally express the MHC IIB gene. When 50 µg of pGL3IIB2.6 was co-injected with 50 µg of pRL-CMV into the soleus muscle, firefly luciferase activity was significantly less than in the TA (Fig. 3). This was also true for pGL3IIB1.3 and pGL3IIB0.3. The firefly luciferase activity derived from both pGL3IIB0.06 and pGL3IIB0.88 was similarly very low in the TA and the soleus. Because it is possible that the soleus muscle may not express the firefly luciferase gene well, it was critical to inject a different promoter to drive the firefly luciferase. As a positive control, a skeletal a-actin promoter driving firefly luciferase (HSA2000, a kind gift from Dr. Robert Wade) was co-injected with pRL-CMV into the soleus and TA muscles. Unlike the MHC IIB gene, the endogenous skeletal a-actin is expressed equally in both fast- and slow-twitch muscle. The firefly luciferase activity from this reporter gene construct was not significantly different between the soleus and TA muscles (Fig. 3, P > 0.05). The lack of expression of the pGL3IIB-derived constructs in the slow-twitch soleus muscle was thus likely due to the fiber-type-specific expression pattern of this gene and suggests that at least one cis-acting element(s) required for fiber-type-specific expression of this promoter lies downstream of position −295.

For visual confirmation that luciferase is expressed in a muscle-type-specific manner, extracts from muscles that were injected with pGL3IIB1.3 were immunoprecipitated with a luciferase antibody, followed by detection on a Western blot. As Fig. 4 shows, the relative activity of firefly luciferase in muscle extracts correlated with the relative abundance of immunoreactive firefly luciferase protein.

Induction of activity of the IIB promoter with hindlimb suspension. The lack of weight bearing in the soleus of a rat undergoing hindlimb suspension induces an increase in MHC IIB mRNA expression in the soleus muscle (Fig. 5A and Refs. 12 and 14). Animals were placed into two groups: normal and hindlimb suspended. After 3 wk of suspension, soleus muscle weight to body weight ratios significantly declined (0.417 ± 0.017 vs. 0.233 ± 0.012 mg/g, P < 0.05). IIB mRNA was not detected in any of the control soleus muscles but was expressed in the unweighted soleus muscles at a level ~10% of that in the TA (Fig. 5, A and B). In animals not used for RNA analysis, pGL3IIB0.3 and pRL-CMV were co-injected into the soleus muscles of normal and hindlimb-suspended rats after 2 wk of suspension, and the hindlimb-suspended rats were suspended for one additional week. Renilla luciferase

![Figure 3](http://ajpcell.physiology.org/) Muscle type-specific expression of the exogenous IIB MHC promoter and its derivatives. One hundred micrograms of each shown construct with 100 µg of pRL-CMV was injected into TA of rat. Similarly, 50 µg of the shown constructs with 50 µg of pRL-CMV were injected into soleus, a slow-twitch muscle that does not express endogenous MHC IIB gene. Firefly luciferase activity and renilla luciferase activity were measured in muscle extracts 1 wk after injections. pGL3IIB2.6 (n = 20 for TA, n = 10 for soleus), pGL3IIB1.3 (n = 9 for TA, n = 10 for soleus), and pGL3IIB0.3 (n = 18 for TA, n = 11 for soleus) were expressed in soleus at ~1/10 the level found in TA. pGL3IIB0.06 (n = 8 for TA, n = 4 for soleus) and pGL3IIB0.88 (n = 4 for TA, n = 4 for soleus) were not expressed at appreciable levels in either muscle. Skeletal a-actin promoter, HSA2000 (n = 7 for TA, n = 7 for soleus), was expressed equally in soleus and TA. Injection of pGL3basic (n = 7 for TA, n = 5 for soleus) led to negligible firefly luciferase activity in both muscles. These data suggest that the region that lies between −295 and −63 of the MHC IIB gene was required for high-level expression in vivo and contained necessary cis-acting element(s) required for fiber-type-specific expression of MHC IIB gene constructs. Data shown as means ± SE. *P < 0.05 vs. soleus.

![Figure 4](http://ajpcell.physiology.org/) Immunoprecipitation of luciferase from muscles injected with pGL3IIB1.3. With use of an antibody directed against firefly luciferase protein, immunoreactive luciferase from injected muscle extracts was immunoprecipitated and detected using a Western blot. Samples shown were selected because transfection efficiency was nearly the same for these 2 muscles (see renilla luciferase activity at bottom of each lane). Firefly luciferase activity is also shown at bottom of each lane, demonstrating muscle-specific expression of pGL3IIB1.3. Lane 1, 1 ng of recombinant firefly luciferase; lane 2, immunoprecipitate from TA injected with pGL3IIB1.3; lane 3, immunoprecipitate from soleus injected with pGL3IIB1.3; lane 4, immunoprecipitate of uninjected TA. Arrows indicate firefly luciferase protein and immunoglobulin G (IgG) heavy chain from immunoprecipitation.
Fig. 5. Expression of endogenous MHC IIB gene and exogenous IIB MHC promoter (pGL3IIB0.3) in normal soleus, unweighted soleus, and normal TA muscles. A: Northern blot of 10 µg of normal soleus total RNA (N sol) and 10 µg of unweighted soleus total RNA (S sol) demonstrates de novo expression of MHC IIB mRNA in unweighted soleus muscle. One microgram of total RNA from TA muscle is also shown. B: mRNA levels; blots like those shown in Fig. 5A were scanned, quantitated, and expressed relative to 28S rRNA. MHC IIB mRNA was not detected (ND) in normal soleus. Suspension induced expression of MHC IIB mRNA in soleus to >1/10 of level in normal TA. Luciferase activities; pGL3IIB0.3 was injected into normal (n = 10) soleus muscles and soleus muscles that underwent 2 wk of hindlimb suspension (n = 11). After an additional week of suspension in soleus group, luciferase activities were determined in muscle extracts. Luciferase activity in unweighted soleus muscle was significantly above that of normal muscle and significantly lower than that in normal TA. Thus the proximal 295 bp of MHC IIB promoter contain at least one element that is responsive to unweighting in soleus muscle. Data shown as means ± SE. Individual data points are shown by open circles. *P < 0.05 vs. normal soleus; **P < 0.05 vs. normal TA.

DISCUSSION

Major findings. The primary findings of this study were 1) demonstration that only 295 bp of the proximal upstream regulatory region of the MHC IIB gene were required for accurate reproduction of endogenous gene expression in vivo and 2) that same region contained at least one element that was responsive to unweighting in the soleus muscle.

Use of DNA injection in vivo for the study of gene regulation. Recently, the use of somatic gene transfer into muscle has been shown to be a viable technology for the study of transcriptional regulation of gene expression in muscle (1, 5–7, 9, 17, 19, 23, 35, 36, 38). Initial studies by Wolff et al. (38) demonstrated an inherent variability of reporter gene activity after injection into muscle. It was also shown that a second vector co-injected could correct for that variability (36, 38), a finding confirmed here (Fig. 1). On analysis of the data presented in Table 1, it is apparent that the variability of the firefly luciferase activity was substantial, with standard errors ranging from one-third to one-half of the mean value. This was also true for renilla luciferase activities. After normalization of the firefly luciferase activity to the renilla luciferase activities, it became quite apparent that the variance in firefly luciferase activity from any given promoter was a function of the efficiency of gene transfer. Thus somatic gene transfer appears to be a feasible tool for probing transcriptional control in vivo. In fact, other investigators using DNA injections have demonstrated an element of the skeletal α-actin gene responsive to muscle overload (7, 8), a slow-muscle-specific element within the troponin I slow promoter (9), a pressure overload response element within the c-fos promoter (1), cardiac expression of the β-MHC gene (36), differential expression of the creatine kinase M gene in cardiac and skeletal muscle (35), and thyroid responsiveness of the IIB MHC gene (23) and the α-MHC gene (5, 17, 19).

Genetic control of fiber-type-specific expression of IIB reporter genes. Three constructs tested here, pGL3IIB2.6, pGL3IIB1.3, and pGL3IIB0.3, all generated luciferase activities well above background, as determined by injections of the parent vector, pGL3basic (Table 1 and Fig. 3). As Fig. 3 shows, this IIB promoter elicited greater activity than a previously tested skeletal α-actin promoter (9), although some of this difference may be attributable to different parent vectors (pGL3 vs. pGL2). No significant decrease in promoter activity in the TA was detected between pGL3IIB2.6 and pGL3IIB0.3, suggesting that the regulatory elements that drive high-level expression in the TA are likely located downstream of –295 (the most 5′-end of pGL3IIB0.3). The pGL3IIB0.3 construct, as well as the larger constructs, displayed fiber-type specificity. Expression in the soleus muscle, which does not express the IIB mRNA or protein at detectable levels (Fig. 5A), was only one-tenth of the level in the TA (Fig. 3). Thus the region upstream of the most 5′-end of the pGL3IIB0.6 must contain at least one element required for high levels of reporter gene activity and may contain an element that drives expression specifically in IIB-expressing fibers. Certainly there may be elements upstream of the 5′-end of the pGL3IIB0.3 promoter that may be able to direct high levels of muscle
FIBER-TYPE-SPECIFIC REGULATION OF THE MHC IIB PROMOTER

The data obtained in the current study suggest that the region between –295 and –63 of the IIB promoter is necessary for muscle fiber-type-specific expression of reporter genes. How- ever, the data obtained in the current study suggest fiber-type-specific expression of reporter genes. However, the data obtained in the current study suggest that the region between –295 and –63 of the IIB promoter is necessary for muscle fiber-type-specific expression of reporter genes.

Genetic control of fiber-type adaptation. When exposed to various loading conditions, skeletal muscle has the ability to alter gene expression (20, 25). The MHC IIB gene is exquisitely regulated by weight bearing in the soleus muscle. The normal rat soleus does not express detectable levels of the MHC IIB mRNA by Northern blot analysis, whereas after 3 wk of hindlimb suspension, the unweighted soleus muscle expresses the MHC IIB mRNA (Fig. 5). Although this mRNA is de novo expressed, the level of expression in the unweighted soleus of the MHC IIB mRNA was much less than in the normal TA muscle (Fig. 5). Firefly luciferase expression off of the injected pGL3IIB0.3 reporter construct was also upregulated significantly in the unweighted soleus muscle above that of the normal TA muscle. The proximal 295 bp of the MHC IIB promoter is required for appropriate upregulation in the functionally overloaded plantaris muscle (32) and downregulation in the unweighted soleus muscle (18). Interestingly, those elements of the β-MHC promoter shown to be important for high levels of expression in cell culture (31) are not required for either the upregulation (32) or downregulation (18) of expression from this promoter. Differences in analysis of cis-acting elements within the rat α-MHC promoter have also been found between cell culture and whole animal studies (5). Thus the present data suggest that the use of somatic gene transfer to identify important cis-acting elements for fiber-type-specific expression of the MHC IIB gene appears to be both feasible and the appropriate approach.

Elements within the MHC IIB gene. Because only the proximal 295 bp are required for fiber-type-specific activity, the region downstream of –295 likely contains at least one cis-acting element that drives fiber-type-specific expression. Within this region are numerous candidate elements that may bind transcription factors in a fiber-type-specific manner. The MHC IIB gene has a typical TATA box located –27 relative to the TSS which cannot be exchanged for another TATA box, implicating the importance of the context with which the TATA box resides (11). There is also a CArG box, found in numerous genes expressed in a muscle-specific fashion (35), located at –109 to –88. The MHC IIB gene also has three A-T-rich regions within the proximal 220 bp of the TSS which may bind members of the myocyte enhancer factor 2 family (4, 13, 39). An E-box (centered around –63) which serves as an element for transcription factors of the basic helix-loop-helix family, including the four identified myogenic proteins (MyoD, MRF-4, Myf-5, myogenin) also lies within the proximal 295 bp. Interestingly, MyoD and myogenin have been shown to preferentially accumulate in fast and slow adult muscles, respectively (15, 16, 37). The A-T elements, CArG box, and the E-box are missing from both pGL3IIB0.06 and pGL3IIBΔ0.88. Both of these reporter gene constructs demonstrate low activity in all muscle (Fig. 3) and in cell culture (data not shown). It remains to be determined which, if any, of these elements is required for the high-level activity in skeletal muscle, and further, high-level activity in fast-twitch muscle.

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