The CACC Box and Myocyte Enhancer Factor-2 Sites within the Myosin Light Chain 2 Slow Promoter Cooperate in Regulating Nerve-specific Transcription in Skeletal Muscle*

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Previous experiments showed that activity of the ~800-base pair MLC2slow promoter was 75-fold higher in the innervated soleus (SOL) compared with the noninnervated SOL muscles. Using in vivo DNA injection of MLC2slow promoter-luciferase constructs, the aim of this project was to identify regulatory sites and potential transcription factors important for slow nerve-dependent gene expression. Three sites within the proximal promoter (myocyte enhancer factor-2 (MEF2), E-box, and CACC box) were individually mutated, and the effect on luciferase expression was determined. There was no change in luciferase expression in the SOL and extensor digitorum longus (EDL) muscles when the E-box was mutated. In contrast, the MEF2 mutation resulted in a 30-fold decrease in expression in the innervated SOL muscles (10.3 versus 0.36 normalized relative light units (RLUs)). Transactivation of the MLC2slow promoter by overexpressing MEF2 was only seen in the innervated SOL (676,340 versus 2,225,957 RLUs; p < 0.01) with no effect in noninnervated SOL or EDL muscles. These findings suggest that the active MLC2slow promoter is sensitive to MEF2 levels, but MEF2 levels alone do not determine nerve-dependent expression. Mutation of the CACC box resulted in a significant up-regulation in the EDL muscles (0.23 versus 4.08 normalized RLUs). With the CACC box mutated, overexpression of MEF2 was sufficient to transactivate the MLC2slow promoter in noninnervated SOL muscles (27,536 versus 1,605,797 RLUs). Results from electrophoretic mobility shift and supershift assays confirm MEF2 protein binding to the MEF2 site and demonstrate specific binding to the CACC sequence. These results suggest a model for nerve-dependent regulation of the MLC2slow promoter in which derepression occurs through the CACC box followed by quantitative expression through enhanced MEF2 activation.

The acquisition and maintenance of an adult skeletal muscle phenotype is regulated, in part, by the activity of specific motor neurons (1–3). An adult skeletal muscle fiber normally expresses a relatively homogeneous pattern of either fast or slow contractile protein isoforms consistent with its type of innervation. These proteins include fast and slow isoforms from the myosin heavy chain, myosin light chain (MLC), 1 tropomyosin, and the troponin I, T, and C gene families (3, 4).

It has been well established that chronic low frequency electrical stimulation of fast muscle fibers, by stimulating either through the nerve or by direct stimulation of the muscle, induces the slow skeletal muscle phenotype (2–4). This phenotype is characterized by an increased expression of the slow contractile protein isoforms. Conversely, if a slow nerve is removed or electrical activity is blocked, there is a transition from a slow to fast muscle phenotype. These transitions in phenotype have been determined at the mRNA level, which suggests that alterations in slow neural activity transcriptionally regulate slow isoform gene expression (3, 5, 6). Regions of the MLC1slow, TnIslow, and MLC2slow genes have been identified that are responsive to slow innervation; however, the specific elements involved have not been isolated (7–10). Potential cis-acting elements include those that are known to regulate qualitative and quantitative skeletal muscle gene expression. Some examples are E-box, MCAT, MEF2, CACC, and CArG box sequences (11–16).

The MLC2slow gene, in particular, is one of the contractile protein isoform genes that is largely regulated by slow innervation. Unlike many of the other contractile proteins genes, the MLC2slow gene is not expressed in newly formed myotubes either in vivo or in vitro (17–20). During skeletal muscle development, in humans and rats, induction of MLC2slow expression occurs late and correlates with the establishment of mature innervation patterns. The requirement of specific neuromuscular activity for MLC2slow gene expression is most evident when studying skeletal muscle regeneration in vivo. Expression of the MLC2slow gene is only detected in the soleus muscle regenerating in the presence of its own nerve (6). In this system, induction of MLC2slow mRNA occurs at a time that is coincident with the establishment of functional neuromuscular junctions (21). Because of this relatively clear on/off pattern of control, the MLC2slow promoter serves as a good molecular tool for the identification of promoter elements responsible for slow nerve-dependent regulation of transcription.

Recently, it was determined that the 800-bp promoter of the rat MLC2slow gene was capable of directing slow nerve-dependent expression in regenerating muscles in vivo (9). As in the previous work, this study combined skeletal muscle regeneration with in vivo injection of plasmid DNA constructs into muscle. The main objectives were 1) to find a smaller region of...
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the 800-bp MLC2slow promoter capable of directing slow nerve-dependent expression, 2) to determine specific cis-acting regulatory sites, and 3) to determine site-specific factor binding and assess potential transcription factor involvement in regulation of the MLC2slow promoter. Using site-directed mutagenesis, three sites (E-box, MEF2, and CACC box) were mutated to investigate their functional role in MLC2slow expression. It was determined that both the CACC box and MEF2 sites were important for appropriate slow nerve-dependent promoter activity during regeneration. These data suggest that the slow nerve acts through the CACC box to derepress the promoter. Once repression is lifted, then high level expression is regulated by MEF2. Interestingly, the proximal combination of the CACC box and MEF2-like sites is seen in other previously defined slow muscle specific regions of contractile protein genes, including TnIslowl, TnCslow, MLC1slow, myoglobin, and slow myosin heavy chain. This suggests that a conserved mechanism may exist by which the CACC box and MEF2 sites contribute to slow nerve-dependent regulation of the subset of slow contractile protein isoform genes.

EXPERIMENTAL PROCEDURES

Generation of the −270MLC2slow Construct—Previous work had generated the −800MLC2slow construct that contains from −800 to +12 of the rat MLC2slow gene inserted upstream of the luciferase cDNA in the pGL3basic vector (Promega Corp.). This template was used to amplify a shortened promoter region from −270 to +12 of the MLC2slow gene. For the polymerase chain reaction, the 3′ primer overlapped a natural EcoRi site at position +12 (5′-CTGCTGTCTTCTGAAGATTC-3′). The 5′ primer at position −270 contained a 5′ KpnI restriction site (5′-GGGATCCCCCATTAGACAATGGCAGG-3′). Standard polymerase chain reaction conditions were followed (22, 23), and the product was ligated into a modified pGL3basic luciferase expression vector (Promega Corp.) containing the desired restriction sites. Identification and orientation of the −270MLC2slow clone was verified by restriction digestion analysis.

Generation of the Mutated Constructs—The QuikChange site-directed mutagenesis kit (Stratagene) was utilized to mutate the MEF2 site, E-box, and CACC box of −270MLC2slow (see Figs. 1 and 3). Complementary primers (mutated bases underlined) containing the mutated MEF2 promoter (Promega Corp.) were designed. The PCR reactions were performed using 5 μl of nuclear extracts, 1 μg of poly(dI-dC)-poly(dI-dC), and 1 ng of labeled probe (40,000 cpm/ng) in binding buffer as outlined elsewhere (23). Cold mutant specific or non-specific (mutated E-box) oligos were added at a 200-fold molar excess, and DNA binding reactions were carried out at 4 °C. For the supershift experiments, preimmunum serum or antibody (1 μl) was added 30 min after the initial incubation, and these samples were incubated for an additional 30 min. The Sp1 and MEF2 antibodies were obtained from Santa Cruz Biotechnology. MEF2A- and MEF2D-specific antibodies were generously provided by Dr. R. Prywes (29), the muscle LIM anti-myosin antibody—TnCslow (Promega Corp.) containing the desired restriction sites. Identification and orientation of the −270MLC2slow clone was verified by restriction digestion analysis.

In Vivo DNA Injection—For promoter and overexpression assays, in vivo DNA injection during muscle regeneration was utilized following the protocol described previously (9). Briefly, 5 μg of the promoter construct (−270MLC2slow, −270MEF2, −270Ebox, or −270CACC) and 3 μg of β-galactosidase control plasmid DNA (vector: SV40 promoter upstream of the β-galactosidase cDNA; CLONTECH Laboratories, Inc.) were mixed in a 10% sucrose-phosphate-buffered saline solution, pH 7.4. For the overexpression studies, 3 μg of the expression vector MEF2A (24), MEF2C (25), or myogenin (26) and 5 μg of the promoter vector were prepared for injection. At day 0, muscle regeneration was induced by injecting bupivacaine hydrochloride (Marcaine®). On day 3, plasmid DNA was injected (40 μl/muscle) with a disposable 0.3cc syringe (28G/1/2 needle). At this time, the nerve to the soleus was cut (innervated SOL) or left intact (innervated SOL). On day 14, the SOL and EDL muscles were collected, immediately frozen in liquid N2, and stored at −80 °C.

Luciferase and β-Galactosidase Assays—Muscles were homogenized in 750 μl of Reporter Lysis buffer (1×, Promega Corp.) and centrifuged at 5000 × g for 20 min at 4 °C. Supernatants were removed for analysis of luciferase and β-galactosidase activity. Luciferase activity was measured using the luciferase assay system from Promega Corp. Each homogenate was tested in duplicate, using 10 μl per reaction. Relative Light Units (RLUs) were integrated over 10 s and were within the linear range. Normalized luciferase activity was expressed as RLUs per RLU and was determined as the average of three independent experiments. All results were statistically analyzed using analysis of variance with significance set a priori at p < 0.05.

Preparations of Nuclear Extracts, Western Blots, and Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared according to recently published techniques (27). Frozen skeletal muscle tissue was minced on ice, homogenized (10 ml HEPES, pH 7.5, 10 mM MgCl2, 5 mM KCl, 0.1 mM EDTA, pH 8.0, 0.1% Triton X-100, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, aprotonin (2 μg/ml), and leupeptin (2 μg/ml)) and spun down for 5 min at 3000 × g. The pellet was resuspended in ice cold lysis buffer (20 mM HEPES, pH 7.9, 25% glycerol, 500 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, pH 8.0, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, aprotonin (2 μg/ml), and leupeptin (2 μg/ml)) and incubated for 30 min on ice. To exchange the high salt buffer, an equal volume (−500 μl) of binding buffer (20 mM HEPES, pH 7.9, 40 mM KCl, 2 mM MgCl2, 10% glycerol, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, aprotonin (2 μg/ml), and leupeptin (2 μg/ml)) was added, and this was spun using filters from Millipore (Ultrafree 5K NWMIL membranes). This centrifugation step was repeated two times. The protein concentrations of the nuclear enriched extracts were determined using the Bradford protein assay.

Western blots were performed as described elsewhere (28). Briefly, equivalent amounts of nuclear enriched protein (from 15 to 50 μg) were separated by 7% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. MEF-2 protein levels were detected using the polyclonal rabbit anti-MEF2 antibody (Santa Cruz Biotechnology) with an enhanced chemiluminescence kit (Amerham Pharmacia Biotech). For the electrophoretic mobility shift assays (EMSA), standard conditions were used (23). Double stranded oligonucleotides specific for the MEF2 and CACC box sites within the MLC2slow promoter were end-labeled using T4 polynucleotide kinase (Promega, Madison, WI). Binding reactions were performed using 5 μg of nuclear extracts, 1 μg of poly(dI-dC)-poly(dI-dC), and 1 ng of labeled probe (40,000 cpm/ng) in binding buffer as outlined elsewhere (23). Cold mutant specific or non-specific (mutated E-box) oligos were added at a 200-fold molar excess, and DNA binding reactions were carried out at 4 °C. For the supershift experiments, preimmunum serum or antibody (1 μl) was added 30 min after the initial incubation, and these samples were incubated for an additional 30 min. The Sp1 and MEF2 antibodies were obtained from Santa Cruz Biotechnology. MEF2A- and MEF2D-specific antibodies were generously provided by Dr. R. Prywes (29), the muscle LIM antibody—TnCslow (Promega Corp.) containing the desired restriction sites. Identification and orientation of the −270MLC2slow clone was verified by restriction digestion analysis.

RESULTS

Deletion and Mutational Analyses of the MLC2slow Promoter—Previous studies determined that the −800-bp promoter of MLC2slow was sufficient to elicit slow nerve-dependent expression in regenerating skeletal muscle (9). Approximately 100-fold higher expression was detected in the SOL+ compared with SOL− or innervated EDL muscles. As can be seen in Fig. 1 (bottom panel), the −270-bp promoter of the MLC2slow gene maintains slow nerve-dependent expression in vivo. Relative luciferase activity for −270MLC2slow was significantly correlated with that for −800MLC2slow across all SOL and EDL muscles studied. This promoter region was then used to identify potential nerve-dependent regulatory elements using site-specific mutations.

Work on other slow isoform contractile protein genes, such as the TnIslowl and TnCslowl genes, has demonstrated that MEF2, E-box, and CACC box sites are important in directing slow skeletal muscle specific expression (15, 16, 33, 34). To address
potential functional roles of these three sites within the 270-bp MLC2slow promoter, we individually mutated the E-box, MEF2, and CACC sites (Fig. 1). As can be seen in Fig. 1, mutation of the E-box (−270Ebox) within the MLC2slow promoter did not result in a change in expression in the SOL+ and EDL muscles. Specifically, relative luciferase expression in the SOL+ muscles was 10.3 ± 1.5 RLUs for −270MLC2slow compared to 8.12 ± 1.1 RLUs for −270Ebox construct. In EDL muscles, expression of the −270MLC2slow (0.23 ± 0.05) versus −270Ebox (0.12 ± 0.04) construct was also not significantly different. These results suggest that the E-box is not required for MLC2slow promoter activity or slow nerve specificity. The decrease in expression with the −270Ebox construct in the SOL− muscles was statistically significant (0.135 ± 0.03 versus 0.06 ± 0.005 RLUs; p < 0.05). However, expression of −270MLC2slow construct is already very low in the SOL− muscles (75-fold lower than SOL+ muscles), so it is not clear what the functional significance of this drop in expression means.

In contrast to the E-box mutation, mutation of the MEF2 site (−270MEF2) does result in a significant reduction in relative luciferase expression in all the innervated and noninnervated muscles (Fig. 1). In the SOL+ muscles, the average luciferase/β-galactosidase ratio decreased 30-fold between the −270MLC2slow construct and the −270MEF2 construct (10.3 ± 1.5 versus 0.36 ± 0.05 respectively; p < 0.01). The MEF2 mutation also resulted in significant decreases in relative luciferase levels in the SOL− (0.135 ± 0.03 versus 0.024 ± 0.005 RLUs; p < 0.01) and EDL muscles (0.23 ± 0.05 versus 0.024 ± 0.006 RLUs; p < 0.01).

The next site within the MLC2slow promoter chosen for mutation was the CACC box because it has been implicated in slow muscle specific expression of the TnIslow and TnCslow gene (7, 15, 34). As can be seen in Fig. 1, expression of the mutated CACC construct results in no change in expression compared with the intact −270-bp MLC2slow promoter in SOL+ muscles (7.4 ± 0.83 versus 10.33 ± 1.5 RLUs, respectively). Compared with −270MLC2s, the CACC mutation resulted in a nonsignificant increase in expression in the SOL− muscles (0.4 ± 0.095 versus 0.135 ± 0.03 RLUs; p = 0.07). A dramatic increase in luciferase expression was evident in the EDL muscles, with the −270CACC construct expressing 18-fold higher compared with the −270MLC2slow construct (4.08 ± 1.1 versus 0.23 RLUs respectively; p < 0.01). These results indicate that the CACC box site on the MLC2slow promoter acts as a repressor element in fast EDL muscle and possibly in the SOL− muscle as well. The lack of repressor activity detected in the SOL+ muscle also suggests that the slow nerve may regulate the MLC2slow promoter, in part, by derepression at the CACC box site.

Transactivation of the MLC2slow Promoter in Vivo—Because the MEF2 site is important for high level promoter activity in SOL+ muscle, we tested whether forced overexpression of MEF2 could transactivate the MLC2slow promoter in SOL− or EDL...
muscles in vivo. For these experiments, the −270MLC2slow construct was co-injected with either a MEF2C, MEF2A, or myogenin expression vector. As can be seen in Fig. 2, overexpression of either MEF2A or MEF2C was not sufficient for transactivation of the −270MLC2slow promoter in the SOL− or EDL muscles. However, in the SOL+ muscles, overexpression of MEF2A or MEF2C increased luciferase expression 3–4-fold (−270MLC2slow 676,340 ± 105,169 RLU; +MEF2C 2,225,957 ± 346,793 RLU; +MEF2A 2,104,446 ± 793,125 RLU; p < 0.01). This enhanced expression was specific for MEF2 as the overexpression of myogenin did not result in any change in luciferase activity in either the SOL+ or EDL muscles (Fig. 2). Co-injection of the MEF2 expression vector with the −270MEF2 mutant construct did not enhance luciferase activity in SOL+ muscles (Fig. 2), which argues that the MEF2 protein, through binding at the MEF2 site, is functionally important for MLC2slow promoter activity in vivo.

Because overexpression of MEF2 was not sufficient by itself to induce MLC2slow promoter activity in the SOL− and EDL muscles, we next tested whether derepression, through mutation of the CACC box, would permit MEF2 transactivation. SOL− and EDL muscles were co-injected with the −270CACC construct and the MEF2 expression vector. As can be seen in Fig. 3, once the promoter is derepressed in the SOL− muscle, it can be strongly transactivated by overexpressing MEF2C. Luciferase activity was increased almost 70-fold when MEF2C was overexpressed with the −270CACC construct in SOL− muscles (1,605,797 ± 582,998 RLU for −270CACC; p = 0.05). It was surprising that overexpressing MEF2C with the −270CACC construct in the EDL did not result in a significant increase in luciferase activity. The luciferase activity was about 1.6-fold higher with the overexpression of MEF2C, but this difference was not statistically significant. This suggests that either MEF2 levels are not limiting for transcription in the EDL or that the EDL regulates transcription of the −270CACC construct through a different mechanism compared with that in the SOL muscle.

Analysis of MEF2 Protein Levels and EMSA with Extracts from Regenerating SOL and EDL Muscles—Because MEF2 levels were implicated in the transcriptional regulation of the MLC2slow promoter, quantitative assessment of MEF2 protein levels in nuclear extracts from SOL−, EDL, and SOL+ muscles was made by Western blot analysis. A representative blot is presented in Fig. 4 using the polyclonal MEF2 antibody from Santa Cruz Biotechnology that was raised against human MEF2A but does recognize both MEF2C and MEF2D isoforms. As can be seen from Fig. 4, EDL and SOL−, MEF2 protein levels are similar between SOL− and EDL nuclear extracts (15 μg of total protein loaded). However, there was a dramatic decrease in MEF2 levels in the extracts from SOL− muscles.
When analyzing 15 μg of nuclear extract, MEF2 levels were undetectable in the SOL− extracts (Fig. 4, SOL−). MEF2 protein levels were detected in the SOL− extracts when the total protein loaded was increased over 3-fold (to 50 μg). Although not conclusive, this finding suggests that MEF2 levels are lower in SOL− extracts when compared with levels in SOL+ and EDL extracts. Extracts obtained from SOL− muscles transfected with the MEF2 expression vector were also analyzed on Western blots (Fig. 4). When comparing the right lane of Fig. 4 to the lane just to the left, it is evident that in vivo transfection of SOL− muscles with the MEF2 expression vector does result in detectable increases in MEF2 protein levels. This observation was also verified in the extracts from in vivo transfected EDL muscles (data not shown).

To assess transcription factor binding to the MEF2 and CACC sites within the MLC2slow promoter, EMSA and supershift assays were performed with nuclear extracts from the regenerating SOL+, SOL−, and EDL muscles (Figs. 5 and 6). As can be seen in Fig. 5A (lanes 1, 4, and 7), there was one primary complex formed with the labeled MLC2slow MEF2 oligonucleotide and nuclear extracts from either SOL+, EDL, or SOL− muscles. This complex was specific for the MEF2 site as it was competed off by excess cold MEF2 oligo (lanes 2, 5, and 8) but was not affected by excess nonspecific oligo (E-box mutant; Fig. 5A, lanes 3, 6, and 9) or the mutant MEF2 oligo (Fig. 5B, compare lanes 1–4). Supershift assays using extracts from SOL+ muscles are seen in Fig. 5B with antibodies to transcription factors MEF2A and MEF2D, Sp1, and a translational regulatory factor, 4E-BP1. Results from this series of experiments confirmed that MEF2 and Sp1 proteins contribute to complex binding at the MEF2 site. Interestingly both MEF2 antibodies, which are specific to the 2A and 2D forms of MEF2, were very effective in competing away the binding activity in these samples. This suggests that both MEF2A and MEF2D contribute to complex formation in extracts from skeletal muscle. Addition of the Sp1 antibody resulted in a classic supershift of the MEF2 complex as seen in Fig. 5B, lane 7. The competition and supershift activities with the MEF2 and Sp1 antibodies were specific because addition of an antibody to a protein that regulates translation, 4E-BP1/PHAS-I, did not affect DNA binding (Fig. 5B, lane 8). We also saw no effect on DNA binding to the MEF2 site with an antibody to another A+T-rich binding transcription factor, Oct-1 (35) (data not shown). It is important to note that there was no muscle specific or nerve specific binding to the MEF2 DNA because we saw the same mobility shift and supershift pattern when extracts from the SOL− and EDL muscles were used (data not shown).

Binding to the CACC box site of the MLC2slow promoter was also analyzed using EMSA and supershift assays (Fig. 6). As with the MEF2 probe, the same pattern of binding was seen with extracts from SOL+, SOL−, and EDL muscles (Fig. 6, lanes 1–12). At least two complexes were apparent, with the faster migrating complex being most predominant. Both complexes, however, were specific to the intact CACC site as competition for binding was seen with excess cold CACC oligo but not with either excess mutated E box or the mutated CACC box oligos. This pattern of protein-DNA binding was also seen with extracts from the SOL− muscles (data not shown). Supershift experiments (Fig. 6, lanes 5–8) using antibodies to the MEF or muscle Lim protein suggest that neither of those proteins are part of the complexes binding to the MLC2slow CACC sequence (compare lanes 2, 5, and 6). This was seen using extracts from
Fig. 6. Mobility shift and supershift analyses of CACC box binding with extracts from regenerating SOL+ and EDL muscles. Two protein-DNA complexes are seen using extracts from SOL+ and EDL muscles and they are similar for both muscles (compare lanes 2 and 12). The complexes for all extracts are specific to the CACC box sequence as excess cold CACC sequence does compete for binding but neither mutated CACC or mutated Ebox oligos effectively compete for binding (lanes 2–4 and 9–12). Supershift experiments using an antibody to MNF and muscle Lim protein indicate that neither of those proteins is part of the complexes formed with the CACC oligo (lanes 5–8).

SOL+ and EDL muscles as well as SOL− muscles (data not shown). This suggests that even though MNF was originally isolated by its binding to the CACC sequence of the myoglobin promoter, it is not part of the complex binding at the MLC2slow CACC box site.

discussiOn
From the results of this study, we describe a two-step model for slow nerve-dependent regulation of the MLC2slow promoter. The first step of the model is derepression at the CACC box followed by high level expression regulated through the MEF2 site. Because overexpressing MEF2 is not sufficient to enhance transcription in SOL− and EDL muscles, this suggests that repression of the MLC2slow promoter may be a dominant effect. The appearance of closely linked CACC box and MEF2-like sites within the regulatory regions of a number of previously identified slow contractile protein genes raises the possibility that this two-step model for slow nerve regulation of the MLC2slow promoter may represent a general mechanism for controlling slow isoform contractile protein gene expression.

A two-step model of gene regulation has been previously proposed for a-actin and keratin genes (36–38). With this model, the activation of transcription is controlled by one set of transcription factors and high level expression is regulated by a different set of transcription factors. When applying this model to the MLC2slow promoter, derepression is the first step, and it is mediated by the slow nerve through binding at the CACC box. However, because there is no difference in EMSA results with SOL+, SOL−, or EDL extracts, that would argue that the slow nerve does not act by altering the number of factors binding to the CACC box. This suggests either that slow innervation leads to derepression through posttranslationally modifying the existing protein(s) binding to the CACC box or that the CACC box site, in the context of the full −270-bp promoter, has a different binding behavior compared with what was seen with the oligonucleotide in an in vitro assay.

To date, there is very little known about CACC box-binding proteins. One candidate protein that was considered is the winged-helix transcription factor, MNF. This factor was initially isolated using the CACC box sequence of the human myoglobin gene (32), and its expression is up-regulated in muscles subjected to chronic motor nerve stimulation (32). However, in this study, negative results from supershift assays suggest that MNF is not part of the complex binding to the CACC box sequence as either a repressor or derepressor.

It was interesting to find that derepression through the CACC site is not sufficient for high level expression in SOL− muscles (see Fig. 1). This finding led to the identification of the second step of regulation in which activation through the MEF2 site is necessary for high level expression of the MLC2slow promoter. The findings from the in vitro transfection experiments (mutational and overexpression experiments) were consistent with those from the in vitro DNA binding and supershift assays. This strongly suggests that MEF2 proteins are specifically binding at the MEF2 site within the MLC2slow promoter and that this binding is critical for complex formation and subsequent transcriptional activity. The supershift experiments also indicated that the ubiquitous factor, Sp1, but not Oct-1 contributes to the binding activity on the MEF2 site. This is seen with extracts from all muscles studied and is consistent with reported Sp1 and MEF2 interaction seen with nuclear extracts from myotubes in culture (39) and nerve cells (40).

Another potential factor that has been implicated in regulating transcriptional activity through the MEF2 site of muscle specific promoters is the nuclear factor of activated T cells, NFAT. In a recent study, Chin et al. (41) demonstrated that overexpression of activated calcineurin, which leads to increased NFAT translocation to the nucleus, can enhance transcription of slow muscle type genes. They propose that NFAT and MEF2 are involved in a combinatorial mechanism to regulate slow muscle type genes in skeletal muscle. Although intriguing, the results from this study are not consistent with that NFAT-MEF2 model. If the slow nerve does activate transcription of slow muscle type genes through a calcineurin/NFAT mechanism, then it would be predicted in our experiments that the increase in nuclear NFAT and its interaction with MEF2 should be evident in EMSA experiments with SOL+ versus SOL− nuclear extracts. However, results from electrophoretic mobility shift assays did not detect any differences between the MEF2 complexes in SOL+ and SOL− extracts. In addition, if NFAT translocation to the nucleus was required for transcriptional activation, then overexpressing MEF2 in the SOL− muscles would not be effective in transactivating the promoter. However, overexpression of MEF2 did induce significant transactivation in the SOL− muscles when the CACC site was mutated within the MLC2slow promoter. This indicates that there are multiple levels of nerve-dependent regulation, of which modulation of repressor/derepressor activity through the CACC site is, in part, a critical step. Finally, unlike the promoters discussed by Chin et al. (41), the MLC2slow promoter does not contain a consensus NFAT site. These arguments do not rule out NFAT involvement in some aspect of regulation, but clearly, further experiments must be done to determine its function in this system.

A unique aspect of this study came from the in vitro transfection studies that provided important insight into potential mechanisms by which MEF2 transcriptional activity is regulated in adult skeletal muscle in vivo. To date, most of the information regarding regulation of contractile proteins genes by either MEF2 or the bHLH myogenic regulatory factors have
been made during embryonic development or in cell culture (11, 26, 42–45). In this in vivo study, overexpressing MEF2 (both MEF2A and MEF2C) enhanced MLC2slow promoter activity in the SOL+ muscles. This suggests that MEF2 levels are limiting for quantitative transcriptional output from the MLC2slow promoter during skeletal muscle regeneration and that the MLC2slow promoter does not seem to discriminate between these two MEF2 isoforms. The additional observation from Western blots that MEF2 protein levels are significantly decreased in these two MEF2 isoforms. The additional observation from Western blots that MEF2 protein levels are significantly decreased in the SOL+ muscles compared with either the SOL+ or EDL muscles also suggests that innervation is important for appropriate regulation of MEF2 levels in adult skeletal muscle.

In contrast to the regulation of other skeletal muscle specific contractile protein genes (7, 15, 16, 44, 46), neither the E-box site nor one of the basic helix loop helix myogenic regulatory proteins, myogenin, was important for expression of the MLC2slow promoter. This is consistent with the lack of MLC2slow gene expression in myotubes at times when expression of the myogenic regulatory factors is high (18). However, recent in vitro work has elegantly shown that the basic helix loop helix myogenic regulatory factors, myoD and myogenin, can cooperate with MEF2 proteins and transactivate muscle specific genes (47). Therefore, it was somewhat surprising in this study that MEF2 overexpression could only enhance transcription in SOL+ muscles through the MEF2 site and that overexpression of myogenin did not enhance MLC2slow promoter activity. These results suggest that within the MLC2slow promoter, myogenin (and perhaps the other basic helix loop helix proteins) is not transcriptionally important for either induction or high level expression.

Results from this study have implicated the CACC box of the MLC2slow promoter as a dominant site through which the slow nerve mediated expression. The identification of the CACC box as a repressor site within the MLC2slow promoter was a surprise because mutational studies of the CACC box within the MLC2slow promoter showed that mutation of the CACC box alone resulted in increased transcription (33, 34, 53–55). Although speculative, this raises the possibility that the two-step model for slow nerve-dependent regulation of the MLC2slow promoter, proposed in this paper, may be shared by other slow isoform genes. Future experiments are required to test the applicability of this two-step model to other slow isoform genes.

In summary, the results from in vivo transfection experiments indicate that both the CACC box and MEF2 sites are important for mediating slow nerve-dependent regulation of the MLC2slow promoter. Consistent with the in vivo findings, results from electrophoretic mobility shift and supershift assays confirmed MEF2 and Sp1 binding to the MEF2 site and demonstrate specific factor binding to the CACC sequence. From these results, a model for MLC2slow transcriptional regulation is proposed in which derepression occurs through factor(s) binding at the CACC box followed by quantitative expression through enhanced MEF2 activation. The prevalence of MEF2-like sites in close approximation to CACC sites within specific expression in adult animals is not clear at this time.

It was not surprising, however, that a repressor element was identified within the rat MLC2slow promoter. Previous reports had noted that the rat MLC2slow gene had an upstream repressor site (HF3) that when mutated resulted in up-regulation in nonmuscle tissues (49). In addition, two separate repressor elements have been identified in the chicken MLC2slow promoter: the CSS and NMS sites (50). These two sites within the chicken gene share a common sequence motif, GAAG/CTTC, which is associated with the repressor activity. Whereas the HF3 site of the rat MLC2slow gene does contain a GAAG sequence, the CACC box of the same gene does not. This suggests that the use of the CACC box within the MLC2slow promoter may be critical for fiber type specificity in skeletal muscle, whereas the HF3/CSS/NMS type site may be important for providing striated muscle restriction.

The induction of slow contractile protein isoforms in response to slow innervation or low frequency chronic stimulation in vivo has been well characterized (3, 5, 51). These studies have contributed to the concept of a coordinated pattern of isoform gene expression and have raised the possibility of a shared mechanism of gene regulation. Within the last 4 years, slow nerve-dependent regions of the MLC1slow, TnIslow, and MLC2slow genes have been identified (7–10). Therefore, we compared the DNA sequences of previously identified slow muscle specific regions of slow contractile protein genes (Fig. 7). What is apparent is that each of these regions contains a CACC box site with a MEF2-like site in close approximation, within 50 base pairs (33, 34, 53–55). Although speculative, this raises the possibility that the two-step model for slow nerve-dependent regulation of the MLC2slow promoter, proposed in this paper, may be shared by other slow isoform genes. Future experiments are required to test the applicability of this two-step model to other slow isoform genes.

In summary, the results from in vivo transfection experiments indicate that both the CACC box and MEF2 sites are important for mediating slow nerve-dependent regulation of the MLC2slow promoter. Consistent with the in vivo findings, results from electrophoretic mobility shift and supershift assays confirmed MEF2 and Sp1 binding to the MEF2 site and demonstrate specific factor binding to the CACC sequence. From these results, a model for MLC2slow transcriptional regulation is proposed in which derepression occurs through factor(s) binding at the CACC box followed by quantitative expression through enhanced MEF2 activation. The prevalence of MEF2-like sites in close approximation to CACC sites within
regulatory regions of many slow contractile protein isomorph genes raises a question about the applicability of this two-step model for those other slow isomorph genes.

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