Different Pathways Regulate Expression of the Skeletal Myosin Heavy Chain Genes*

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Mammalian skeletal muscles are a mosaic of different fiber types largely defined by differential myosin heavy chain (MyHC) expression. Little is known about the molecular mechanisms regulating expression of the MyHC gene family members in different fiber types. In this work, we identified several cis- and trans-elements that regulate expression of the three adult fast MyHC genes. Despite multiple DNA-binding motifs for well characterized muscle transcription factors upstream of all three fast MyHC genes, expression of MyoD/Myf-5, calcineurin, or NFAT3 had different effects on the three promoters. MyoD or Myf-5 overexpression preferentially activated the IIb promoter, whereas NFAT or activated calcineurin overexpression preferentially activated the IIA promoter. Calcineurin had a 50–100-fold stimulatory effect on the IIa promoter, and the known downstream effectors of calcineurin (myocyte enhancer factor-2 and NFAT) cannot completely account for this activation. Finally, we identified two elements critical for regulating MyHC-IIId/x expression: a 130-base pair enhancer element and a CArG-like element that inhibited IIId/x promoter activity in vitro. Thus, we have found specific regulatory pathways that are distinct for the three adult fast MyHC genes. These elements are logical candidates for fiber-specific control of skeletal muscle gene expression in vivo.

The sarcomeric myosin heavy chain (MyHC) gene family consists of eight known isoforms, each encoded by separate genes exhibiting a complex pattern of spatial and temporal regulation (1). Of the eight isoforms, four are expressed in adult skeletal muscle: type I or slow MyHC and three fast isoforms, IIa, IIId/x, and IIb. Greater than 90% of the MyHC in adult skeletal muscle is composed of these latter three gene products.

The three adult fast MyHC isoforms are expressed in different types of skeletal muscle fibers that have different physiological characteristics, with IIA fibers being smaller, slower, and more oxidative; IIB fibers typically being the largest, fastest, and most glycolytic; and IIId/X fibers falling between these extremes (2). A greater understanding of the mechanisms regulating MyHC gene transcription would provide tremendous insights into how these individual fiber types are established and maintained.

Several axes of regulation exist for the members of the MyHC gene family, including tissue-specific (muscle versus non-muscle), muscle type-specific (striated versus smooth muscle), fiber type-specific (fast versus slow), and fiber subtype-specific (fast IIA versus IIId/x versus IIB). Many of these regulatory decisions are likely to be determined by different transcription factor-binding motifs within the upstream promoter regions of the different MyHC genes. For example, the adult fast MyHC-IIA, -IIId/x, and -IIB genes undoubtedly share similar pathways for conferring muscle-specific and fast fiber-specific expression; but because they are expressed in distinct fiber subtypes (IIA, IIId/X, and IIB), they must also have unique regulatory circuits as well. However, because there are no data directly comparing the sequence or physiological regulation of the three adult fast MyHC gene regulatory regions, nothing is known about the mechanisms that regulate the differential expression of these genes in distinct fast fiber subtypes.

Of the three adult skeletal fast MyHC genes, only the promoter region of the mouse MyHC-IIb gene has been analyzed to date (11–16). Several muscle-specific regulatory elements have been found within the proximal IIb promoter, including potential binding sites for the myogenic regulatory factors (MRFs), serum response factor (SRF), and myocyte enhancer factor-2 (MEF-2) (11–16). Overexpression of any of the four MRFs greatly increases IIb promoter construct activity in differentiating C2C12 myotubes (15), whereas gene transfer studies have demonstrated that an E-box just upstream of the transcription start site is necessary for high-level expression of the IIb gene in vivo (16). In addition, mutation of the proximal AT-rich element AT-1 abolishes MEF-2 binding and greatly reduces promoter activity (12). These studies have established that members of the MRF and MEF-2 families of myogenic transcription factors appear to be necessary for high-level, muscle-specific expression of the MyHC-IIb gene.

More recently, progress has been made in elucidating the cis-regulatory sites that contribute to slow versus fast muscle gene expression. One pathway that appears to play a role in specifying slow fiber-specific gene expression is the calcineurin pathway (17, 18). Calcineurin is a phosphatase that dephosphorylates and allows nuclear transport of the NFAT (nuclear factor of activated T-cells) transcription factors (17). NFAT can then bind to and activate the slow fiber-specific gene promoters...
(17). A recent report also implicated MEF-2 transcription factors in calcineurin-dependent, slow fiber-specific gene expression (19). Other elements, including the CACC box (20) and the SURE and FIRE clusters of regulatory elements (21, 22), have also been implicated in slow versus fast fiber gene expression. However, the role of these elements has been largely unexplored with respect to the MyHC gene family.

Thus, a considerable amount of research has been done on the role of various muscle-specific transcription factors and on the factors specifying slow versus fast fiber gene expression. To date, however, there have been no data on the regulation of fiber-specific gene expression within the IIA, IID, and IIB fast fiber subtypes. Although members of the MyHC family are the only genes to date that have distinct fast fiber isoforms, other muscle-specific genes show a quantitative difference in expression between different fast fiber subtypes (23, 24). Understanding the factors regulating the differential expression of the three adult fast MyHC genes should provide insights into the subspecialization of different fiber types.

The purpose of this work was to isolate and compare the activities of the upstream promoter regions of the three adult fast MyHC genes and to begin to dissect the factors responsible for their differential expression. We have isolated the upstream regulatory regions of the mouse MyHC-IIa, -IIB, and -IID/x genes and show that −1 kb is sufficient to direct high-level, muscle-specific expression in vitro. Moreover, the promoters showed differential levels of activity in vivo (IIa > IIB > IIda), and the relative expression levels were identical to the expression pattern of the endogenous MyHC genes. We have identified several cis- and trans-regulatory elements with distinct effects on each of the adult fast MyHC promoter regions that may play a role in determining fiber-specific gene expression in vivo.

MATERIALS AND METHODS

Plasmid Construction—Generation of mouse genomic clones containing MyHC gene sequences was described previously (25). Approximately 1 kb of sequence putative MyHC-IIa promoter sequence was deposited in the GenBank under accession numbers AF081358 (IIa) and AF081359 (IID/x). The cytomegalovirus (CMV) promoter-firefly luciferase plasmid VR1255 (Vical) was used as a backbone for all constructs. The CMV promoter was removed by Ball and SacII digestion, and MyHC promoters were inserted using SalI-SacII sites on each of the three adult fast promoters. Plasmids contained MyHC-IIa sequences from −670 to +7 bp (IIaLuc), MyHC-IIb sequences from −781 to +5 bp (IIbLuc), and MyHC-IID/x sequences from −977 to +5 bp (IID/xLuc). Although the length of all three promoter constructs was somewhat less than 1 kb, we observed identical results in preliminary transfections with constructs containing 1000 bp of each promoter linked to a chloramphenicol acetyltransferase promoter (data not shown); and for ease of communication, these constructs will be referred to as the −1 kb constructs. The promoter deletion constructs were cloned by inverse polymerase chain reaction using the type IIa, IIB, and IID/x VR1255 plasmids as templates and primers with EcoRI restriction sites. The Rous sarcoma virus-MyoD expression vector was a gift of Harold Weintraub; the CMV-Myf-5 expression vector was a gift of Stephen Konieczny; and the CMV-GATA-4, CMV-MEF-2C, CMV-constitutively nuclear NFAT, and CMV-constitutively active calcineurin expression vectors were all kindly provided by Dr. Eric Olson. All plasmid DNA used for transfections was purified by cesium chloride gradient centrifugation.

Cell Culture Transfections—Mouse C1C12 myoblasts and L-cells were obtained from American Type Culture Collection. Mouse C2C12 myoblasts were grown on gelatin-coated dishes in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 4.5 g/liter glucose, 1.5 g/liter sodium pyruvate, 5 μg/ml insulin, 5 μg/ml transferrin, 10% fetal bovine serum (Hyclone Laboratories), and 10% j2% bovine serum (HyClone Laboratories). Cells were transfected at 70–80% confluence with 4 μg of DNA and 15 μl of LipofectAMINE transfection reagent (Life Technologies, Inc.) according to the manufacturer's protocol. In all transfections, 1 μg of a thymidine kinase-Renilla luciferase construct (Promega) was used as an internal control. After a 5-h incubation, the transfection medium was removed and replaced.

Western Blotting and Gel Electrophoresis—For Western blotting and gel electrophoresis, C1C12 myoblasts and myotubes were scraped into myosin extraction buffer (27) and incubated on ice for 1 h. Following centrifugation for 5 min to remove cell debris, the lysate was concentrated using the Centronic-10 microconcentrator system (Amicon, Inc.). Protein concentration was determined using the Bradford assay (BioRad) and was adjusted to 3 mg/ml. Samples were stored at −20 °C until use. High-resolution gel electrophoresis was used to separate the different isoforms as described by Talmadge and Roy (28). Proteins were transferred to polyvinylidene difluoride membrane overnight using a mini blot transfer apparatus (Bio-Rad) and blotted for total MyHC using F-59, an antibody that recognizes all skeletal isoforms of MyHC (29).

RESULTS

Structure and Sequence of the MyHC-IIa and MyHC-II/d/x Promoters—Fig. 1A shows that the upstream regulatory regions of the mouse MyHC-IIa, -IIB, and -IID/x sequences share significant homology within the proximal 200–250 bp. The identity between the proximal promoters is as follows: IIa and IIB, 61% identity in 230-bp overlap; IIa and IID/x, 61% identity in 190-bp overlap; and IIB and IID/x, 66% identity in 201-bp overlap. The identity for all three genes is 45% within the proximal 250 bp; over the next −750 bp, the homology drops to 10.2%.

The proximal 250 bp of the mouse adult fast MyHC promoters share four highly conserved elements within the first 250 bp of upstream promoter sequence (Fig. 1A). First, the TATA sequence (TATAAAAG) is identical in all three fast MyHC promoters and is identical to the TATA sequence described previously for other MyHCs (30, 31). The MyHC-IIa gene contains a second TATA box located at −25 bp (CTTAAAAG) that appears to be the functional TATA box in mouse diaphragm muscle, as several clones were sequenced with this start site (data not shown). Second, a motif is found at approximately −100 bp in all three promoters that, in the IIb promoter, contains a consensus CArG box in its core, although there are base pair substitutions in both the MyHC-IIa and MyHC-II/d/x genes that likely eliminate SRF binding (32–36). Nonetheless, the first 7 nucleotides, TTGGCCA, and the last 8 nucleotides, TTGTGCCA, are 100% conserved among all three fast MyHC genes (Fig. 1A). The third shared motif is a 20-bp region designated AT-1 (Lakicevic et al. (12) that is 100% conserved in all three fast MyHC genes (Fig. 1A; see below). This proximal AT-1 element contains a consensus binding site for MEF-2 (CT(A/T)2/GA), shown to be critical in expression of the MyHC-IIb promoter (available under GenBankTM/EBI Data Bank accession number M92099) (12, 13). Finally, a second AT-rich region at approximately −250 bp is also highly conserved among all three fast MyHC genes, with IIB and IID/x sharing 15 of 15 nucleotides and IIB and IIda sharing 15 of 13 (Fig. 1A).

In addition to the CArG-like and AT-rich regions mentioned above, the upstream promoter regions of the MyHC-IIa, -IIB, and -IID/x genes also contain several potential sites for NFAT and MRF binding (Fig. 1B). There are two E-boxes in the proximal MyHC-IIa promoter, seven E-boxes in the IID/x promoter, and two E-boxes in the IIB promoter (Fig. 1). There are
Fig. 1. A, comparison of the first 250 bp of the proximal promoter regions of the mouse MyHC-IIb, -IIa, and -IId/x genes. Asterisks indicate the same base compared with IIb, and spacing (dashes) was introduced to align regions of maximum homology. The sequences were aligned using the ClustalW alignment program in the Vector NTI Suite software package and then adjusted manually to produce optimal alignment. Transcribed bases are underlined. Boxes indicate four conserved elements: a myosin-like TATA box, a CArG box, and two AT-rich motifs. B, schematic of the various putative cis-regulatory elements found in the promoters of the MyHC-IIa, -IIb, and -IId/x genes. MatInspector Version 4.0 and Vector NTI were used to identify putative binding motifs. The exact length of each promoter is given at the end of each promoter.
Expression of all three adult fast MyHC promoters in C_{2}C_{12} myoblasts and myotubes and non-muscle L-cells. Negative controls included a firefly luciferase plasmid with the CMV promoter removed (empty vector) and pCATbasic, a chloramphenicol acetyltransferase reporter construct not expressing luciferase to control for background. Results are expressed as means ± S.E. from three to five experiments. A, C_{2}C_{12} myoblasts and myotubes showing the increase in promoter activity for all three MyHC constructs upon muscle differentiation, as well as differential expression among the IId/x, Iib, and IIa promoters. B, non-muscle fibroblastic L-cells that did not express any of the MyHC promoters above background levels. *, significantly different from L-cells; †, significantly different from myoblasts (p < 0.05). C, expression of MyHC isoforms in C_{2}C_{12} myoblasts and myotubes in vitro. The gel shows MyHC gene expression in C_{2}C_{12} myotubes and adult tibialis anterior (TA) muscle (as a reference) as determined by high-resolution gel electrophoretic separation of the different MyHC isoforms, followed by Western blotting with antibody F-59 to all sarcromeric MyHC isoforms. Emb., embryonic; Peri., perinatal.

Approximately 1 kb of the Upstream Promoter Regions of the Three Adult Fast MyHC Genes Confers Muscle-specific Expression in Vitro—Expression of all three adult fast MyHC promoters was low in undifferentiated C_{2}C_{12} myoblasts and was significantly increased in differentiated C_{2}C_{12} myotubes (Fig. 2A). Differentiation increased promoter activity by 35-fold for the Iib promoter, by 10-fold for the IId/x promoter, and by 5-fold for the Iia promoter (Fig. 2A). Expression was extremely minimal in non-muscle L-cells (Fig. 2B), suggesting that ~1 kb of the three adult fast MyHC promoters is sufficient to confer muscle-specific expression and differentiation-sensitive expression in vitro.

Moreover, activity in myotubes was significantly different among the three adult fast promoters, with IId/x demonstrating the greatest expression, followed by Iib and then Iia (Fig. 2A). Western blotting revealed that, in differentiated C_{2}C_{12} myotubes, the IId/x isoform was the most highly expressed adult isoform (73% of total adult fast MyHC), followed by Iib (17%) and Iia (<9%) (Fig. 2C). These data demonstrate that the adult fast MyHC promoters show differential activity that is consistent with the expression pattern of the endogenous MyHC genes in C_{2}C_{12} myotubes.

Role of the AT-rich and CArG-like Elements in Adult Fast MyHC Promoter Activity—As mentioned above, the 20-bp AT-rich region at approximately ~200 bp is identical in the three mouse adult fast MyHC genes (Fig. 1A). We therefore created internal deletion constructs lacking this 20-bp sequence for all three fast MyHC promoters (Fig. 3A). Expression of all three fast MyHC promoter constructs was significantly reduced to a similar extent by deletion of the AT-rich region (Fig. 3A), suggesting that this element is not involved in the differential expression of the adult fast MyHC genes, but that it plays a role in the overall expression level of all three genes. Moreover, deletion of the AT-rich region did not result in increased expression in non-muscle L-cells, indicating that it is not essential for restriction of muscle-specific expression (Fig. 3B).

Another element found in all three adult fast promoters is the CArG-like element at approximately ~100 bp (Fig. 1A). Deletion of this element in the context of the ~1-kb promoter resulted in a 5-fold decrease in expression of the Iia promoter, whereas Iib promoter activity was not significantly affected (Fig. 4). Surprisingly, deletion of the CArG-like element from the IId/x promoter resulted in a 6-fold increase in IId/x promoter activity (Fig. 4). Thus, the CArG-like element has differential effects on MyHC promoter activity: for Iia, it is an activator; for Iib, it has no effect; and for IId/x, it is inhibitory.

MRF Responsiveness Differs among the Three Adult Fast MyHC Promoters—Previous studies have revealed that the activity of the Iib promoter is sensitive to MRF overexpression (15, 16). In C_{2}C_{12} myotubes, the ~1-kb Iib promoter region behaved as previously described for 192 bp of the Iib promoter by Takeda et al. (15): activity was enhanced ~10-fold by overexpression of MyoD and ~3-fold by overexpression of Myf-5 (Fig. 5A). This 10-fold increase as a result of MyoD cotransfection increased Iib promoter activity such that it was significantly greater than either Iia or IId promoter activity (Table I). In contrast, cotransfection with either MyoD or Myf-5 had no significant effect on the activity of the MyHC-IIa or MyHC-IId/x promoter (Fig. 5A and Table I), despite the presence of multiple E-boxes in each promoter. Infection with a myogenin adenovirus resulted in a 7.5-fold increase in expression of the Iib promoter and no significant increase in the activities of the Iia and IId/x promoters (Fig. 5B). Thus, Iib is the only promoter sensitive to overexpression of all three MRFs.

Differential Sensitivity of the Adult Skeletal Fast MyHC Promoters to Calcineurin and NFAT Overexpression in Vitro—Recent work has supported a role for the calcineurin/NFAT signaling pathway in slow versus fast fiber gene expression (17, 18). Recent studies have shown that calcineurin-dependent activation of the calcineurin/NFAT signaling pathway in slow versus fast fiber gene expression (17, 18).
Among the three adult fast MyHC genes, the progression from faster to less fast is IIb, IId/x, and IIa, with IIa being expressed in the most oxidative fast fibers. We tested the hypothesis that the IIa promoter would be more responsive to calcineurin or NFAT compared with the IIb and IId/x promoters. Cotransfection with a constitutively active calcineurin expression plasmid resulted in a much greater augmentation of IIa promoter activity compared with IIb or IId/x promoter activity; calcineurin increased IIa promoter activity by 50–100-fold, but increased IIb and IId/x promoter activities by only 5–10-fold (Fig. 6A). The increase in IIa promoter activity resulted in IIa promoter activity that was significantly greater than that of IIb and not significantly different from that of IId in response to calcineurin cotransfection (Table I). Similarly, cotransfection with a constitutively nuclear NFAT expression plasmid resulted in a preferential augmentation of expression of IIa compared with IIb or IId/x promoter constructs; IIa promoter activity was increased by ~50% by NFAT, whereas constitutively nuclear NFAT overexpression resulted in a decrease in both IIb and IId/x promoter activities (Fig. 6B). Finally, cotransfection with an MEF-2C expression construct resulted in a 3-fold activation of the IIa promoter, but only minimally affected IIb or IId/x; but because the effect on IIa was highly variable, the results were not significant (Fig. 6A).

Our results demonstrate that the MRF and calcineurin/NFAT systems have differential effects on adult fast MyHC promoter activity when overexpressed in muscle cells. However, the effect of a signaling molecule may be obscured by the presence of cofactors expressed in muscle cells that potentiate or inhibit its effects. We therefore tested the effects of overexpression of MyoD and activated calcineurin on adult fast MyHC promoter activity in non-muscle L-cells. Alternatively, a signaling molecule may already be highly expressed in muscle cells such that overexpression may not produce an effect on promoter activity. Because we have observed, using an MEF-2 “sensor” construct (38), that MEF-2C activity is already extremely high in C2C12 myotubes (data not shown), we tested the effects of MEF-2C overexpression on adult fast MyHC promoter activity in L-cells as well. Overexpression of activated calcineurin showed the same differential effects in L-cells as it did in C2C12 myotubes, with IIa > IId/x > IIb, although the overall magnitude of the response was much less than observed in C2C12 myotubes (Fig. 7, upper panel). Overexpression of MyoD increased the activities of all three adult fast promoters; however, overexpression of MyoD in L-cells increased MyHC-IIb promoter activity to a greater extent than IIa or IId/x promoter activity (Fig. 7, middle panel). Overexpression of MEF-2C in L-cells increased the activities of all three adult fast MyHC promoters by 2–3-fold (Fig. 7, lower panel). These data support the hypothesis that MyoD and calcineurin have differential effects on the adult fast MyHC promoters, whereas MEF-2 has more or less equivalent effects on their expression.
TABLE 1

| Condition | IIa | IIb | IId |
|-----------|-----|-----|-----|
| β-gal control | 3.8 ± 1.7 | 86.7 ± 9.5 | 9.1 ± 1.3 |
| MyoD | 2.0 ± 0.7 | 74.1 ± 9.3 | 99.2 ± 12.3^b |
| β-gal control | 0.67 ± 0.08 | 19.5 ± 1.06 | 1.61 ± 0.06 |
| ca-Cn | 98.3 ± 13.65^a | 112.3 ± 14.91^a | 14.9 ± 1.66^ab |

^a Significantly different from unstimulated constructs (p < 0.05).
^b Significantly different from other stimulated constructs (p < 0.05).

**FIG. 5. Differential MRF responsiveness of the adult skeletal fast MyHC promoters in vitro.** A, MyoD and Myf-5 overexpression. Data are reported as means ± S.E. of the relative luciferase level. MRF overexpression resulted in a preferential increase in IIb promoter activity compared with IIa or IId/x promoter activity. B, myogenin overexpression using an adenovirus system. Data are reported as means ± S.E. of the relative luciferase level. *p < 0.05.

**TABLE 1. Relative activities of MyHC-IIa, -IIb, and -IId promoter constructs in response to MyoD or calcineurin cotransfection.**

Results are mean ± S.E. from four to six different experiments. The data from MyoD and calcineurin experiments are from different experiments done under different experimental conditions, which is why the β-gal control values are different. β-gal, β-galactosidase; ca-Cn, constitutively active calcineurin.

**DISCUSSION**

In the past 10 years, many studies have examined the cis-regulatory elements regulating expression of individual members of the MyHC gene family. The upstream promoter regions of the α- and β-cardiac MyHCs have been extensively characterized both in vitro and in vivo (3–10), as has the promoter region of the adult skeletal fast MyHC-IIb gene (11–16). In many cases, cis- and trans-regulatory elements have been identified that are necessary for muscle-specific and even fast versus slow fiber-specific gene expression. Although these studies have provided invaluable insights into the regulation of individual MyHC isoform genes, there have been no data to date that have directly compared either the promoter sequences or the activities of the different MyHC isoform promoters in the same physiological context. Such a comparison is necessary if

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the elements conferring the sophisticated in vivo expression patterns of the adult fast MyHC genes are to be identified.

In this study, we isolated and sequenced the upstream promoter regions of the mouse MyHC-IIa and MyHC-IIb genes (14), and, along with the previously characterized mouse MyHC-IIb gene, have provided the first data directly comparing the sequences and activities of these promoter regions in vitro.

These data provide the initial basis for determining the regulatory circuits conferring differential expression of these three
genes in distinct muscle fiber types and conferring responsiveness to different physiological stimuli such as altered muscle activation and loading in vivo. As a prelude to studying these complex in vivo conditions, we have first attempted 1) to determine the putative cis-regulatory elements both common to and distinct for each adult skeletal fast MyHC gene; 2) to examine the expression pattern of the three adult fast MyHC promoters in muscle and non-muscle cell types in vitro; and 3) to begin to identify specific cis- and trans-regulatory elements that may direct differential expression of the three adult skeletal MyHC genes.

Comparison of the activities of \(-1\) kb of each of the MyHC promoters in myoblasts, myotubes, and non-muscle cell lines revealed that 1) this length of promoter region was sufficient to confer muscle-specific expression; 2) the activities of all three promoters were much greater in differentiated \(C_2C_12\) myotubes than in myoblasts (Fig. 2A); and 3) differential expression of the three adult skeletal muscle promoters was observed in \(C_2C_12\) myotubes (Fig. 2A) in the order IId/x > Iib > Iia, which correlates with the endogenous MyHC isoform expression pattern in these cells (Fig. 2C). Together, these observations support the contention that \(-1\) kb of upstream promoter contains the regulatory element(s) sufficient to confer muscle-specific, differentiation-sensitive, and differential expression of the three adult skeletal fast MyHC genes in vitro.

Several elements are conserved in the proximal promoter regions of the three adult fast MyHC upstream promoter sequences. One in particular is the AT-rich motif at approximately \(-200\) bp in all three MyHC upstream promoter sequences that shares 20 of 20 nucleotides among all three fast

- Fig. 8. Role of MEF-2- and proximal NFAT-binding sites in the Ila responsiveness to calcineurin. Deletion of the proximal AT-rich element or mutation of the proximal NFAT site in the Ila promoter resulted in an \(-5\)-fold decrease in Ila responsiveness to calcineurin (Cn). Elimination of both elements in the same construct resulted in a further \(-2\)-fold decrease in Ila responsiveness to calcineurin. Elimination of the NFAT site in the 150-bp minimal Ila promoter also reduced calcineurin responsiveness to \(-10\)% of that of the parent 1-kb Ila construct. *, significantly different from \(\beta\)-galactosidase (B-gal)-co-transfected constructs; †, significantly different from all other constructs (\(p < 0.05\)). mNFAT, mutated NFAT.

- Fig. 9. Deletion analysis of the adult fast MyHC promoters. Data are reported as means ± S.E. from four to five experiments. A schematic of putative binding sites for each respective fast MyHC promoter is shown below the respective graph; arrows indicate 5'-deletion sites, and the arrowhead indicates the TATA box. Note that the scale is different for IId/x compared with Iib and Iia.

MyHC genes, but is not found in other members of the MyHC gene family. It is attractive to speculate that it may be involved in conferring fast fiber-specific expression of the MyHC genes.
Deletion of this 20-bp element in the context of the 1-kb promoters resulted in a significant and equivalent decrease in expression of all three fast MyHC promoters in vitro (Fig. 3), consistent with the hypothesis that this element is necessary for high-level expression of all three adult fast promoters in vitro. This element reportedly binds to members of the MEF-2 family of transcription factors (12). Overexpression of MEF-2C resulted in an identical 2-fold increase in promoter activity for all three adult fast MyHC constructs when cotransfected into L-cells (Fig. 7A). Binding of MEF-2 to this element may therefore be critical to the specification of fast fiber-specific expression of all three MyHC genes. However, searches of several fast muscle promoter regions, including muscle creatine kinase, fast troponin C, and myosin light chain-1/3f, failed to find any sequences matching this 20-bp sequence from the MyHC genes (data not shown); so even if this AT-rich region is responsible for activating fast-fiber-specific MyHC gene expression, other fast-fiber-specific genes do not appear to share this same pathway.

Another element that is somewhat conserved is the CArG-like element at approximately 100 bp in all three adult fast MyHC promoters (Fig. 1). For Iib, this sequence is a perfect consensus for binding of SRF, which has previously been implicated in striated muscle-specific gene expression (7). We hypothesized that deletion of this element would have a preferential effect on Iib promoter activity compared with Iia or IId/x promoter activity. Surprisingly, the Iib promoter was the least affected by deletion of this element; its activity was essentially unchanged, whereas the activity of the Iia promoter was decreased by 5-fold, and that of the IId/x promoter was increased by 6-fold (Fig. 4). Both the Iia and IId/x promoters contain base pair substitutions that should greatly reduce or totally eliminate SRF binding (32–36). Together, these data suggest that SRF is probably not involved in this process and suggest that some other transcription factor is binding in this region and having differential effects on MyHC gene expression. The differential effect on Iia versus IId/x expression suggests that this element and its cognate binding protein may be involved in suppressing MyHC-IIId/x expression in IIA fibers. The homology among the three adult fast MyHC promoters extends both upstream and downstream of the core CArG-like element. A recent study elegantly demonstrated that the flank-

ing "arms" of the CArG/serum response element are critical for conferring the specific effects of this element (38). Since these flanking regions were also deleted in this construct, it is possible that they play a role in the function of this element.

Approximately 1 kb of upstream promoter was also sufficient to produce differential responses to MRFs and calcineurin/NFAT. Cotransfection with a MyoD or Myf-5 overexpression construct significantly increased MyHC-IIb promoter activity, but not Iia or IId/x promoter activity (Fig. 5). MyoD cotransfection increased Iib promoter activity such that it was significantly greater than Iia or IId promoter activity (Table I). This preferential effect on the Iib promoter was particularly surprising given that sequence analysis of the IId/x promoter revealed seven potential E-boxes, compared with two for the Iia promoter and two for the Iib promoter. Thus, the absolute number of potential MyoD-binding sites per se does not affect the sensitivity of these promoters to the MRF family members. Conversely, cotransfection with an activated form of calcineurin resulted in an increase in the activities of all three fast MyHC promoters, but the effect was most dramatic on the Iia promoter, with a 100-fold activation (Fig. 6). Since type IIA fibers typically are closer to slow type I fibers in their contractile and biochemical properties, it is possible that the MyHC-IIa gene is regulated more similarly to a slow fiber-specific gene. Our results also demonstrate a prominent role for the MEF-2- and proximal NFAT-binding sites in conferring calcineurin responsiveness of the MyHC-IIa promoter, although even with both of these elements removed/mutated, the Iia promoter still retained substantial sensitivity to calcineurin overexpression. Thus, the MEF-2- and NFAT-binding sites cannot completely account for the activation of Iia promoter activity by calcineurin. Regarding the differential responsiveness of the three adult fast MyHC promoters to MRF and calcineurin signaling, we have also observed a preferential effect of these factors on endogenous MyHC isoform expression such that MyoD overexpression resulted in a preferential increase in MyHC-Iib protein expression, whereas calcineurin overexpression resulted in a preferential increase in MyHC-IIa protein expression.2 Together, these data suggest that the balance between these two

2 D. L. Allen and L. A. Leinwand, unpublished observations.
signaling pathways may play a role in the fiber-specific expression of the three adult fast MyHC genes.

Deletions to between −600 and −300 bp resulted in a significant decrease in the differential expression pattern of the three adult skeletal fast MyHC promoters in vitro. These data support the conclusion that elements between −600 and −300 bp are responsible for conferring high-level and differential expression in muscle cells. Sequence analysis revealed that this deletion would eliminate two NFAT sites and two E-boxes in the IIa promoter, two NFAT sites in the IIb promoter, and four NFAT sites and seven E-boxes in the IId/x promoter. Given the differential sensitivity of the three adult fast promoters to members of these two transcription factor families, it is possible that the loss of these sites was responsible for the loss of differential expression, although it is possible that as yet unidentified regulatory pathways are also important.

Moreover, 5′-deletions in the IId/x promoter suggested that the region between −600 and −450 bp is necessary for the high-level activity of this promoter in vitro (Fig. 9). This region corresponds almost exactly with one that is highly conserved between the mouse and human IId/x promoters (data not shown). An internal deletion of this 130-bp region resulted in a 5-fold decrease in IId/x promoter activity compared with the full-length construct (Fig. 10), in agreement with the 5′-deletion data above. Moreover, addition of this 130-bp region of the IId/x promoter to the 300-bp IIb promoter resulted in a significant increase in promoter activity. Together, these data demonstrate that this 130-bp region is both necessary and sufficient to confer high-level expression in vitro. Deletion of any 30 bp within this region resulted in a decrease in IId/x promoter activity, but to different extents. Deletion 30-1 or 30-3 resulted in a 5-fold decrease in IId/x promoter activity, similar to that obtained by deletion of the entire 130-bp region, whereas deletion 30-2 decreased IId/x promoter activity by only 50% (Fig. 10). However, the final 30 bp of this region resulted in a massive decrease in IId/x promoter activity, reducing activity by 100-fold (Fig. 10). Together, these data suggest that, whereas all parts of this 130-bp element appear to be necessary for high-level IId/x promoter activity, this final 30-bp element, which we have termed a 5-box for its effect on IId/x activity, is absolutely critical. Examination of the sequence of this region revealed no binding sites for currently identified muscle-specific transcription factors; however, TRANSFAC analysis (37) identified possible binding sites for members of the SOX and ternary complex factor family of transcription factors. These factors are known to play roles in diverse developmental processes (40), but have not been directly implicated in muscle-specific gene expression. Alternatively, it is possible that other, as yet unidentified transcription factors are involved in this process. We are currently undertaking studies to identify the factor(s) binding to this region.

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