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Paired MyoD-binding sites regulate myosin light chain gene expression

(muscle/enhancer)

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ABSTRACT **The muscle-specific enhancer element located downstream of the myosin light chain (MLC) locus encoding MLC1 and MLC3 contains three binding sites (A, B, and C) for the myogenic determination factor MyoD. A 173-base-pair region of the MLC gene enhancer, including these three sites, retains full enhancer function when transfected into muscle cells. Whereas mutation of either upstream MyoD binding site (A or B) has a mild effect on muscle-specific enhancer activity, mutation of the third MyoD binding site (C) substantially weakens the enhancer, both in muscle cells or in nonmuscle cells cotransfected with a MyoD, myogenin, or myf5 expression vector. Site C is necessary but insufficient, since double mutation of two MyoD binding sites (A plus B) abrogates enhancer activity. Thus, site C requires either site A or B for enhancer function. This study shows a hierarchy of function among the three MyoD binding sites in the MLC enhancer. We propose that a protein–DNA complex is formed with at least two of these sites (A and C or B and C) to effect activation of the locus encoding MLC1/3 during myogenesis.**

The activation of the myogenic program in muscle precursor cells involves a hierarchy of regulatory factors, which coordinate the expression of multiple contractile proteins. Among the structural genes that are induced during muscle differentiation, the myosin alkali light chain proteins MLC1 and MLC3 are generated from a single genetic locus by transcription from two different promoters and alternate splicing of the pre-mRNAs (1–3). The muscle-specific regulation of both rat and human gene expression of MLC1/3 depends upon a strong enhancer element, located ≈24 kilobases (kb) downstream of the MLC1 gene promoter in both species (4, 5). (The human locus has been assigned the symbol *MYL1*.) This element causes a dramatic increase in the transcription of linked reporter genes exclusively in myotubes, independent of its distance, position, or orientation relative to the promoter (4, 5). In addition, transgenic mice carrying multiple copies of a MLC1 promoter-chloramphenicol acetyltransferase (CAT) gene transcription unit linked to the MLC enhancer activate the transgene exclusively in skeletal muscles, concurrently with the onset of endogenous MLC1 transcription (6). These studies have identified the transcriptional control elements necessary to activate the locus encoding MLC1/3 at the appropriate fetal stage and indicated that in rodents, the MLC gene enhancer is sufficient to induce developmentally regulated expression from the MLC1 promoter exclusively in skeletal muscle cells.

The sequence conservation between the rat and human MLC enhancers (5) suggests that they represent an original component of the ancestral mammalian MLC1/3 locus and are targets for factors involved in the developmental and tissue-specific regulation of muscle gene transcription. Potential candidates for these regulatory proteins include a related group of myogenic factors—MyoD (7), myogenin (8, 9), myf5 (10), and MRF4 (11)—some of which autoregulate their own expression by a positive feedback mechanism (9, 12). A conserved basic domain and an adjoining helix–loop–helix motif is present in all members of this family and, in the case of MyoD, has been shown to be necessary and sufficient for myogenic conversion of C3H/10T½ fibroblasts (13, 14). MyoD binds cooperatively but with different affinities to two sites in the muscle creatine kinase (MCK) gene (*CKMM* in human) enhancer, both of which are important for enhancer activity (15, 16). One of these sites is also a target for myogenin (17). In addition to their ability to determine the myogenic phenotype, these factors may activate genes expressed exclusively in terminally differentiated muscle cells.

In this study we have identified three distinct MyoD binding sites (A, B, and C) in the conserved MLC enhancer sequence by gel-shift and footprinting analyses that closely resemble the consensus binding site for MyoD1 in other muscle-specific regulatory elements (15, 18–20). By deletion and mutation analysis, we show that retention of at least two intact MyoD binding sites is crucial for the muscle-specific activity of the enhancer. Mutations in these sites also affect the ability of the MLC enhancer to be trans-activated by several myogenic factors in a nonmuscle cell background, suggesting that the multiple MyoD1 binding sites in the MLC enhancer are targets for the action of these factors during muscle cell differentiation.

MATERIALS AND METHODS

Mutagenesis. The 0.9-kb MLC enhancer fragment (4) was bisected at a unique *Ava* II site producing a 538-base-pair (bp) 3′ fragment containing full enhancer activity and MyoD binding sites A, B, and C. Subfragments of the 538-bp MLC enhancer were generated with either restriction endonucleases or exonuclease BAL-31. The deletion fragments in ΔBC and in BC were made by restriction enzyme digestion of the pCAT173 insert with *Dra* I and *Fsp* I, respectively. Enhancer subfragments were subcloned downstream of the MLC1 promoter-CAT transcription unit (pCAT, see Fig. 1*a* and ref. 4). Mutations were introduced into the 173-bp MLC enhancer (see Fig. 2) by using oligonucleotides with the following mutated (m) sense-strand sequences: mA:TCATAACTCAAGTCG*GTTACC*GTGCAAAAATGGAGC; mB:TTTTAAAATCCCCAC*GGTAACC*CGAAGCAACAGGTG;mC:CACCAGCTGGCGAAG*GTTACC*GTGCCTAATTCCTCA (mutations in italic).

Transfections. Transient transfections of CAT expression plasmids were carried out by established procedures (4) using

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Abbreviations: CAT, chloramphenicol acetyltransferase; MLC, myosin light chain; MCK, muscle creatine kinase; GS, glutathione.

pMSVβGAL (gift of C. Smith, Children's Hospital, Boston) as a transfection control. The activity of each fragment is represented as the percentage of chloramphenicol converted to acetylated forms in 1 hr at 37°C and is based on the numeric average of three experiments.

Trans-activations. NIH 3T3 cells were seeded at 5×10^5 cells per 100-mm plate 1 day before transfection. Each transfection contained 20 μg of each myogenic factor expression plasmid combined with 5 μg of MLC-CAT reporter plasmid, and 2 μg of pSV2βGAL (N.R., unpublished data) for normalization. The DNAs were added to the cultured cells as described earlier (4, 7), and the medium was changed 16 hr later to differentiation medium. The cells were harvested 48 hr later, and enzyme assays were performed.

Northern Blots. Total cellular RNA was isolated from cultured cells (21) and treated with 1 unit of RQ1 RNase-free DNase (Promega) for 15 min at 37°C. Ten micrograms of RNA was analyzed on Northern blots (22) hybridized with ^{32}P-labeled DNA probes as indicated.

Mobility-Shift Assays. Fragments of the 173-bp MLC enhancer (see ABC and mABC in Fig. 2) were ^{32}P-end-labeled on the sense or antisense strand at vector polylinker restriction sites. Smaller DNA fragments were generated by secondary digestions with *Fsp* I (fragments A and BC) and *Pvu* II (fragment C). Fragment B was a synthetic double-stranded oligonucleotide including the sequence (CCCCACCAGCTG-GCGAG, sense strand) end-labeled after subcloning into puc19. Analytical mobility-shift reactions were prepared by combining ≈1–5 ng of labeled DNA (≈5×10^6 cpm/nmol) with glutathione (GS)-MyoD fusion protein extract (0.05–0.4 μg) in a final volume of 20 μl containing 10 mM Hepes (pH 7.9), 50 mM NaCl, and poly[d(I-C)] (0.05–1 μg). Samples were size fractionated at 10°C through a 6% acrylamide gel (1:79 methylenebisacrylamide/acrylamide) containing 6.7 mM Tris, 3.3 mM sodium acetate, 1 mM EDTA (pH 7.9), and 4% glycerol at 10 V/cm, with buffer recirculation.

Methylation Interference Footprinting. Methylation interference footprinting was performed by published procedures (23). DNA fragments containing MyoD binding sites A, B, and C were 3′-end-labeled using Klenow fragment and dephosphorylated 5′ ends with polynucleotide kinase. Preparative gel-shift reactions represented a 5-fold scale-up of the analytical reactions including 5×10^5 to 2×10^6 cpm of end-labeled probe.

RESULTS

MLC Enhancer Activity Depends on a Short, Highly Conserved Sequence. The original 0.9-kb fragment containing the rat MLC enhancer (4) was located ≈2.3 kb downstream of the MLC1/3 DNA polyadenylylation site. To define the functional domains in this region, successively smaller fragments were tested downstream of an MLC1 promoter-CAT transcription unit (pCAT; Fig. 1*a*). Transient assays of CAT activity in C2C12 undifferentiated myoblasts, differentiated myotubes, or NIH 3T3 fibroblasts established that full muscle-specific enhancer activity was retained within the 3′ 538 bp of the 0.9-kb segment. A smaller fragment comprising the first 173 bp of this sequence (pCAT173) retained most of the activity of pCAT538 and represented the region of highest homology to the cognate enhancer from the human locus encoding MLC1/3 (5). Notably, the larger insert in pCAT465 (Fig. 1*a*) was the most active in these assays, even though the first 90 bp of homologous sequence was deleted. This suggests that a negative regulatory element may lie in the proximal end of the MLC enhancer. The sequence of the 173-bp enhancer includes several motifs found in other muscle-specific regulatory elements (underlined in Fig. 1*b*).

MyoD Binds to Multiple Sites in the MLC Enhancer. A striking similarity between one of the MyoD binding sites in the MCK enhancer (15) and several sequences in the MLC enhancer led us to investigate what role multiple MyoD binding sites might play in the activity of muscle-specific regulatory elements. In the MCK enhancer, a 10-bp motif, CACCTGCTGC, constitutes the strongest of the two MyoD binding sites (15). This 10-bp motif is represented, either perfectly (site A) or as a related sequence (sites B and C), in three distinct positions in both the rat and human MLC enhancers (Fig. 1*b*). A fourth motif, CATGTG, at position 23 in the 173-bp enhancer (underlined in Fig. 1*b*) is less closely related but includes the consensus CANNTG sequence present in all reported binding motifs for helix–loop–helix proteins, including MyoD.

To determine whether these motifs in the MLC enhancer could bind MyoD protein, mobility shift assays were performed with the 173-bp rat MLC enhancer fragment and bacterial extracts containing a GS-MyoD fusion protein (15). Multiple protein–DNA complexes were obtained (Fig. 2*a*). Formation of the higher complexes, which may represent MyoD binding to more than one site (16), could be preferentially blocked by competition with excess nonspecific poly[d(I-C)] DNA (Fig. 2*a*) and were not consistently obtained with all bacterial extract preparations. The lower complex presumably represents MyoD binding to individual sites within the enhancer, as confirmed by footprinting analysis (see below).

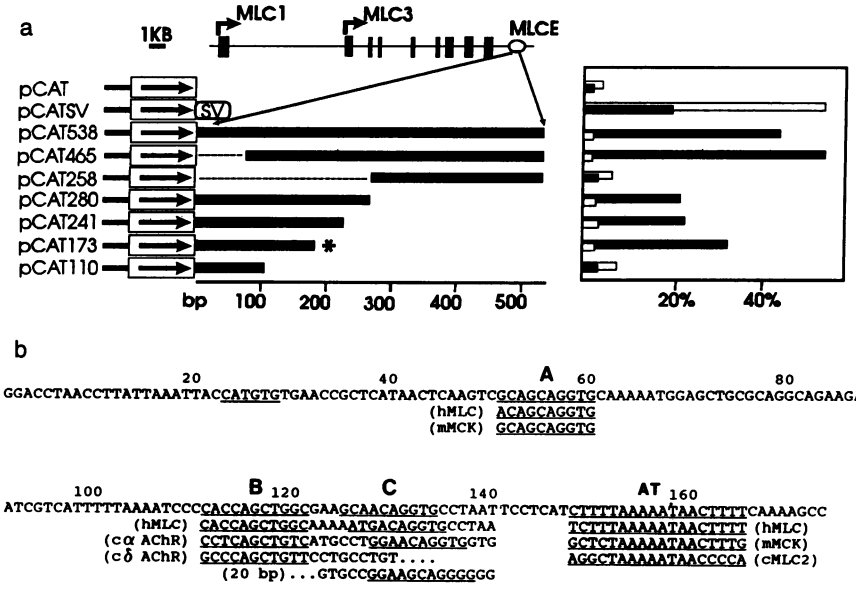

FIG. 1. (*a*) Functional assays of MLC enhancer fragments. The genomic organization of the locus encoding MLC1/3 shows the sites of MLC1 and MLC3 transcription initiation (arrows) and the position of the 538-bp MLC enhancer contained in pCAT538 (open circle). Deletion fragments below were tested for enhancer activity, and the results are shown to the right. ■, C2C12 myotubes; □, NIH 3T3 fibroblasts; SV, SV40 enhancer. (*b*) Sequence of the rat 173-bp MLC enhancer in pCAT173 (see asterisk in *a*). MyoD binding sites A, B, and C are indicated. Homologous muscle-specific DNA regulatory elements (underlined) are: hMLC, human MLC enhancer (5, 8); mMCK, mouse muscle creatine kinase enhancer (15, 18); αAChR, chicken acetylcholine receptor α-subunit enhancer (19, 20, 24); δAChR, chicken acetylcholine receptor δ-subunit enhancer (25); cMLC2, chicken cardiac MLC2 promoter (26).

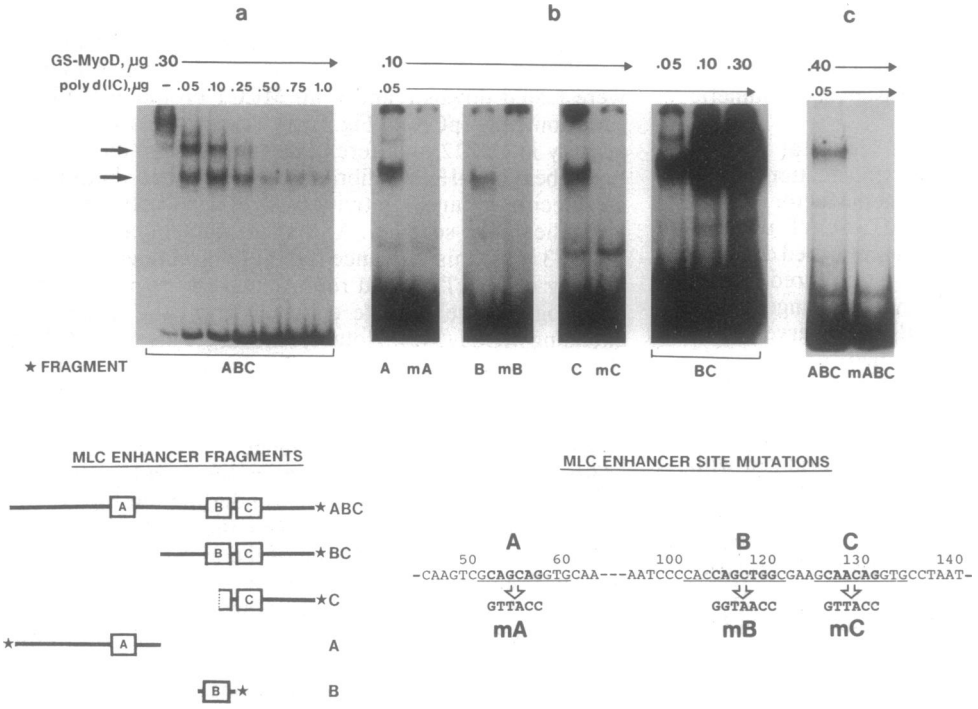

FIG. 2. Mobility shift assays with GS-MyoD bacterial fusion protein. (*Upper*) ^{32}P-labeled MLC enhancer fragments were combined with GS-MyoD fusion protein (15) and analyzed by gel electrophoresis. (*a*) Competition for MyoD protein with the 173-bp MLC enhancer and increasing amounts of poly[d(I-C)]. Higher and lower complexes are indicated (arrows). (*b*) Mobility shift assays with MyoD extract and MLC enhancer subfragments containing wild-type (A, B, C, and BC) or mutant (mA, mB, and mC) MyoD binding sites with similar specific activities. (*c*) Mobility shift assay with full-length MLC enhancer containing wild-type (ABC) or all three mutated (mABC) MyoD binding sites. (*Lower Left*) A diagram of MLC enhancer subfragments used in mobility shift assays in which stars indicate the position of the ^{32}P label. (*Lower Right*) The sequences of wild-type and mutated sites A, B, and C.

A series of DNA subfragments derived from the 173-bp enhancer (Fig. 2, fragment ABC), was generated by restriction enzyme digestion and used to establish the presence of the individual MyoD binding sites in the MLC enhancer. The resulting deletion fragments retained sites BC, C, and A, respectively. The subfragment representing site B alone was a double-stranded synthetic oligonucleotide (see *Materials and Methods*). These subfragments each formed a complex with the MyoD extract (Fig. 2*b*). The subfragment containing both sites B and C (BC) produced the strongest interaction with the MyoD extract, also forming an additional larger complex not visible with either site B or C alone.

To confirm that the interactions of MyoD with the MLC enhancer were sequence-specific, corresponding subfragments containing enhancer site mutations at consensus sites A, B, or C (Fig. 2) were tested in parallel. The sequences of the site mutants corresponded as closely as possible to the substitutions introduced into the MCK enhancer MyoD binding sites, which eliminated MyoD binding *in vitro* and dramatically reduced MCK enhancer activity in cell transfection assays (15, 18). None of the subfragments containing mutated sites (mA, mB, mC) were capable of forming MyoD complexes (Fig. 2*b*). This shows that the interaction of MyoD with the MLC enhancer is specific for at least three sequence motifs: A, B, and C. These three sequences appear to be the only MyoD binding sites in the enhancer, since a 173-bp enhancer fragment in which all three sites were mutagenized *in situ* (Fig. 2*c*; mABC) did not form a complex with MyoD extract, when compared with the wild-type enhancer fragment (Fig. 2*c*; ABC). Thus, the upstream CATGTG consensus sequence at position 23 does not appear to be a fourth MyoD binding site. Additional gel shifts using a minimal enhancer subfragment including only this upstream site produced a weak complex with MyoD only at high extract concentrations (>0.5 μg; data not shown).

Localization of MyoD Binding Sites in the MLC Enhancer. The specific nucleotides involved in the binding of MyoD with the MLC enhancer were mapped by methylation interference assays. Comparison of the methylation pattern seen in the protein-bound DNA sample (Fig. 3, lanes B) to the free DNA (Fig. 3, lanes F) established the position of MyoD interaction in subfragments containing site A directly over the conserved 10-bp motif. The pattern of methylated guanosine residues, which interfered with MyoD binding to the MLC enhancer, overlapped that obtained from a similar analysis of the identical 10-bp motif in the MCK enhancer (15), despite differences in the surrounding DNA sequence. A similar analysis was performed with an MLC enhancer subfragment comprising site B. When sites B and C were included in the subfragment, a footprint consistently appeared over site C alone, suggesting that in the context of the intact enhancer, site B has significantly lower affinity for the MyoD fusion protein. The larger complex seen in mobility shifts with subfragment BC (see Fig. 2*b*) did not yield a reproducible footprint. Comparison of the MyoD footprint over sites, A, B, and C (Fig. 3) revealed that, at least *in vitro*, considerable flexibility in protein–DNA contact points allows MyoD to interact with three related but distinct sequence motifs in the MLC enhancer.

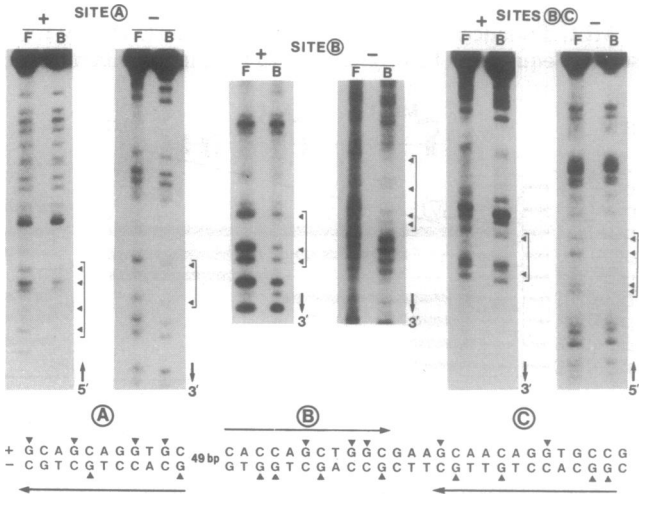

FIG. 3. Methylation interference footprinting of the GS-MyoD fusion protein bound to the MLC enhancer. Fragments containing sites A, B, or BC (Fig. 2) were labeled on the sense (lanes +) and antisense (lanes –) strands, and binding to GS-MyoD was assayed by methylation interference on bound (lanes B) and free (lanes F) DNA. MyoD consensus binding sites are bracketed. The MyoD footprint patterns are summarized below the lanes.

Mutation of MyoD Binding Sites Affects MLC Enhancer Activity. Based upon the functional assays shown in Fig. 1*a* (see pCAT465), we deduced that the first 90 nucleotides of the 173-bp enhancer, including site A, are not essential for muscle-specific expression of the linked CAT gene. This was an unexpected conclusion, since the identical 10-bp motif appears in the MCK enhancer and is essential for the function of that element (15, 18). In addition, deletion of sites B and C from the MLC enhancer (pCAT110) abrogated expression of the linked CAT gene, indicating that site A alone is insufficient to activate muscle-specific gene expression. To confirm these observations, a series of MLC enhancer mutants was generated in which each of the three MyoD binding motifs were individually destroyed by oligonucleotide-directed mutagenesis, incorporating the mutant DNA sequences used in the competition assay in Fig. 2*b*. The three resulting 173-bp fragments, each containing a mutated MyoD site (mA, mB, or mC in Fig. 4), were tested downstream of an MLC1 promoter-CAT transcription unit in transient transfections of muscle and nonmuscle cells. Mutation of either site A or B (Fig. 4; construct mA and mB) decreased enhancer activity by ≈50% compared with the wild-type enhancer fragment (Fig. 4; pCAT173), whereas destruction of both sites reduced CAT activity to the background levels seen with the MLC1 promoter alone (Fig. 4; construct mAB). Mutation of the third site C alone (Fig. 4; construct mC) also dramatically reduced the level of CAT expression. An additional construct, which contained a 50-bp subfragment including only sites B and C (construct ΔBC, positions 103–152 in Fig. 1*b*), displayed an intermediate level of activity, while a larger 97-bp subfragment including sites B and C flanked by two A+T-rich motifs (construct BC, positions 76–173 in Fig. 1*b*) reconstituted wild-type activity. It is notable that construct mA, in which site A was mutated, was less active than construct BC, in which sequences including site A were deleted. This implies that a negative element, in addition to site A, has been removed from the 5′ end of the enhancer. These data show that MyoD binding site C is necessary but not sufficient, requiring the addition of either the adjacent site B or the upstream site A for MLC enhancer function. The relative contribution of the paired MyoD binding sites to MLC enhancer activity is further modulated by the presence of adjacent sequences, such as the A+T-rich motifs flanking sites B and C.

Trans-activation of the MLC Enhancer Is Dependent on MyoD Binding Sites. The specific interactions of MyoD with the MLC enhancer *in vitro* (Fig. 2) suggested that it may be directly involved with enhancer activation during myogenesis. To investigate this possibility, we used a transient trans-activation assay in which a MyoD expression vector was cotransfected with the various MLC enhancer-CAT vectors described in Fig. 4 into NIH 3T3 fibroblasts. The effect of exogenous MyoD expression on MLC enhancer activity was tested by measuring CAT activity in the cotransfected cells after 60 hr. Two other myogenic determination factors, myogenin (8, 9) and myf5 (10), were also tested for their ability to trans-activate the MLC enhancer in a nonmuscle cell background (Fig. 5). Vectors carrying myogenin or MyoD genes activated CAT expression 60- to 90-fold over background, compared with an enhancerless MLC1 promoter-CAT transcription unit, which was 3- to 7-fold over background. In contrast, a vector carrying the *myf5* gene activated CAT expression only 10-fold over background. Similar experiments were performed with the three CAT vectors mA, mB, and mC, cotransfected with either MyoD, myogenin, or myf5 expression vectors into NIH 3T3 fibroblasts. Mutation of site A decreased the ability of these myogenic factors to trans-activate the enhancer by ≈50%, whereas mutation of either site B or site C essentially inactivated the enhancer in this assay (Fig. 5). Thus, the ability of myogenic factors to trans-activate the mutant mA and mC enhancers corresponded to the relative activity of these mutants in muscle cells (Fig. 4). In contrast, the mB mutant was active in muscle cells (Fig. 4) yet could not be trans-activated with any of the myogenic factors in fibroblasts. The difference in the behavior of the mB mutant enhancer in the two assays may be attributed to additional regulatory factors, present exclusively in muscle cells, that are required for the function of the mB mutant in which only sites A and C are left intact.

MLC Enhancer Is Rapidly Trans-activated by Coexpressed Myogenic Factors. We examined the time course of MLC enhancer-driven CAT gene expression after cotransfection of NIH 3T3 fibroblasts with a MyoD or myogenin expression vector to determine whether the induction of CAT activity (Fig. 5) was caused by the exogenous myogenic factor or by the activation of endogenous myogenic genes. MLC enhancer-driven CAT activity was detected after 24 hr in cells cotransfected with either myogenic factor (Fig. 6). Northern blot analysis of RNA prepared from parallel NIH 3T3 cultures as late as 48 hr after cotransfection with MyoD failed to detect significant amounts of either myogenin or myf5 endogenous transcripts, which normally cross-activate each other during myogenesis (12). A similar analysis of RNA from the NIH 3T3 cultures cotransfected with myogenin failed to detect MyoD or myf5 endogenous transcripts. Although NIH 3T3 fibroblasts can be converted to a myogenic phenotype at a low percentage by overexpression of these factors (ref. 7; M.D., unpublished data), it appears that a full myogenic

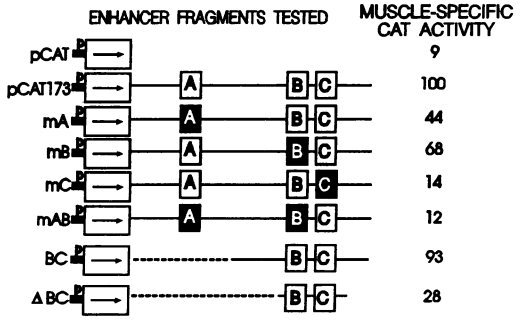

FIG. 4. Muscle-specific activity of MLC enhancer mutants. MyoD binding sites in the 173-bp MLC enhancer were individually mutated to nonbinding forms (Fig. 2) and tested for their ability to direct muscle-specific expression of a linked CAT gene, relative to the activity of the wild-type enhancer. Black boxes indicate mutated MyoD binding sites. The CAT activity of the 173-bp wild-type enhancer is arbitrarily set at 100% (see Fig. 1*a*, pCAT173). None of the constructs were active over background levels in undifferentiated C2C12 myoblasts or in NIH 3T3 fibroblasts (data not shown).

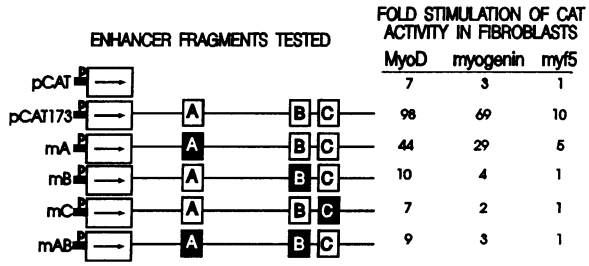

FIG. 5. Trans-activation of the MLC enhancer by myogenic factors. Mutant MLC enhancer-CAT reporter plasmids (Fig. 4) were transiently cotransfected with pEMSVScribeα2 expression vectors encoding MyoD (15), myogenin (8, 9), or myf5 (10) into NIH 3T3 fibroblasts. The fibroblasts were then shifted into fusion media for 48 hr after which cellular extracts were assayed for CAT activity.

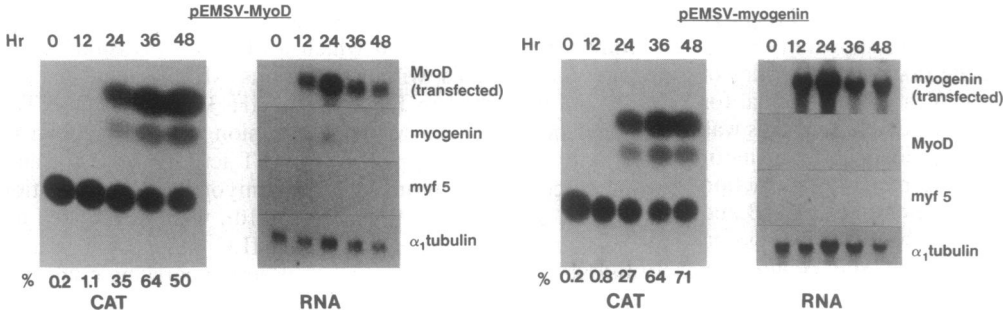

FIG. 6. Time course of MLC enhancer trans-activation. NIH 3T3 fibroblasts were cotransfected with pCAT173 and MyoD (*left*) or myogenin (*right*) expression vectors, and CAT assays were performed at indicated time points. Total RNA was analyzed by Northern blots with MyoD, myogenin, myf5, and α_1-tubulin cDNA probes.

program is not activated in the transfected cells during the transient assays described here.

DISCUSSION

In this study we have characterized three sequences in the rat MLC enhancer that constitute binding sites for the myogenic factor MyoD. The expression of truncated and mutated MLC enhancer fragments in differentiated muscle cells shows that a minimum of two MyoD binding sites are required for enhancer function. A more restricted set of paired MyoD binding sites is responsive to trans-activation by several myogenic factors in nonmuscle cells. Thus, three sites in the MLC enhancer are potential targets for the action of multiple myogenic factors that may coordinate gene expression during muscle cell differentiation. This is supported by the close resemblance of these three sites to functionally important sequences in other muscle-specific regulatory elements. Specifically, site A in the MLC enhancer is identical to a sequence comprising both a MyoD (15, 18) and myogenin (17) binding site in the mouse MCK enhancer, mutation of which reduces MCK enhancer activity by a factor of 25 (15, 18). By contrast, deletion or mutation of this site had only a two-fold effect on MLC enhancer activity, suggesting that the function of the A motif in muscle-specific enhancers is dependent on its context. In addition, the region comprising sites B and C in the MLC enhancer resembles a sequence in the muscle-specific enhancers upstream of the genes encoding the chicken α and δ-acetylcholine receptors (see Fig. 1; refs. 19, 20, 24, 25). Notably, the relative position of the paired B and C motifs in the MLC enhancer would place them on opposite sides of the DNA helix, whereas an extra 3 bp between similar sequences in the enhancer upstream of the α-acetylcholine receptor gene positions them on the same helical face. Therefore, potential interactions between factors binding to the paired motifs may be different in the two enhancers.

The behavior of the MLC enhancer mutants in differentiated muscle cells shows that there is flexibility in the way different MyoD binding sites can operate in pairs and that adjacent sequences provide additional modulatory functions. An A+T-rich motif downstream of site C appears to play a role in MLC enhancer activity, since its inclusion increases enhancer activity ≈3-fold (see Fig. 4; ΔBC vs. BC). This motif is closely related to a similar sequence in the MCK enhancer and MLC2 promoter (refs. 26–29; see Fig. 1*b*). In both the MLC and MCK enhancers, the A+T-rich sequence constitutes a binding site for the myocyte-specific enhancer-binding factor, MEF2, which may also participate in the regulation of multiple muscle-specific genes during myogenesis (30). Finally, the transient transfection assays used in this study may not fully reflect the activity of the MLC enhancer *in vivo*. It remains to be determined what role each of these sites play, individually or collectively, in the complex regulation of MLC1/3 gene expression during skeletal muscle development.

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