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

Received for publication, August 20, 2001, and in revised form, September 6, 2001
Published, JBC Papers in Press, September 10, 2001, DOI 10.1074/jbc.M108017200

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

David L. Allen, Carol A. Sartorius, Laura K. Sycuro‡, and Leslie A. Leinwand§

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-IId/x expression: a 130-base pair enhancer element and a CARG-like element that inhibited IId/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, IId/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 IId/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 IId/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, -IId/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, IId/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 differenti-
(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 approximately 1 kb is sufficient to direct high-level, muscle-specific expression in vitro. Moreover, the promoters showed differential levels of activity in vitro (IIa > IIB > IIa), 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 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 each of the mouse MyHC-IIa and MyHC-IId/x promoter sequences was deposited in the GenBank under accession numbers AF0813585 (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 Bsr I and Sac II digestion, and MyHC promoters were inserted using Sall-Sac II 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 (IIb/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 Sal I and H11002 restriction sites. The Rous sarcoma virus-Myc expression vector was a gift of Harold Weintraub; the CMV-Myc-5 expression vector was a gift of Stephen Koncizewsky; 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 C57BL/mice myoblasts and L-cells were obtained from American Type Culture Collection. Mouse C57BL myoblasts were grown on gelatin-coated dishes in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 4.5 g/liter D-glucose, 1.5 g/liter sodium bicarbonate, 1 m M sodium pyruvate, and 10% fetal 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 experiments, 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 with growth medium; differentiation medium (Dulbecco’s modified Eagle’s medium plus 1% fetal bovine serum or horse serum) was added 12–24 h after transfection. Myoblasts were harvested 24–36 h after transfection, and myotubes were harvested 3–4 days after transfection.

For cotransfection studies, a total of 4 µg of DNA, 1.5 µg of MyHC reporter vectors, and either 1.5 µg of a control vector (CMV-Bgalactosidase) or 1.5 µg of the expression vectors were transfected.

Luciferase Assays—A commercially available dual luciferase assay system was used (Promega). Briefly, cells were lysed in 1× Passive Lysis Buffer, and 10 µl of the cell lysate was assayed for both firefly (MyHC promoter constructs and positive controls) and Renilla (internal control) luciferase activities using a standard luminometer. Values are reported as firefly luciferase levels divided by Renilla luciferase levels.

Western Blotting and Gel Electrophoresis—For Western blotting and gel electrophoresis, C57BL 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 Centricon-10 microconcentrator system (Amicon, Inc.). Protein concentration was determined using the Bradford assay (Bio-Rad) 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 miniblot 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-IId/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; IId/x and IIB, 61% identity in 190-bp overlap; and IIA 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 (TATAAAAAG) 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 (TTTAAAAG) 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-IId/x genes that likely eliminate SRF binding (32–36). Nonetheless, the first 7 nucleotides, TTGGCAA, 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 by Lakici 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)4(G/A)), shown to be critical in expression of the MyHC-IId promoter (available under GenBank™/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 IIA and IId/x sharing 13 of 15 (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.
As mentioned above, the 20-bp AT-rich region resulted in a 200% increase in expression of the 1.0-kb pro-moter in C2C12 myoblasts and myotubes and non-muscle L-cells. Negative controls included a firefly luciferase plasmid with the CMV promoter removed (empty vector) and pCAIgat, 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, CACC box, myoblasts and myotubes showing the increase in promoter activity for all three MYHC constructs upon muscle differentiation, as well as differential expression among the IIId/x, IIb, and IId 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 C2C12 myoblasts and myotubes in vitro. The gel shows MYHC gene expression in C2C12 myotubes and adult tibial 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 sarcomeric MYHC isoforms. Emb., embryonic; Peri., perinatal.

Three putative NFAT-hindering sites in the IId promoter, five in the IId/x promoter, and two in the IIb promoter (Fig. 1B). Searches for the consensus sequences (CCCCACCC) of the CACC box (20) and for the SURE and FIRE elements (21, 22) failed to produce any matches in any of the three adult fast MYHC genes (data not shown).

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 C2C12 myoblasts and was significantly increased in differentiated C2C12 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 IId 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 IId (Fig. 2A). Western blotting revealed that, in differentiated C2C12 myotubes, the IId/x isoform was the most highly expressed adult isoform (73% of total adult fast MYHC), followed by IId (17%) and IId/x (<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 C2C12 myotubes.

Role of the AT-rich and CACC-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 CACC-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 IId promoter, whereas IIb promoter activity was not significantly affected (Fig. 4). Surprisingly, deletion of the CACC-like element from the IId/x promoter resulted in a 6-fold increase in IId/x promoter activity (Fig. 4). Thus, the CACC-like element has differential effects on MYHC promoter activity: for IId, 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 C2C12 myoblasts, 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 IId or IId/x promoter activity (Table 1). In contrast, cotransfection with either MyoD or Myf-5 had no significant effect on the activity of the MYHC-IId or MYHC-IIId/x promoter (Fig. 5A and Table 1), despite the presence of multiple E-boxes in each promoter. Infection with a myogenin adenovirus resulted in a 5-fold increase in expression of the IIb promoter and no significant increase in the activities of the IId 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,
Effects of deletion of the CArG-like element from the adult fast MyHC promoters. The activities of the AT-rich region-deleted constructs in C6C12 myotubes showed that deletion of this 20-bp sequence in the context of the –1-kb promoters resulted in a 50% decrease in the activities of all three adult fast MyHC promoters. B, the activities of the AT-rich region-deleted constructs in non-muscle L-cells showed no increase. Data are reported as means ± S.E. from three to five experiments. *, significantly different from full-length constructs containing the AT-rich region (p < 0.05).

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 C6C12 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 C6C12 myotubes, with IIa > IId/x > IIdb, although the overall magnitude of the response was much less than observed in C6C12 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-2C has more or less equivalent effects on their expression. Because calcineurin is thought to act via both the NFAT and MEF-2 families of transcription factors to achieve its effect on gene expression (17, 18), we tested the effects of calcineurin on MyHC-IIa promoter constructs containing deletions or mutations of the MEF-2- and NFAT-binding sites. Deletion of the proximal AT-rich region or mutation of the proximal NFAT site in the IIa promoter resulted in a 5-fold decrease in the calcineurin responsiveness of the full-length IIa promoter (Fig. 8). Mutation/deletion of both elements together resulted in a further decrease in IIa promoter responsiveness to calcineurin to a level ~10% of that of the parent IIa construct (Fig. 8). The activities of the IIa promoters with the AT deletion, the NFAT mutation, or both were not significantly different from the activity of the unstimulated control (Fig. 8). Mutation of the proximal NFAT-binding site within the context of a 150-bp minimal IIa promoter construct, which does not contain the MEF-2-binding site, reduced calcineurin responsiveness to ~10% of that of the parent 1-kb construct (Fig. 8). However, even this 150-bp minimal construct with a mutated NFAT site still retained a 20-fold increase in IIa promoter activity in
The Region between -600 and -300 bp Is Responsible for the Differential MyHC Promoter Activity Observed in Myotubes in Vitro—We sought to determine whether shorter lengths of upstream promoter sequence would maintain or abolish the differential pattern of expression of the three adult fast MyHC promoters. Deletion of the IIa promoter construct from -670 to -520 bp had only minimal effects on IIa promoter activity, but deletion to -300 bp resulted in an -2-fold decrease in promoter activity (Fig. 9). For the IIb promoter construct, deletion from -1000 bp down to 750 bp resulted in a 10-fold decrease in promoter expression, but deletion to -600 bp resulted in an increase in IIb promoter activity back to levels not significantly different from those of the 1000-bp promoter. Deletion to -450 bp again decreased IIb promoter activity by -10-fold, whereas deletion to -300 bp did not further decrease IIb promoter activity (Fig. 9). Deletion of the IId/x promoter construct from -780 to -600 bp resulted in a 2-fold decrease in IId/x promoter activity; further deletion to -450 bp resulted in a 2-fold increase in promoter activity back to levels comparable to those of the -780 bp construct (Fig. 9). Deletion to -300 bp of upstream promoter dramatically reduced expression compared with the -450 bp IIb promoter (Fig. 10). Further deletion to -150 bp did not result in a further decrease in expression of any of the adult fast constructs (Fig. 9), and the -150 bp constructs were still not expressed above background levels in non-muscle L-cells (data not shown), suggesting that muscle-specific expression was maintained. In summary, the region between approximately -600 and -300 bp appears to contain elements necessary for high-level and differential muscle expression of all three adult fast MyHC promoters, whereas the sequence(s) conferring muscle-specific expression still reside within the proximal 150-bp region of the three adult skeletal fast promoters.

Identification of an IId/x-specific Enhancer Element—The results demonstrate that deletion from -600 to -450 bp of the MyHC-IIId/x promoter resulted in a significant decrease in MyHC-IIId/x promoter activity (Fig. 9, upper panel). This corresponds to a region identified by BLAST2 analysis extending from -589 to -460 bp that has high homology between the mouse and human MyHC-IIId/x promoters (data not shown). When this 130-bp region was internally deleted in the IId/x promoter, activity was reduced by -5-fold compared with the parent construct (Fig. 10A). Conversely, addition of one copy of the 130-bp IId/x region to a 300-bp minimal MyHC-IIb promoter resulted in a 4-fold increase in Iib promoter activity (Fig. 10B); addition of two copies of this IId/x domain resulted in an -20-fold increase in IIb promoter activity. Thus, the 130-bp region of the IId/x promoter is both necessary and sufficient to confer high-level expression on a heterologous promoter construct in vitro. We then created four 30-bp internal deletions within this region to identify specific sequences responsible for this effect. All four deletions had deleterious effects on MyHC-IId/x promoter activity; however, deletion of the final 30 bp of this 130-bp region resulted in almost total abolition of MyHC-IId/x promoter activity, reducing it by almost 100-fold (Fig. 10A).

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 both 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
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-IIId/x genes and, along with the previously characterized mouse MyHC-IIb gene (14), 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 MyHC genes.

**Fig. 6.** Differential responsiveness of the adult skeletal fast MyHC promoters to transcription factor overexpression in vitro. C2C12 myoblasts were transfected with the respective adult skeletal fast MyHC promoters (IIa, IIb, and IIId/x) and cotransfected with plasmids overexpressing MEF-2C (A), constitutively active calcineurin (ca-Cn; B), or constitutively nuclear NFAT (cn-NFAT; C). Data are reported as means ± S.E. of the fold increase compared with control cotransfected with pCATbasic. MEF-2C, constitutively active calcineurin, and constitutively nuclear NFAT resulted in preferential stimulation of IIa promoter activity compared with IIb or IIId/x promoter activity. Results are the means of four to six experiments. *, significantly different from IIb (p < 0.05). B-gal, β-galactosidase.

**Fig. 7.** Effects of overexpression of MEF-2C, calcineurin, or MyoD on adult fast MyHC promoter activity in L-cells. Upper panel, overexpression of MEF-2C resulted in an equivalent 2–3-fold increase in the activities of all three adult fast MyHC promoters. Middle panel, overexpression of a constitutively active form of calcineurin (ca-Cn) resulted in increased activity of the IIa promoter only. Lower panel, overexpression of MyoD increased the activities of all three adult fast promoters, although the MyHC-IIb promoter was the most affected. *, significantly different from IIb; †, significantly different from IIa. B-gal, β-galactosidase.
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 fast 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 C2C12 myotubes than in myoblasts (Fig. 2A); and 3) differential expression of the three adult skeletal muscle promoters was observed in C2C12 myotubes (Fig. 2A) in the order IIId/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 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-IId/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 flanking “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

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 deletion of 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 3-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.

Acknowledgments—We thank Brooke Harrison for helpful comments on this manuscript and Jesse Weber and Christopher Miller for help in the cloning and analysis of the deletion constructs.

REFERENCES

26. Deleter in proof