Myocyte Enhancer Factor-2 and Serum Response Factor Binding Elements Regulate Fast Myosin Heavy Chain Transcription in Vivo*

Received for publication, February 2, 2005, and in revised form, February 16, 2005
Published, JBC Papers in Press, February 22, 2005, DOI 10.1074/jbc.M501207200

David L. Allen‡, Jesse N. Weber§, Laura K. Sycuro§, and Leslie A. Leinwand¶

From the Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, Colorado 80309-0347

Adult fast muscle fibers express distinct myosin heavy chains (MyHC) in differing proportions, but the mechanisms underlying their differential expression remain undefined. We used a variety of in vitro and in vivo approaches to explore the contribution of transcriptional regulation to adult fast MyHC expression. Here we show that 800–1000 bp of a sequence upstream of the three mouse adult fast MyHC genes (Ia, Iib, and IId/x) are sufficient to drive muscle-specific and fiber-specific expression in vivo. We show that the upstream promoter region of the gene most abundantly expressed in mouse skeletal muscles, Iib MyHC, retains binding activity and transcriptional activation for three positive transcriptional factors, the serum response factor, Oct-1, and myocyte enhancer factor-2, whereas the other two genes (Iia and IId/x) have nucleotide substitutions in these sites that reduce binding and transcriptional activation. Finally, we demonstrate that regions upstream of 300 bp modulate the effects of these elements. Together, these data demonstrate that the quantitative differences in MyHC expression in mouse skeletal muscle have evolved at least in part through the elimination of positive-acting transcription factor binding sites.

The primary function of skeletal muscle is to produce contractile force via the formation and maintenance of a highly specialized contractile apparatus. The myosin heavy chain (MyHC) protein contains both the α-helical rod domain necessary for thick filament formation and the ATPase domain necessary for converting chemical energy into mechanical force (1). The formation of different types of muscle fibers depends on differential expression of a suite of contractile and metabolic proteins specialized for each fiber type (3). The myogenic regulatory factor family of transcriptional activators, which includes myf-5, MyoD, myogenin, and myogenic regulatory factor-4 (4, 5), and the myocyte enhancer factor-2 (MEF-2) family of transcriptional factors (6) are believed to underlie the expression of most if not all muscle-specific genes. Other muscle-enriched transcriptional factors, including the serum response factor (SRF) (7), myocyte nuclear factor (8), and MEF-3 (9), have been shown to contribute to muscle-specific gene expression, either alone or by interacting with ubiquitously expressed factors such as nuclear factor-1 (NF1), Sp-1, and Oct-1 (10–12).

In addition, the calcineurin/NFAT (nuclear factor of activated T cells) pathway appears to be involved in the slow-specific expression of the myoglobin and troponin I slow genes (13–17). Calcineurin is a phosphatase that dephosphorylates the NFAT family of transcription factors, allowing them to enter the nucleus, bind to specific sequences in slow-specific genes, and activate their expression (13). MEF-2 is also activated by calcineurin as well as by calcium-calmodulin kinase and may activate transcription of some slow fiber-specific genes (18, 19). Finally, studies on the Iib MyHC promoter have identified a proximal MyoD binding E-box that is necessary for fast muscle-specific expression of the Iib gene in vivo (20). However, little is currently known about the factors that regulate fiber-specific expression of type I versus Iia versus IId/x versus Iib MyHC within individual muscle fibers.

We reported previously the isolation of the upstream promoter regions of the three adult fast skeletal MyHC genes and their activity in C2C12 myotubes (21). These studies demonstrated that ~1 kb of the upstream promoter sequence was sufficient to confer muscle-specific, differentiation-sensitive expression in vitro. But although tissue culture systems are excellent models for studying muscle differentiation and for screening the quantitative effects of individual promoter mutations on gene expression, cultured myotubes cannot adequately mimic the complexity of skeletal muscle in vivo. Thus in vivo analyses are necessary to determine the role of specific regulatory elements in regulating MyHC gene expression in fully mature muscle fibers. In addition, fiber-specific gene expression cannot be modeled in vitro, because myotubes in culture do not mature to the point where fiber type specialization occurs.

The purpose of the present study was to evaluate the activity and specificity of the three adult fast MyHC promoters within skeletal muscle in vivo and to identify elements involved in their regulation.
differential expression in different muscle and fiber types in vivo. We show that ~1 kb of the upstream promoter sequence of the adult fast MyHC genes is sufficient to confer muscle-specific and fiber-specific expression in vivo. Moreover, we determined the quantitative role of specific cis-regulatory domains on expression of the adult fast MyHC promoters in vivo.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—Isolation of the three adult fast MyHC promoters from murine genomic DNA has been described previously (21). Briefly, ~0.8–1 kb (1000 bp for IIa, 990 bp for IId/x, and 780 bp for IIb) was cloned into the firefly luciferase reporter plasmid VR1255 (Vical, San Diego, CA). A reporter plasmid containing a Renilla luciferase gene driven by a cytomegalovirus promoter (pRL-CMV; Promega, Madison, WI) was used as an injection control for all in vivo injections. We also created reporter constructs containing an enhanced green fluorescent protein gene (eGFP) by excising the firefly luciferase reporter gene from the promoter-VR1255 constructs using the SalI and BamHI sites and generating eGFP by high fidelity PCR with these sites added to the ends. All clones were checked by sequencing at the DNA Sequencing Core of the University of Colorado to ensure that they contained the appropriate deletions or mutations.

**Cell Culture and Transfection**—C2C12 myoblasts were plated and cultured as described previously (21, 22). Promoter-reporter plasmids were transfected into proliferating myoblasts using Lipofectamine 2000 (Invitrogen) per the manufacturer’s instructions. Two days after transfection, myoblasts were differentiated into myotubes as described previously (22). Double-stranded oligonucleotides were labeled with [α-32P]dCTP, and unlabeled radiolabeled oligonucleotides were removed by spin column purification. Binding reactions were carried out using 100 μCi of labeled probe, 10 μg of nuclear extract.

**Intramuscular Plasmid DNA Injections**—Plasmid DNA injections were carried out using standard techniques (20). Plasmid DNA was purified by cesium chloride gradient centrifugation; 25 μg of DNA was injected per muscle in 25 μl of total solution for the tibialis anterior (TA), and 100 μg of DNA in 100 μl of total solution was injected into the calf. For all studies a control plasmid was co-injected (1.25 μg of pRL-CMV) to control for injection efficiency. Seven days after injection, muscles were surgically removed and homogenized in lysis buffer (25 mM glycylglycine, pH 7.8, 15 mM MgSO4, 4 mM EGTA, and 1 mM dithiothreitol), and the lysate was centrifuged for 30 min at 3000 × g. The supernatant was removed and stored at −20 °C until use. Dual luciferase assays were carried out using a commercially available kit (Promega, Madison, WI). For all experiments, firefly luciferase values were divided by the Renilla value to normalize for differences in transfection efficiency.

**GFP Analysis**—For constructs containing a GFP reporter, muscles were excised 7 days after injection, frozen in isopentane cooled in liquid nitrogen, and stored at −70 °C until use. Muscles were sectioned and stained with anti-MyHC antibodies as described previously (24). The primary antibodies used were MHCs (Novocastra, Newcastle-on-Tyne, England) specific for type I MyHC, SC-71 specific for IIa MyHC, BF-F3 specific for IIb MyHC, and 6H1 specific for IId/x MyHC (23). Sections were incubated in a secondary antibody, namely goat anti-mouse IgG or IgM tetramethylrhodamine isothiocyanate (TRITC) conjugate for MyHC and goat anti-rabbit TRITC for laminin at 1:50 dilution for 1 h at room temperature. Sections were again washed in phosphate-buffered saline. After staining, the GFP-positive fibers were found by using the original video images as a guide, and their reaction with the various antibodies was scored.

**Binding Assays**—Nuclear extracts were isolated from 10–20 15-cm dishes of cultured non-muscle L cells, C2C12 myoblasts, and C2C12 myotubes as described previously (22). Double-stranded oligonucleotides were labeled with [α-32P]dCTP, and unlabeled radiolabeled oligonucleotides were removed by spin column purification. Binding reactions were carried out using 100 μCi of labeled probe, 10 μg of nuclear extract.

**FIG. 1. Muscle-specific expression of the three adult fast MyHC promoters.** A, absolute firefly luciferase levels for heart and skeletal muscle in vivo. Equal amounts of each promoter plasmid were injected into either the heart or the TA muscle of adult mice, and activity was evaluated using a luminometer 7 days after injection. B, Western blot analysis of MyHC isoform expression from the TA muscles of eight different adult mice. C–F, relative luciferase activity of the three adult fast MyHC promoter constructs following injection into the TA (C), gastrocnemius (Gastroc.) (D), plantaris (Plant.) (E), and soleus (Sol.) (F) muscles of adult mice. For all animals, MyHC promoter-driven firefly luciferase values were normalized to Renilla luciferase levels driven by the CMV promoter. Luciferase data in panels A and C are expressed as mean ± S.E. for 3–5 animals per construct. *, significantly different from heart; p < 0.05.
Activity of the Adult Fast MyHC Promoters

**RESULTS**

The Adult Fast MyHC Promoters Are Skeletal Muscle-specific—We sought to determine whether the upstream promoter sequences of the three adult fast MyHC genes were sufficient to restrict reporter activity to skeletal muscle in vivo. Activity of the adult skeletal fast MyHC promoters was considerably greater when injected into the TA muscle than when injected into the heart. Absolute luciferase values for the IIA, IID/x, and IIB MyHC promoters were 5, 1600-, and 3200-fold greater, respectively, in skeletal muscle versus heart (Fig. 1). The level of IIA MyHC promoter activity was much lower in the TA than that of IID/x or IIB, presumably because ~10% of the fibers in the TA muscle express IIA MyHC (24). These data suggest that ~1 kb of an upstream promoter sequence is sufficient to confer expression of the three adult fast MyHC genes in skeletal but not cardiac muscle in vivo.

The adult fast MyHC promoters also show muscle-tissue specificity in skeletal muscle in vivo. In the TA muscle, IIB MyHC accounts for ~70% of the total MyHC protein, whereas IID/x and IIA MyHC account for ~20% and 10%, respectively (Fig. 1B). Quantification of the activity of the IIA, IID/x, and IIB promoters following injection into the TA muscle revealed a similar pattern, with IIB > IID/x > IIA (Fig. 1C). Injection into muscles containing different percentages of the three adult fast MyHCs further revealed that MyHC promoter activity mimicked endogenous MyHC protein expression. For example, the gastrocnemius, which has a fiber percentage similar to that of the TA, showed high levels of IIB promoter activity (Fig. 1D), whereas the soleus, which expresses ~50% type IIA, had the highest IIA promoter activity (Fig. 1F). The plantaris, which is composed of a mixture of primarily IIB and IID/x fibers, had comparable IIB and IID/x MyHC promoter activities (Fig. 1E). Thus, the three adult skeletal fast MyHC promoters were sufficient to confer muscle type-specific expression in vivo.

Fiber-specific Promoter Activity—To test whether the adult fast MyHC promoter regions were sufficient to drive muscle fiber-specific expression, we created constructs in which the adult skeletal fast promoters were linked to an eGFP reporter gene. These were injected into mouse TA muscles, and the relationship between GFP expression and endogenous MyHC protein expression was examined using immunohistochemistry. Expression of the IID/x MyHC promoter was fiber-specific; of 21 IID/x-GFP-positive fibers, 100% (21/21) were found to express IID/x MyHC (Table I and Fig. 2). Similarly, the IIB MyHC promoter was also fiber-specific; of 15 IIB-GFP-positive fibers, 100% (15/15) were positively stained for an antibody specific for IIB MyHC (Table I). In contrast, a positive control plasmid containing a CMV upstream enhancer driving GFP did not show fiber specificity and was expressed in all fiber types (Table I and Fig. 2).

Initially, we were unable to obtain any GFP-positive fibers following injection of the 1000-bp IIA-GFP construct into untreated control mouse TA; this was probably due to the small number of IIA-expressing fibers ordinarily present in the adult mouse TA muscle. We therefore injected the soleus muscle, which has more IIA-expressing fibers, and pretreated muscles with the myotoxin Marcaine, which has been shown to increase the uptake of plasmid DNA (25). When the IIA MyHC promoter construct was injected into Marcaine-treated calf muscles, >90% of the 1000-bp IIA-GFP fibers (42/46) were positive for IIA MyHC protein by immunohistochemistry (Table I). Thus, 800–1000 bp of the three adult fast MyHC promoters was sufficient to drive fiber-specific expression in vivo.

The AT2 Element and Promoter Activity in Vitro—Based on the observation that these sequences were sufficient to direct fiber-specific expression, we sought to identify regulatory elements within the IIA, IID/x, and IIB MyHC promoter regions that are involved in their differential expression. For example, as shown above, >70% of the MyHC expressed in the TA muscle is the IIB isoform, suggesting that the IIB MyHC gene is more transcriptionally active in this muscle than the IID/x or IIA genes. We therefore searched for potential transcriptional activator binding sites present in the IIB but not in the IID/x or IIA MyHC genes. We focused on two elements in the proximal promoter regions of the IIB MyHC gene, an AT-rich region called AT2 (13) and a CArG element.

The AT2 region has been shown previously to bind to both the muscle-enriched transcription factor MEF-2 and the ubiquitously expressed transcription factor Oct-1 (13). In the IIB MyHC promoter, a TT to CC substitution has occurred in this

---

**TABLE I**

Summary of GFP injection experiments

| Construct | No. of animals | No. of GFP-positive fibers | No. of fibers expressing same MyHC | Percentage |
|-----------|----------------|---------------------------|-----------------------------------|------------|
| CMV       | 4              | Hundreds                  | Variable                          | ~25%       |
| IIA (soleus) | 6            | 43                        | 47                                | 91%        |
| IID/x     | 8              | 22                        | 22                                | 100%       |
| IIB       | 8              | 15                        | 15                                | 100%       |

---

**FIG. 2. The MyHC promoters are muscle fiber type-specific in vivo.** Representative cross-sections from adult mouse TA muscle following injection with GFP reporter plasmids driven by either the IID/x, IIB, or CMV promoters followed by immunohistochemistry for specific MyHC isoforms. GFP-positive fibers have been numbered for easier identification between the GFP images (top row) and the tetramethylrhodamine isothiocyanate (TRITC)-labeled immunostaining (bottom row). For both the IID/x promoter and the IIB promoter, GFP-positive fibers were always positive for the corresponding MyHC isoform despite the presence of negative fibers nearby; three IID/x-GFP-positive fibers and two IIB-GFP-positive fibers are numbered in the respective panels for illustration. For CMV-GFP, no fiber specificity was evident; the representative section was immunostained with antibodies to both IIA (red) or IIB (green) to better illustrate this observation. The three CMV-GFP-positive fibers numbered in the right hand panel showed IIA immunostaining (fiber 1), IIB immunostaining (fiber 2), and immunostaining of neither (fiber 3). Note that the IIB images were taken at a slightly higher magnification than the IID/x or CMV images.
A. **MyHC Isoform** | **AT2 Sequence**
---|---
Mouse IIb | 5′-TCAAATATTATAGATGA-3′
Mouse IId/x | 5′-TCAAATATTATAGATGA-3′
Mouse IIa | 5′-TCAAATATTACATAGAGA-3′

**FIG. 3.** The AT2 element activity is decreased in the IIa and IId/x promoters. A, the sequence of the AT-rich region AT2 for the mouse IIa, IId/x, and IIb genes. The binding sites for the transcription factors Oct-1 and MEF-2 as determined by electrophoretic mobility shift assays and DNA footprinting by Lakich et al. (12) are boxed. Nucleotides in the IIa and IId/x MyHC promoters that differ from the IIb AT2 sequence are underlined. B, relative luciferase activity of the wild type IIa MyHC promoter (left bar), the IIa MyHC promoter with the two nucleotides found in the IId/x promoter (middle bar), and the IIa MyHC promoter with all four nucleotides changed so that they are identical to the IIb AT2 sequence (right bar). Activity of the promoters was normalized to the mean of the wild type. C, relative luciferase activity of the wild type IId/x promoter (left bar) and the IId/x promoter with the either the IIa (middle bar) or IIb AT2 sequence (right bar) inserted via mutagenesis. D, relative luciferase activity of the wild type IIb promoter (left bar) and the IIb promoter with the same sequence as the IIa MyHC AT2 (right bar). Activity of the wild type and mutated promoters was normalized to the mean of the wild type for all experiments. All values are mean ± S.E. of 3–6 experiments. *, significantly different from wild type promoter construct; p < 0.05.

B. **IIa MyHC**

C. **IId/x MyHC**

D. **IIb MyHC**

**FIG. 4.** The IIb AT2 binds Oct-1 and MEF-2. Electrophoretic mobility shift assays were run with double-stranded oligonucleotides containing the IIa (lanes 1–4), IId/x (lanes 5–8), IIb (lanes 9–12), or IId-Int AT2 hybrid (lanes 13–15). Lanes 1, 5, 9, and 13, probe alone; lanes 2, 6, 10, and 14, probe plus C12C12 myotube nuclear extract; lanes 3, 7, and 11, probe plus myotube extract plus polyclonal antibody to Oct-1; and lanes 4, 8, 12, and 15, probe plus myotube extract plus polyclonal antibody to MEF-2. The position of the Oct-1 and MEF-2 bands is shown by arrows; an arrowhead marks the position of the supershifts (SS) induced by the addition of Oct-1 or MEF-2 antibodies.

tested the three different AT2 sequences for protein binding with nuclear extracts from C12C12 myotubes. Several complexes formed with the IIb AT2 oligonucleotide (Fig. 4, lane 10). The addition of an antibody to Oct-1 resulted in elimination of the second slowest migrating complex and the formation of a band shift (Fig. 4, lane 11), and the addition of an antibody to MEF-2 resulted in elimination of the topmost, slowest migrating complex and the formation of another supershift (Fig. 4, lane 12). These data confirm that the IIb AT2 element can bind both Oct-1 and MEF-2 as described previously (13). In contrast, the IIa AT2 showed little protein binding (Fig. 4, lanes 1–4). The faint, slowly migrating complex in the IIa AT2 lanes ran at the same position as the Oct-1 complex and was slightly decreased by the addition of an Oct-1 antibody (Fig. 4, lane 3). No complex was observed at the MEF-2 position, and the addition of an MEF-2 antibody did not produce a band shift (Fig. 4, lane 4). Thus, MEF-2 binding is eliminated, and Oct-1 binding is greatly reduced in the IIa AT2 element as compared with the IIb AT2.

The IId/x AT2 oligonucleotide did bind to both Oct-1 and MEF-2, but the binding was consistently less strong for both factors than that of the IIb AT2 (Fig. 4, lanes 5–8). We therefore created an oligonucleotide that contained the TG sequence found in the IId/x AT2flanking region in place of the GA found in the endogenous IId/x AT2 and found that this restored Oct-1 and MEF-2 binding as compared with the endogenous IId/x AT2 sequence (compare lane 6 with lane 14 in Fig. 4). In summary, the nucleotide substitutions in the IIa and IId/x AT2 regions result in impairments in Oct-1 and MEF-2 binding.

region during the divergent evolution of these genes (Fig. 3A). In addition, both the IIa and the IId/x MyHC genes contain an AT to GA substitution in the region immediately flanking the MEF-2 binding site (Fig. 3A). Mutagenesis of the IIa AT2 element so that it was identical to the IId/x AT2 motif resulted in a significant increase in IIa MyHC promoter activity in C12C12 myotubes compared with the wild type IIa MyHC construct (Fig. 3B). Further mutagenesis of the IIa MyHC AT2 region so that this element was then identical to that of the IIb AT2 resulted in a further increase in IIa MyHC promoter activity by ~150% that of the wild type IIa MyHC promoter (Fig. 3B).

Conversely, mutation of the IId/x MyHC AT2 to the sequence of the IIa AT2 resulted in a significant 50% decrease in activity of this promoter, whereas mutation to the IIb AT2 sequence resulted in a significant 50% increase in activity of the IId/x promoter (Fig. 3C). Finally, mutagenesis of the IIb AT2 so that it was identical to the IIa AT2 element decreased IIb activity significantly (Fig. 3D). Thus, the nucleotide substitutions in the IIa and IId/x MyHC AT2 elements relative to the IId/x promoter result in significant reductions in promoter activity relative to the IIb MyHC promoter sequence such that IIb > IId/x > IIa.
compared with the IIb AT2 sequence.

The CArG Element and Promoter Activity—A second potential activator element in the IIb promoter is the CArG box at approximately -80 to -120 bp upstream from the transcription start site. CArG boxes (CC(A/T)6GG) have been shown to bind SRF, a potent transcriptional activator (26). In the mouse IIb gene this element consists of an SRF consensus binding sequence flanked by two TTGCCN consensus sequences for the NF1/CCAAT binding factor (Fig. 5A). However, both the IIa and the IId/x MyHC promoters contain nucleotide substitutions within the SRF consensus core.

Mutation of the IIb SRF consensus site to that of the IIa or IId/x results in a significant decrease in IIb promoter activity. C, mutation of the IId/x CArG-like sequence to that of the IIa or IId/x MyHC sequence significantly increases IId/x promoter activity, whereas mutation of the IIb CArG site has no significant effect. B, injection of the IId/x MyHC promoter construct and a IId/x MyHC construct in which the CArG-like element has been deleted into mouse TA muscle. All results are the mean ± S.E. of 3–6 experiments each, 4–6 wells per experiment. *, significantly different from wild type construct; p < 0.05.

Sensor Constructs Reveal That the IId/x CArG-like Element Is a Repressor—To test whether the IId/x CArG-like element acts as a repressor in a heterologous context, we created “sensor” constructs in which four tandem copies of either the IIb CArG element or IId/x CArG-like element were placed upstream of a minimal 100-bp thymidine kinase promoter (TKp100) driving luciferase expression (Fig. 6A). As expected, placing four copies of the IIb CArG element upstream of TKp100 dramatically increased activity by 5-fold (Fig. 6A). In contrast, adding four copies of the IId/x CArG-like element upstream of TKp100 significantly decreased promoter activity (Fig. 6B). Finally, a sensor construct consisting of five tandem copies of the c-fos SRE driving luciferase increased activity by ~5-fold (Fig. 6B). Together, these data suggest that the IIb CArG MyHC promoter results in an increase in promoter activity suggests that the IId/x CArG-like element acts as a repressor.
element is a relatively weak activator, whereas the II/d/x CArG-like element acts as a repressor both in the context of the MyHC promoters as well as in a heterologous promoter context.

The II/d/x CArG-like Element Binds a Protein That Is Not SRF—We then examined the protein binding pattern of the IIa, II/d/x, and IIb CArG-like elements. As a positive control, we first examined the binding pattern of an oligonucleotide containing the c-fos consensus SRF binding sequence. This oligonucleotide showed at least four distinct binding activities, namely a slow migrating doublet and two faster migrating activities (Fig. 7A, lane 2). The topmost, slowest migrating complex was supershifted by addition of an antibody to SRF (Fig. 7A, lane 3). The IIb CArG element also showed four distinct binding activities, although these were typically less strong and less distinct than those observed in the c-fos SRE construct (Fig. 7B, lane 10). Again, the slowest migrating complex in the IIb CArG reactions appeared to be SRF, based on supershifting with an anti-SRF antibody (Fig. 7B, lane 11).

The middle complex seen in the IIb CArG reactions was also observed with the IIa and II/d/x CArG-like elements and was not supershifted with an anti-SRF antibody (Fig. 7B, lanes 2 and 6). However, the II/d/x CArG-like element also demonstrated a slower migrating activity (asterisk in Fig. 7B) that was also not supershifted by the addition of an anti-SRF antibody (Fig. 7B, lane 7) or an antibody to the repressor YY1,2 which often binds to CArG elements, although it was almost completely eliminated by the addition of a 50-fold excess of cold II/d/x CArG-like element probe (Fig. 7B, lane 8). This binding activity was not observed in reactions with the IIa CArG-like element (Fig. 7B, lane 2). We tested several other nuclear extracts to see whether they contained proteins that would produce binding activities similar to that of myotube nuclear extracts with the II/d/x CArG-like element. Interestingly, the complex was present in all extracts tested, including myoblasts, myotubes, neonatal skeletal muscle, non-muscle fibroblastic L cells, and liver.2 Finally, the II/d/x CArG2 element also appeared to bind to SRF, but the binding was extremely weak (Fig. 7C).

Combined AT2 and CArG-like Element Mutations and MyHC Promoter Activity—The data presented above show that nucleotide substitutions in the AT2 and CArG-like elements of the IIa and II/d/x MyHC promoters eliminate activator sites found in the IIb MyHC promoter and that mutation of these elements individually affects MyHC promoter activity. We therefore tested the effects of mutations to both of these elements on MyHC promoter activity in C2C12 myotubes in vitro and in mouse TA muscle in vivo. Mutation of the IIa MyHC promoter so that both the AT2 and CArG-like elements were identical to the IIb sequences resulted in a significant increase in promoter activity compared with that of the wild type constructs in C2C12 myotubes and a 2-fold increase in mouse TA muscle, whereas changing both the IIb AT2 and CArG elements to the IIa sequences for these elements resulted in a large decrease in IIb promoter activity to ~10% of wild type in C2C12 myotubes and in TA muscle (Fig. 8A). Our hypothesis from these experiments was that these two elements in the IIb promoter region contribute to the fiber specificity of this gene. However, when the constructs containing the IIa MyHC promoter driving GFP expression were mutated such that the AT2 and CArG elements were identical to those of the IIb MyHC promoter, we did not observe promiscuous expression of GFP in muscle fibers in the IIb-fiber-enriched TA muscle.2

Upstream Regions Modulate the Effects of the AT2 and CArG Elements—Finally, we wished to determine whether the effects of these elements were modified by upstream elements. We therefore created deletion constructs for all three adult fast MyHC promoters and injected them into mouse TA muscle. Deletion to either 300 or 150 bp, which still contain both the AT2 and CArG elements, resulted in a significant decrease in activity.

---

2 D. L. Allen, J. N. Weber, L. K. Sycuro, and L. A. Leinwand, unpublished observations.
of all three adult fast MyHC promoters and eliminated the differential expression of these promoters in vivo (Fig. 9A). This result suggests that elements upstream of 300 bp are necessary to produce high level and differential expression in vivo.

We further tested the role of the upstream regions of the IIa and IId/x MyHC promoters by creating domain swapped constructs in which the distal 500 bp was excised and either religated onto its proximal region (Fig. 9B). Conversely, removing the IIb distal region and replacing it with the IIa distal region resulted in a nearly 3-fold increase in IIb MyHC promoter activity (Fig. 9B). These results suggest that the IIa distal region contains activator elements and/or that the IIb distal region contains inhibitor elements. To confirm this notion, heterologous constructs were created in which the IIa 500-bp distal region was ligated onto a minimal TKp100 promoter (Fig. 9B).

**DISCUSSION**

In adult skeletal muscle, different isoforms of fast MyHC are expressed in distinct fast muscle fiber types. A greater understanding of the factors regulating expression of the fast MyHC isoforms at different levels in different muscles would provide insights into how these different skeletal muscle fiber types are established and maintained. Although considerable progress has been made in elucidating the molecular genetics underlying muscle-specific and slow versus fast gene expression, little is currently known regarding the molecular mechanisms governing gene expression within the fast subfamily. We recently reported the characterization of the upstream promoter sequences of the three adult skeletal fast MyHC genes in C2C12 myotubes (21). This study identified several regulatory elements involved in differential expression of the fast MyHC genes in vitro. The present study is the first to compare directly the sequences and activities of all three adult fast promoters in skeletal muscle in vivo.

In the present study ~1 kb of the upstream promoter regions of the three adult fast MyHC genes was sufficient to confer the following: 1) skeletal muscle-specific expression in vivo, because activity was high in skeletal muscle but minimal in heart (Fig. 1A); 2) muscle type-specific expression, as each promoter was differentially active depending on the level of endogenous MyHC isoform expression in different muscles (Fig. 1, B–F); and 3) fiber type-specific expression, as ~800–1000 bp of the promoter linked to GFP was sufficient to restrict expression to the individual fiber types (Fig. 2 and Table I). Thus, most or all of the cis-regulatory elements necessary for this hierarchy of different regulatory decisions, i.e. skeletal versus cardiac, slow versus fast muscle, and IIa versus IId/x versus IIb, are contained within the first 800–1000 bp upstream of the transcription start site, although this does not preclude regions up and downstream of this from contributing to and/or modifying MyHC gene expression.

We used sequence comparison to identify elements within this 1000-bp region that may be involved in the expression of the adult fast MyHC genes in adult muscle in vivo. We focused on two elements in the proximal promoter region, the AT2 and the CARG-like elements, because these had been demonstrated previously to act as activators of the IIb MyHC promoter (12, 27). The IIb AT2 element binds both Oct-1 and MEF-2, but binding is greatly reduced for the IIa and IId/x AT2 elements (Fig. 4), resulting in decreased activity of the IIa and IId/x MyHC promoters (Fig. 3). Similarly, the IIb MyHC promoter contains a CARG box or consensus SRF binding sequence (CCAAAAATGG), but substitutions in the IIa and IId/x promoters have resulted in elimination of SRF binding (Fig. 7), which, in turn, decreases promoter activation (Fig. 5).

Experiments with sensor constructs demonstrated that the IId/x CARG-like element acts as a repressor in a heterologous context (Fig. 6). This IId/x CARG-like element binds a protein that is not antigenically related to SRF (Fig. 7) but is found in both muscle (Fig. 7) and non-muscle nuclear extracts. Analyses using transcription factor binding motif recognition software such as MatInspector have failed to identify a likely candidate for this protein; we are currently attempting to identify this putative repressor. Nevertheless, these data support the hypothesis that the substitutions that occurred in the IId/x MyHC CARG-like element during its evolution from the ancestral MyHC gene have resulted not just in the elimination of an activator site but also in the creation of a repressor site.

The results of the present study support a role for the AT2 and CARG-like elements in the differential expression of the three adult fast MyHC genes. However, because all of the mutagenesis experiments were done using the ~1000-bp upstream promoter regions for all three MyHC genes, the possibility remains that other, yet unidentified elements within this region may modulate the effects of these elements. Indeed, deletion to ~300 bp resulted in a significant decrease in activity of all three MyHC promoters to a level comparable with one
another both in vitro (21) and in vivo (Fig. 9A), strongly suggesting that these elements within the proximal 300 bp interact with element(s) upstream of 300 bp to establish differential expression of the adult fast MyHC genes.

One puzzling aspect of the present data is the role for MEF-2 and SRF in regulating quantitative expression of the three adult fast MyHC genes in vivo. Quantification of SRF levels (28) or MEF-2 sensor activity (18) in adult mice has suggested that expression and/or activity of these factors is low in adult fast skeletal muscle. How is it that these two factors, which are

A. C_{2}C_{12} Myotubes
B. TA Muscle

![Graph A: C_{2}C_{12} Myotubes](image)

![Graph B: TA Muscle](image)

**FIG. 8. Effects of double mutations in the AT2 and CArG elements on MyHC promoter activity.** A, luciferase activity of wild type and doubly mutated Ila and IIb MyHC promoter constructs in C_{2}C_{12} myotubes. For Ila MyHC, the AT2 and CArG sequences were changed to that of the IIb sequences; for IIb the AT2 and CArG sequences were changed to that of the Ila sequences. All values were normalized to the mean activity of the wild type construct. B, luciferase activity of wild type and doubly mutated Ila and IIb MyHC promoter constructs in mouse TA skeletal muscle in vivo. Constructs are the same as those used in panel A. Values are reported as mean ± S.E. for 4–6 animals each.

**FIG. 9. Upstream elements modulate the effects of the AT2 and CArG elements.** A, deletion constructs of the three adult fast MyHC promoters were created and injected into TA muscles in vivo. *, significantly different from 1 kbp construct; p < 0.05. B, domain-swapping experiments in which the upstream 700 bp of the Ila and IIb MyHC promoter were excised and either relegated onto the endogenous proximal 300 bp or swapped onto the other proximal region and transfected into C_{2}C_{12} myotubes in vitro.
enriched in slow muscles such as the soleus, are involved in the regulation of expression of the fast MyHC genes, particularly Iib MyHC, which is rarely expressed in the soleus under normal conditions? First, there is some evidence that levels of these transcription factors are associated with Iib MyHC levels; dy/dy dystrophic mice, for example, have greatly reduced muscle MEF-2 and SRF concentrations (29) as well as decreased Iib MyHC levels and increased type I MyHC levels (30, 31). Second, it may be that MEF-2 and SRF, which are typically expressed in much higher concentrations in developing muscle, are necessary for the initiation of Iib MyHC expression and that maintenance of fiber specificity requires much lower levels of these proteins in adult muscle. Third, both of these transcription factors associate with a number of other coactivators (32, 33), and these cofactors may be differentially expressed in different fiber types. Moreover, both the MEF-2 and SRF gene are extensively alternatively spliced (26, 34), and different isoforms may be expressed in a fiber-specific manner. Finally, our own data are consistent with the idea that additional elements upstream of the AT2 and CArG elements are also involved in modulating the effect of these elements (Fig. 9). Thus, MEF-2 and SRF appear to be critical for the quantitative expression of the Iib MyHC gene, but other transcription factors and their binding elements are necessary to restrict fast and slow MyHC expression to the appropriate muscle fiber types.

Our data suggest that the AT2 and CArG elements play a central role in the diversification of MyHC isoform expression across different fiber types. Interestingly, comparison of these sites across species reveals that these changes have not been conserved. For example, the Iia AT2 sequence differs in a single nucleotide in pigs from that in mouse or human (5'-TCAATATCATCATAGAGA-3' for mouse/human and 5'-TCAATATCATCATAGAGA-3' for pig, with the differing nucleotide underlined). The CArG-like element in the Iia MyHC promoter also differs between pig, mouse, and human (5'-CCAAGAGGCT-3' for mouse, 5'-CCAAGAGGCTA-3' for human, and 5'-CCAAGACTTT-3' for pig, with the differing nucleotides underlined). Moreover, the consensus AT2 and CArG binding sites found in the mouse Iib MyHC promoter are not conserved in the human promoter and may indeed underlie some of the differences in Iib MyHC expression between mouse and human. Together, these data suggest that these two elements may represent “hot spots” wherein mutations have accumulated that dramatically affect orthologous and paralogous expression of the adult fast MyHC cluster.

In summary, we have demonstrated that ~1 kb of the upstream promoter region of the adult fast MyHC genes is sufficient to confer muscle-specific, muscle type-specific, and fiber-specific activity in vivo. Moreover, we characterized the role of specific cis-elements in the three adult fast MyHC promoters. These studies provide a foundation for the analysis of two questions pertinent to muscle biology: 1) identification of the element(s) responsible for establishing and maintaining fiber-specific gene expression; and 2) elucidating the transcriptional signaling pathways that allow fibers to adapt to changes in neuromuscular activation.

Acknowledgments—We thank Massimo Buvoli for excellent technical assistance in the cardiac plasmid injections and Margaret Isenhart for assistance in maintaining the mouse colony.

REFERENCES

1. Weiss, A., and Leinwand, L. A. (1996) Annu. Rev. Cell Dev. Biol. 12, 417–439
2. Talmadge, R. J., Roy, R. R., and Edgerton, V. R. (1993) Curr. Opin. Rheumatol. 5, 695–705
3. Schiaffino, S., and Reggiani, C. (1996) Physiol. Rev. 76, 371–423
4. Weintraub, H. (1993) Cell 75, 1241–1244
5. Edmondson, D. G., and Olson, E. N. (1993) J. Biol. Chem. 268, 755–758
6. Naya, F. S., and Olson, E. N. (1999) Curr. Opin. Cell Biol. 11, 683–688
7. Vincent, C. K., Gualberto, A., Patel, C. V., and Walsh, K. (1993) Mol. Cell. Biol. 13, 1264–1272
8. Bassel-Duby, R., Hernandez, M. D., Yang, Q., Rochelle, J. M., Seldin, M. F., and Williams, R. S. (1994) Mol. Cell. Biol. 14, 4596–4605
9. Parmacek, M. S., Ip, H. S., Jung, F., Shen, T., Martin, J. F., Vora, A. J., Olson, E. N., and Leiden, J. M. (1994) Mol. Cell. Biol. 14, 1870–1885
10. Koeser, K., Nelson, T., Lupa-Rimball, V., and Blough, E. (1999) J. Biol. Chem. 274, 12095–12102
11. Grayson, J., Bassel-Duby, R., and Williams, R. S. (1998) J. Cell. Biochem. 70, 366–375
12. Lakich, M. M., Diagina, T. T., North, D. L., and Whalen, R. G. (1998) J. Biol. Chem. 273, 15217–15226
13. Chin, E. R., Olson, E. N., Richardson, J. A., Yang, Q., Humphries, C., Shelton, J. M., Wu, H., Zhu, W., Bassel-Duby, R., and Williams, R. S. (1998) Genes Dev. 12, 2499–2509
14. Naya, F. J., Merzer, B., Shelton, J., Richardson, J. A., Williams, R. S., and Olson, E. N. (2000) J. Biol. Chem. 275, 4545–4558
15. Nakayama, M., Staufeler, J., Cheng, J., Bannerjee-Basu, S., Wawrousek, E., and Buonanno, A. (1996) Mol. Cell. Biol. 16, 2408–2417
16. Calvo, S., Venevally, P., Cheng, J., and Buonanno, A. (1996) Mol. Cell. Biol. 16, 1515–1525
17. Calvo, S., Venu, H., Venevally, P., Cheng, J., Karavarakos, I., and Buonanno, A. (2001) Mol. Cell. Biol. 21, 8490–8503
18. Wu, H., Naya, F. J., McInnes, T. A., Merzer, B., Shelton, J. M., Chin, E. R., Simard, A. R., Michel, R. N., Bassel-Duby, R., Olson, E. N., and Williams, R. S. (2000) EMBO J. 19, 1963–1973
19. Wu, H., Rothermel, B., Kanatos, S., Rosenberg, P., Naya, F. J., Shelton, J. M., Hutcherson, K. A., DiMasi, J. M., Olson, E. N., Bassel-Duby, R., and Williams, R. S. (2001) EMBO J. 20, 6414–6423
20. Wheeler, M. T., Snyder, E. C., Patterson, M. N., and Swoap, S. J. (1999) Am. J. Physiol. 276, C1069–C1078
21. Allen, D. L., Sartorius, C. A., Sycuro, L. K., and Leinwand, L. A. (2001) J. Biol. Chem. 276, 43524–43533
22. Allen, D. L., and Leinwand, L. A. (2002) J. Biol. Chem. 277, 45323–45330
23. Lucas, C. A., Kang, I. H., and Hoh, J. F. (2002) Biochem. Biophys. Res. Commun. 297, 303–308
24. Allen, D. L., Harrison, B. C., Sartorius, C., Byrnes, W. C., and Leinwand, L. A. (2000) Am. J. Physiol. 278, C637–C645
25. Wells, D. J. (1993) Annu. Rev. Cell Dev. Biol. 9, 371–423
26. Miano, J. M. (2003) Annu. Rev. Cell Dev. Biol. 19, 2408–2417
27. Takeda, S., North, D. L., Lakich, M. M., Russell, S. D., and Whalen, R. G. (1992) J. Biol. Chem. 267, 16957–16967
28. Gordon, S. E., Fluck, M., and Booth, F. W. (2001) J. Appl. Physiol. 90, 1174–1183
29. Sakuma, K., Nakao, R., Inashima, S., Hirata, M., Kubo, T., and Yasuhara, M. (2004) Acta Neuropathol. 108, 241–249
30. Reggiani, C., Brocks, L., Wirtz, P., Loermans, H., and te Kronnie, G. (1992) Muscle Nerve 15, 199–208
31. Hayes, A., and Williams, D. A. (1996) J. Appl. Physiol. 80, 670–679
32. Naya, F. S., and Olson, E. N. (1999) Mol. Cell. Biol. 19, 2408–2417
33. Wang, D. Z., Li, S., Hockemeyer, D., Sutherland, L., Wang, Z., Schratt, G., Richardson, J. A., Norheim, A., and Olson, E. N. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 14555–14560
34. McDermott, J. C., Cardoso, M. C., Yu, Y. T., Andres, V., Leifer, D., Krainc, D., Lipton, S. A., and Nadal-Ginard, B. (1993) Mol. Cell. Biol. 13, 2564–2577

3 B. C. Harrison, D. L. Allen, A. Hoffman, and L. A. Leinwand, manuscript in preparation.