Distinct Regulatory Elements Control Muscle-Specific, Fiber-Type-Selective, and Axially Graded Expression of a Myosin Light-Chain Gene in Transgenic Mice†

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The fast alkali myosin light chain 1f/3f (MLC1f/3f) gene is developmentally regulated, muscle specific, and preferentially expressed in fast-twitch fibers. A transgene containing an MLC1f promoter plus a downstream enhancer replicates this pattern of expression in transgenic mice. Unexpectedly, this transgene is also expressed in a striking (~100-fold) rostrocaudal gradient in axial muscles (reviewed by J. R. Sanes, M. J. Donoghue, M. C. Wallace, and J. P. Merlie, Cold Spring Harbor Symp. Quant. Biol. 57:451–460, 1992). Here, we analyzed the expression of mutated transgenes to map sites necessary for muscle-specific, fiber-type-selective, and axially graded expression. We show that two E boxes (myogenic factor binding sites), a homeodomain (hox) protein binding site, and an MEF2 site, which are clustered in an ~170-bp core enhancer, are all necessary for maximal transgene activity in muscle but not for fiber-type- or position-dependent expression. A distinct region within the core enhancer promotes selective expression of the transgene in fast-twitch muscles. Sequences that flank the core enhancer are also necessary for high-level activity in transgenic mice but have little influence on activity in transfected cells, suggesting the presence of regions resembling matrix attachment sites. Truncations of the MLC1f promoter affected position-dependent expression of the transgene, revealing distinct regions that repress transgene activity in neck muscles and promote differential expression among intercostal muscles. Thus, the whole-body gradient of expression displayed by the complete transgene may reflect the integrated activities of discrete elements that regulate expression in subsets of muscles. Finally, we show that transgene activity is not significantly affected by deletion or overexpression of the *myoD* gene, suggesting that intermuscular differences in myogenic factor levels do not affect patterns of transgene expression. Together, our results provide evidence for at least nine distinct sites that exert major effects on the levels and patterns of MLC1f expression in adult muscles.

The differentiation of muscle fibers involves complex patterns of gene expression that transform mesodermal precursors into myoblasts and myoblasts into myotubes (10). In parallel, differential gene expression by subsets of precursors, myoblasts, and myotubes leads to acquisition of distinct properties by groups of muscle fibers (26, 76, 89). One useful starting point for gaining insight into the mechanisms that underlie muscle cell differentiation and diversification has been to characterize the regulatory regions of genes that are specifically expressed in muscle fibers. Typically, genomic fragments are linked to a reporter gene whose expression can be monitored following introduction of the construct into cultured cells or transgenic mice. Once active fragments have been identified, critical sequences can be mapped and transcriptional regulators can be identified. Some of the genes that have been analyzed in this way are those for creatine kinase (1, 11, 42), actin (9, 74), the troponins (4, 15, 34, 35, 51, 66), the myosins (3, 8, 18, 19, 21–25, 43, 44, 77, 86, 87), and the myogenic factors (6, 13, 64).

Here, we focus on the myosin light chain 1f/3f (MLC1f/3f) gene. This gene uses discrete promoters and alternative splic-

ing to generate two mRNAs, which encode MLC1f and MLC3f, respectively (63, 73, 77). Both light chains are selectively expressed in fast-twitch muscle fibers (75, 76, 88), although their developmental regulation differs (46, 53, 69, 70). Analysis by transfection into muscle cell lines has led to the characterization of the MLC1f and MLC3f promoters (5, 18, 19, 28, 86) and to the identification of a distal enhancer that can activate both MLC1f and MLC3f promoters as well as heterologous promoters (21, 43, 78, 90). Further studies with transgenic mice showed that a construct containing a 1.2-kb MLC1f promoter fragment plus a 0.9-kb distal MLC enhancer fragment reproduced many features of the expression of the endogenous gene, including appropriate developmental regulation, high levels of expression in muscle, low levels of expression in nonmuscle tissues, and selective expression in fast-twitch muscle fibers (21, 24, 32, 79, 82). In addition, we found that the transgene was expressed in a striking (~100-fold) caudal-high–rostral-low gradient in adult axial muscles (23). This result was unexpected, in that expression of neither MLC1f nor MLC3f varied systematically along the rostrocaudal axis (23). The basis for the graded expression of the transgene remains unknown, but it provided the first molecular correlate of positional differences that had been inferred from electrophysiological studies of segmentally selective reinnervation following nerve damage (47, 48, 91, 92). Moreover, we used the transgene as a marker to show that muscle cells bear a cell-autonomous and heritable memory of their position of origin (24) and to implicate DNA methylation in the establishment and maintenance of the positional memory (25).

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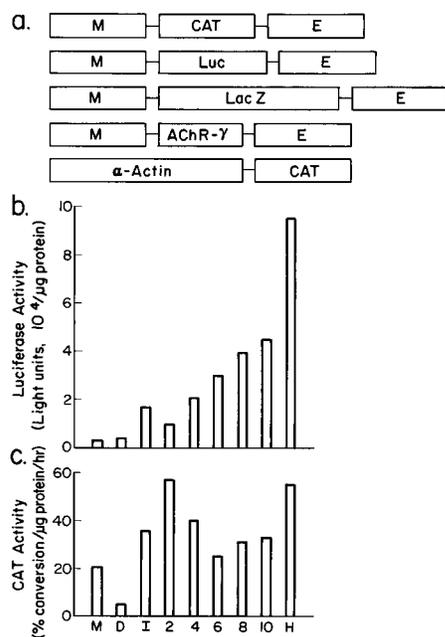§ Present address: Section of Neurobiology, Yale Medical School, New Haven, CT 06510.

FIG. 1. Patterns of MCATE expression are reporter independent. (a) Transgenes used to test dependence of expression on reporter and regulatory elements. The reporter CAT in MCATE was replaced by firefly luciferase (Luc), *E. coli* β-galactosidase (Lac Z), or a cDNA encoding the mouse nicotinic AChR γ-subunit (AChR-γ). Alternatively, regulatory elements from chicken skeletal actin (α-Actin) or mouse creatine kinase (not shown) were linked to CAT. M, 1,158-bp MLC1f promoter; E, 900-bp MLC enhancer. (b) Specific activities of luciferase in muscles of an adult MLucE mouse. (c) Specific activities of CAT in muscles of an adult α-actinCAT mouse. M, masseter; D, anterior plus posterior digastricus; I, infrahyoid muscles (sternomastoid, omohyoid, and sternohyoid); 2, 4, 6, 8, and 10, intercostals of the second-tenth thoracic segment; H, plantaris plus gastrocnemius muscles of the lower hindlimb.

To understand how muscle-specific, fiber-type-selective, and position-dependent patterns of expression are integrated, it will be crucial to learn how each is controlled. The present studies represent a first step toward this goal. Our aims were to identify sites within the MLC promoter and enhancer that are necessary for each aspect of the overall expression pattern and to test binding sites for specific transcriptional activators, including a consensus motif that we demonstrate to be recognized by homeodomain proteins. To these ends, we generated transgenic mice in which the MLC1 promoter or MLC enhancer had been mutated, truncated, or replaced or in which one reporter (chloramphenicol acetyltransferase [CAT]) had been replaced by another (β-galactosidase or luciferase). We then assayed expression of the altered transgenes in muscles and nonmuscle tissues and in muscles that differed in fiber type composition or axial position. In this way, we identified nine distinct sites that regulate quantitative and qualitative aspects of the transgene's expression.

MATERIALS AND METHODS

Construction of transgenes. The MCATE transgene, which has been described previously, contains the bacterial CAT gene flanked by two fragments of the rat MLC1f/3f gene (21–25, 79) (Fig. 1a). Upstream of CAT, the transgene contains a 1,158-bp fragment that includes the promoter and transcriptional start site of the MLC1f RNA (22, 73). We refer to this entire fragment as the MLC1 promoter even though the most proximal 200 bp was sufficient for transcriptional activation in cultured muscle cells (22, 86). Downstream of CAT, the transgene contains a 0.9-kb fragment from the 3′ end of the MLC1f/3f gene which acts as an enhancer (22). In this study, we mutated both the MLC1f promoter and the MLC enhancer in the MCATE transgene and substituted other reporters for CAT.

Site-directed mutagenesis of the enhancer was performed by a modification of the PCR-based method of Hemsley et al. (38). The 1.2-kb *Acc*I-*Hin*dIII MLC1f promoter and the MLC enhancer from MCATE were subcloned into pBluescript II SK+ (Stratagene, La Jolla, Calif.) to provide templates for PCR. Primers used for PCR introduced novel restriction sites, both to permit identification of mutated clones and to facilitate subsequent constructions. Primer sequences and restriction sites for these mutants were as follows: for MCATEmB1, 5′-TAAAATCCC CACGGTACCACGAAGCAAC-3′ (*Kpn*I) and 5′-AAAATGACGATCACTCT TCTGCCTG-3′; for MCATEmC1, 5′-GGCGAAGCAAGTCGACCCTAATTC C-3′ (*Sal*I) and 5′-AGCTGGTGGGGGATTTTAAAAATG-3′; for MCATEmH1, 5′-GACCTAACCTTCGCGAATTACCATG-3′ (*Nru*I) and 5′-CCATGACAGT GTGCTGAAG TTAG-3′; for MCATEmH2, 5′-GACCTAACCTTGCGC AG GCGCCATGTGTGAAC-3′ (*Fsp*I and *Nar*I) and a second primer identical to MCATEmH1; for MCATEmF1, 5′-CCTAATTCCTCATACCGGTAAAAAAA CTTTTCAAAAGCC-3′ (*Age*I) and 5′-CACCTGTTGCTTCGCCAGCTG-3′; and for MCATEmF2, 5′-CCTAATTCCTCATCTTAT GGGGCCCACTTTTC AAAAGCC-3′ (*Apa*I) and 5′-CACCTGTTGCTTCGCCA GCTGGTGGGG-3′.

Deletion mutants were generated by using unique restriction sites that had been introduced by site-directed mutagenesis (in the enhancer) or were naturally occurring (in the promoter). MCATEdC contains nucleotides (nt) 1 to 518 of the enhancer, ending at the *Sal*I site in MCATEmC1. MCATEdH1 contains nt 1 to 398 of the enhancer, ending at the *Nru*I site in MCATEmH1. MCATEdH2 contains nt 399 to 915 of the enhancer, beginning with the *Nru*I site in MCATEmH1. MCAT0 contains no enhancer sequences. M391CATE contains nt −391 to +1 of the promoter, beginning at the *Pvu*II site at nt −391. M568CATE contains nt −568 to +1 of the promoter, beginning with the *Eae*I site at nt −568. M676CATE contains nt −676 to +1 of the promoter, beginning with the *Bfa*I site at nt −676. M835CATE contains nt −835 to +1 of the promoter, beginning with the *Bbs*I site at nt −835.

In three constructs, CAT was removed from the MCATE construct and replaced with other reporters: *Escherichia coli* β-galactosidase (*lacZ*) (56) in MLacE, firefly luciferase (pGL2; Promega) in MLucE, and a mouse acetylcholine receptor γ subunit cDNA (93) in MAChRγE.

Additional constructs were generated as follows. In hspCATE, the MLC1 promoter was replaced with an 835-bp promoter fragment from the mouse heat shock protein 68 (hsp68) gene (45). In MΔhspCATE, sequences from nt −1158 to −377 of the MLC1 promoter were fused to the 5′ end of hspCATE. In MCATEcore, a fragment containing nt 387 to 559 of the enhancer was amplified by PCR using the primers 5′-CGCGGATCCGGACCTAACCTTATTAAAT TACCA-3′ and 5′-CGCGGATCCGGCTTTTGAAAAGTTATTTTTAAA-3′. The product was digested with *Bam*HI and cloned into MCAT0.

Transfections. Sol 8 myoblasts (61) were grown at 37°C in Dulbecco's modified Eagle medium containing 20% fetal calf serum. Myoblasts were plated on gelatinized 6-cm-diameter dishes (4×10^5 cells per dish) on the day before transfection and fed with fresh medium 1 h before transfection. Transfection was carried out by the calcium phosphate method of Chen and Okayama (12), using a mixture of test DNA and a plasmid in which *lacZ* was expressed under the control of a Rous sarcoma virus long terminal repeat (a gift of N. Gautam, Washington University). After transfection, the cells were washed with sterile phosphate-buffered saline and fed with Dulbecco's modified Eagle medium plus 2% horse serum to induce formation of myotubes. Three days later, the myotubes were harvested and extracted with Reporter Lysis Buffer (Promega, Madison, Wis.).

Generation of transgenic mice. Transgenic mice were generated by standard methods (40). In all cases, transgene inserts were excised and purified as described in reference 79, to avoid introduction of vector sequences into the transgenes. Embryos for injection were obtained from (C57BL6/J × CBA)F₁ × (C57BL6/J × CBA)F₁ matings, and transgene-positive offspring were identified by PCR using tail digests (36).

In addition to MCATE transgenic mice and their derivatives, we also assayed several previously generated transgenic strains. In AChRα-CAT, an 850-bp promoter fragment from the chicken nicotinic acetylcholine receptor (AChR) α-subunit gene was fused to CAT (57). In skeletal α-actin-CAT, a 2.2-kb promoter of the chicken α-skeletal actin gene was fused to CAT (74). These mice were a generous gift of S. Hughes and C. Petropoulos (National Cancer Institute, Frederick, Md.). In −3300-MCK-CAT and E2-776-MCK-CAT, promoter and enhancer sequences from the muscle creatine kinase gene were fused to CAT (42). These mice were generously provided by Jane Johnson and Barbara Wold (California Institute of Technology, Pasadena, Calif.). In myogenin-CAT, a 1,565-bp promoter from the mouse myogenin gene was fused to CAT (58). Mice containing a null mutation of the *myoD* gene (80) were generously provided by Rudolf Jaenisch (Massachusetts Institute of Technology, Cambridge, Mass.). A mouse overexpressing *myoD* under the control of a muscle creatine kinase promoter-enhancer (60) was a kind gift of Jeff H. Miner and Barbara Wold.

Adult mice (>4 weeks of age) were euthanized by an overdose of anesthetic, and the following muscles were dissected: masseter, digastricus (anterior plus posterior), infrahyoid (sternomastoid, omohyoid, and sternohyoid), forelimb (pectoralis major, pectoralis minor, triceps, and forearm muscles), diaphragm, thoracic segment 2 (T2) intercostal, T4 intercostal, T6 intercostal, T8 intercostal, T10 intercostal, soleus, plantaris, gastrocnemius, extensor digitum longus, and tibialis anterior. Liver, spleen, kidney, heart, and brain tissues were also collected in most cases. Tissues were sonicated in 0.25 M Tris (pH 7.5) to extract CAT.

TABLE 1. Tissue-specific expression directed by
MLC regulatory elements

Tissue	Result for transgene[a]:		
	MCATE	MLucE	MLacE
Hindleg muscles	26	970,000	+++
Brain	≤0.4	117	−
Heart	≤0.5	170	−
Kidney	≤0.5	18	−
Liver	≤0.5	21	−
Spleen	≤0.5	15	−

[a] Results for MCATE are from line 52 and are expressed as percent conversion per microgram of protein per hour. Similar results were obtained with MCATE lines 7 and 63. Results for MLucE are expressed as light units per microgram of protein. Results for MlacE are from histochemical staining of tissue blocks. +++, intense staining after 1 h of incubation; −, no detectable staining after 16 h of incubation.

Biochemical and histochemical assays. β-Galactosidase activity in cell extracts was assayed fluorometrically by the method of Mercer et al. (56). CAT activity in cell and tissue extracts was assayed according to the method of Gorman et al. (30) as modified by Merlie et al. (58). To assay luciferase activity, tissues were extracted with a solution containing 0.1 M Tris-HCl (pH 7.5) and 1 mM dithiothreitol and extracts were assayed in a luminometer as described by Niederrelther et al. (65). The histochemical stain for *lacZ* activity was performed on fixed tissues as described by Sanes et al. (83, 84), and immunohistochemical staining for lacZ and myosin was as described in reference 21. Total soluble protein levels were assayed by the method of Bradford (7).

DNase I footprinting. Recombinant hox B4 and hox C8 proteins were prepared as described in reference 72. hox element-containing fragments spanning nt 359 to 578 were isolated from wild-type and mutant (EmH2; see above) MLC enhancers by digestion with *Bsa*JI and *Ssp*I. The fragments were labeled with [α-^{32}P]dCTP by using Klenow polymerase and purified on polyacrylamide gels. Recombinant hox proteins were incubated for 1 h at 4°C with labeled DNA fragments (50,000 cpm) in a buffer containing 50 mM Tris-HCl (pH 7.9), 12.5 mM MgCl$_2$, 1 mM EDTA, 20% glycerol, 100 mM KCl, 0.1% Nonidet P-40, 1 mM dithiothreitol, and 0.2 to 1 μg of poly(dI-dC) per ml. Samples were kept at room temperature for 2 min, and then MgCl$_2$ and CaCl$_2$ were added to final concentrations of 10 and 5 mM, respectively. After an additional 1-min incubation, samples were treated with DNase I for 1 min and then the reaction was stopped by addition of a solution containing 200 mM EDTA and 1% sodium dodecyl sulfate. Yeast RNA (50 μg per sample) was added as a carrier to facilitate precipitation, and the solutions were extracted with phenol-chloroform, precipitated with ethanol, washed with 70% ethanol, and dried. Deoxyguanosine sequencing reactions were carried out by the method of Maxam and Gilbert (55). Products were analyzed on urea-polyacrylamide gels (54).

RESULTS

Previous studies revealed several noteworthy properties of the MCATE transgene. First, transgene expression is extremely muscle specific, with no other tissues tested expressing CAT at levels >2% of those detected in limb muscle (79) (Table 1). Second, MCATE is expressed at very high levels in adult limb muscles. This is best illustrated by comparison with other transgenes, assayed under identical conditions. As shown in Table 2, levels of CAT activity in MCATE mice are 2- to 10,000-fold higher than those observed in transgenic mice bearing regulatory elements from the α-actin (74), myogenin (58), AChR α-subunit (57), and muscle creatine kinase genes (42). Third, expression of MCATE is fiber type dependent: levels are higher in type II (fast twitch) muscle fibers than in type I (slow twitch) muscle fibers, and they vary among type II fibers in the order IIB > IIX > IIA (21). Fourth, expression of MCATE varies systematically along the rostrocaudal axis, with the highest levels in caudal muscles of the trunk and hindlimb and the lowest levels in muscles of the head (23). It was the purpose of studies reported here to map genomic regulatory elements responsible for these expression patterns.

Transgene expression is reporter independent. We began our studies by asking whether the cardinal features of MCATE

expression were all attributable to the MLC-derived regulatory sequences in the transgene or whether they were influenced by the supposedly neutral reporter, CAT. To this end, we generated and analyzed transgenic mice in which cDNAs encoding lacZ or luciferase replaced CAT (MLacE and MLucE [Figure 1a]). In brief, patterns of transgene expression in these mice resembled those in MCATE mice in all respects. First, expression was muscle specific, as assayed spectrophotometrically for luciferase or histochemically for lacZ (Table 1). The low background of the assay for luciferase in nonmuscle tissues revealed that levels of reporter were >1,000-fold higher in hindlimb muscle than in all nonmuscle tissues tested. The histochemical stain for lacZ allowed us to test and exclude the possibility that the transgene might be expressed at high levels in small subpopulations of nonmuscle cells (Fig. 2a).

Second, expression levels were high in limb muscle. For the *lacZ* transgene, for example, significant staining of limb muscles was detectable after 5 min of incubation at room temperature in staining solution (Fig. 2i), whereas activity of AChR-α-*lacZ* and AChR-ε-*lacZ* transgenes typically required incubation for 24 h at 30°C (83) and activity of a myogenin-lacZ transgene required incubation for 4 to 12 h at 30°C, depending on the age of the mouse (13).

Third, double staining of muscle sections from MLacE mice with anti-lacZ and antibodies to fast (type II) myosin revealed that lacZ levels were significantly higher in type IIB and type IIX fibers than in type IIA or type I fibers (Fig. 2b to f). This pattern is consistent with that reported previously for MCATE, which is expressed in the order IIB > IIX > IIA > I (21). Endogenous MLC1 is likewise expressed at higher levels in type II fibers than in type I fibers (75, 76, 79), but its relative levels in IIA, IIB, and IIX fibers have not been reported.

Fourth, levels of transgene expression were graded along the rostrocaudal axis. For MLucE, the gradient was ~100-fold (Fig. 1b), similar to that shown previously for MCATE. In contrast, regulatory elements from three other muscle-specific genes, i.e., those for actin, creatine kinase, and the AChR α-subunit, did not direct graded expression (Fig. 1c) (reference 23 and data not shown). We also analyzed mice, generated for unrelated purposes, that bore an AChR γ-subunit cDNA in place of CAT (MAChRγE [Fig. 1a]). For three independently generated lines, RNase protection analysis revealed that transgene RNA was 10- to 20-fold more abundant in hindlimb muscles than in head muscles, with intermediate levels in neck and trunk muscles (data not shown). For MLacE, background activities prevented quantitative assay, but histochemical staining revealed that levels of lacZ activity were very high in hind-

TABLE 2. Transgene activity in hindlimb muscles

Transgene[a]	Line	% Activity[b]
MCATE	7	88
	52	100
	63	81
−3300-MCK-CAT		6.4
E2-776-MCK-CAT-E2		56
AChRα-CAT	2	0.0003
	15	0.001
	32	0.0003
α-Actin–CAT	1	4
	2	0.06
Myogenin-CAT	28	0.72

[a] Transgenes are described in Materials and Methods.
[b] CAT activity in MCATE line 52 was arbitrarily set at 100%, and other values were normalized accordingly.
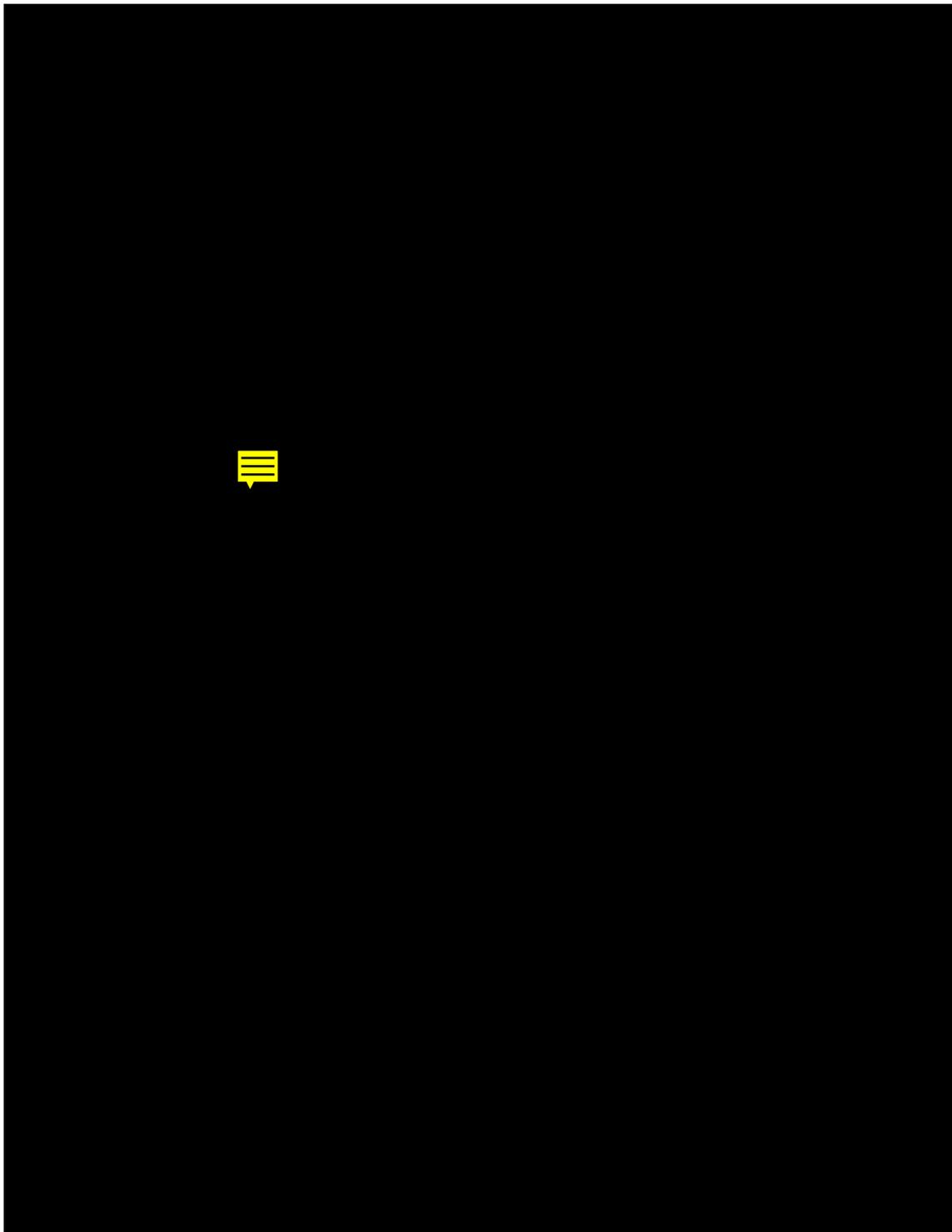
FIG. 2. Position- and fiber-type-dependent expression of the MLacE transgene. (a) An embryonic day 12.5 embryo stained histochemically for lacZ. Note the selective expression in caudal myotomes despite the fact that rostral segments are developmentally more advanced. (b to f) Sections through the tibialis anterior muscle stained histochemically for lacZ (b) or stained with antibodies to lacZ (c and e) plus antibodies to myosin heavy chain IIA (d) or IIB (f). Heterogeneous expression of lacZ represents fiber-type-selective expression of the transgene: type IIB fibers are more intensely lacZ positive than are type IIA fibers. (g to i) Whole muscles from an adult MLacE mouse, stained histochemically for lacZ. A digastricus (g), intercostals (h), and a gluteus (i) are shown. Rostral is up in panel h. Expression is in the caudal-to-rostral gradient hindlimb (gluteus) > caudal intercostals > rostral intercostals > neck (digastricus).

limb muscles, low in most head and neck muscles, and graded along intercostals T2 to T8 (Fig. 2g to i).

Finally, the MLacE transgene allowed us to assess the activity of the MLC-derived regulatory elements in embryos more readily than was feasible with MCATE. MLacE was expressed preferentially in caudal myotomes of embryos at embryonic day 12.5 (Fig. 2a), consistent with previous reports that graded expression of MCATE is established prenatally (24, 32). To test the specificity of this pattern, MLacE embryos were stained in parallel with age-matched embryos bearing a previously described myogenin-lacZ transgene (13); no selective expression in caudal segments was seen in those mice (data not shown; see references 43 and 83 for other examples of ungraded expression). Overall, intermuscular differences appeared to be less pronounced and graded in MLacE mice than in MCATE or MLucE mice; we do not know whether this observation reflects the cellular resolution afforded by the histochemical stain or a genuine influence of the lacZ sequences. It is clear, however, that the high-level, muscle-specific, fiber-type-dependent, positionally graded expression directed by MLC-derived sequences is largely and perhaps entirely reporter independent.

Mutational analysis of MLC1 regulatory sequences. We generated 17 mutant or truncated variants of the MLC-derived sequences present in the MCATE transgene. Constructs were first tested by transfection into a mouse myogenic cell line, Sol 8, and then used to generate transgenic mice, which were analyzed as founders. By sacrificing founders instead of establishing lines, we hoped to maximize both the number of mutants and the number of independent insertions per construct that we could examine. Analysis of multiple founders per construct is important to minimize the chance that an unusual site of integration in a single line might lead to misleading (integration site dependent rather than construct dependent) results. We were concerned, however, that founders might express transgenes at levels markedly different from those of the lines studied previously (Fig. 1 and 2). In an initial study, we therefore compared CAT activities in hindlimb muscles of eight founder (F_0) animals and their F_1 offspring. Six of the animals bore the MCATE transgene and were generated as part of a separate study of the copy number dependence of transgene expression and methylation (82). Two of the animals bore mutant transgenes (MCATE0 and MCATEmF1) described below. Activities in the eight founders varied over a >100-fold range, but the activities of paired F_0 and F_1 mice were highly correlated ($r = 0.91$ by Spearman's rank-difference test) and the average activity for the F_1 animals was 154% of that for the founders. On the basis of these results, we analyzed mutant transgenes in founders exclusively.

Our results are presented in six sections. The first three describe sequences responsible for promoting transgene expression in hindlimb muscle. The next three deal, in turn, with effects of the mutations on muscle specificity of transgene expression, differential expression in slow- and fast-twitch muscles, and position-dependent intermuscular differences.

Mutations of identified binding sites in the MLC enhancer. The MCATE transgene contains a 0.9-kb fragment that was identified as an enhancer by virtue of its ability to activate gene expression in a promoter-, position-, and orientation-independent fashion (22). Sequence analysis of the enhancer revealed several binding sites for transcriptional regulators within the enhancer (Fig. 3a), some of which have been shown to be functional in cultured muscle cells. We chose four sites for mutational analysis: E box B and E box C, both of which bind myoD (90); a binding site for the myocyte-specific enhancer
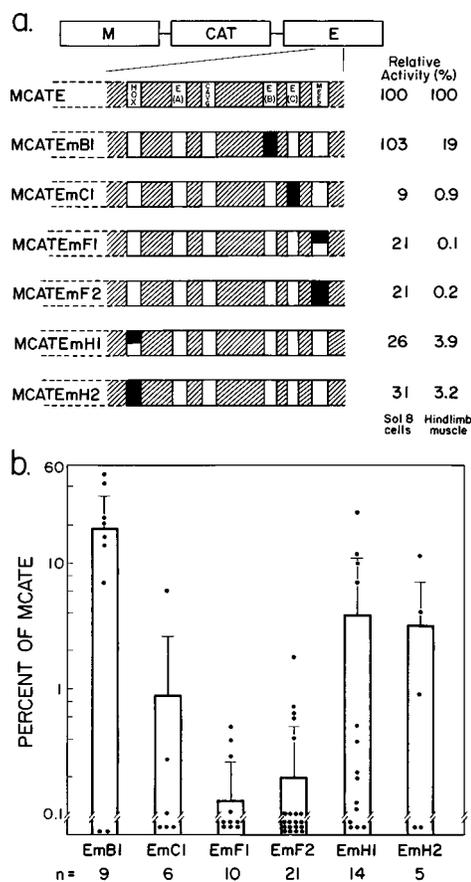

FIG. 3. Mutations of identified protein binding sites in the MLC enhancer. (a) Diagram of transgenes. Positions of the hox-binding site, a CArG box, three E boxes (A to C), and a MEF2 binding site, all within the core enhancer, are indicated. Numbers give average specific activities of CAT in Sol 8 muscle cells and hindlimb muscles of transgenic mice, expressed as percentages of the specific activity of MCATE. (b) Transgene activities in hindlimb muscles of individual mice, expressed as percentages of the specific activity of MCATE and displayed on a log scale. Each point represents a single animal. Open bars and vertical lines show means plus standard deviations. Numbers of animals assayed are given beneath the graph. A break in the scale was arbitrarily set at 0.1% of the specific activity of MCATE. Points beneath the break include animals that expressed significant levels of CAT as well as animals in which CAT activity levels were not significantly above background levels. Because we could not objectively distinguish some low-level expressers from nonexpressers, we have not attempted to specify the fraction of animals that expressed the transgenes and have included all animals in the average values given in panels a and b.

transcription factor 2 (MEF2 [31]); and a putative binding site for homeodomain proteins.

(i) E boxes. E boxes B and C were mutated by changing the recognition sequences for myogenic factors CAGCTGG and CAGGTG to GGTACCA and GTCGAC (MCATEmB1 and MCATEmC1, respectively [Fig. 3a]). Mutation of E box C decreased activity 10-fold, as assayed by transfection into Sol 8 cells, whereas mutation of E box B had no significant effect in this assay (Fig. 3a). These results are consistent with those of Wentworth et al. (90), who showed that mutation of E box C led to a greater decrease of activity in C2 muscle cells than did mutation of E box B (86 and 32% inhibition, respectively).

Expression of these mutated transgenes in hindlimb muscles of transgenic mice paralleled that seen in vitro, although both mutations had more-severe effects in vivo than in vitro. Thus, inactivation of E box B led to a modest but significant decrease in activity (19% ± 15% of the wild-type level [mean ± stan-
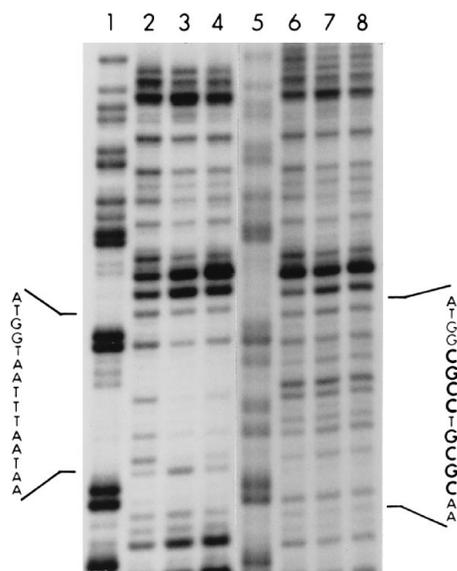
FIG. 4. DNase I footprint analysis of the hox binding site in the MLC enhancer. A 220-bp fragment from the enhancer was incubated with buffer alone (lanes 2 and 6), recombinant hox B4 (lanes 3 and 7), or recombinant hox C8 (lanes 4 and 8). Sequencing standards are shown in lanes 1 and 5. The nucleotide sequence protected is indicated on the left. Note that the sequence TAATTTA AT represents the strand opposite the paired hox sites. In lanes 5 to 8, a fragment in which the hox site was mutated from ATTAAATTA to GCGCAGGCG was used. This mutation abolished binding of hox proteins.

dard deviation; $n = 9$]), whereas inactivation of E box C decreased activity by ~100-fold (Fig. 3b). Taken together, our results with C2 cells, Sol 8 cells, and transgenic mice suggest that binding of myogenic factors to E box C is critical for the strong activity of the MLC enhancer whereas activation of E box B does not greatly affect activity.

(ii) MEF2 site. Two different mutations were introduced into the MEF2 site of the MLC enhancer: the sequence 5′-CTTTTAAAAATA-3′ was changed to ACCGGTAAAATA in MCATEmF1 and to CTTATGGGGCCC in MCATEmF2. Both mutations decreased the activity of the enhancer in Sol 8 cells to ~20% of wild-type levels (Fig. 3a). Likewise, both mutations decreased expression of the transgene in hindlimb muscles, but as was the case for E box C, effects were more severe in vivo than they were in vitro: transgene activity averaged 0.1 to 0.2% of wild-type levels for both mutants (Fig. 3b) and activities were decreased to <0.01% of the wild-type level in 17 of 31 animals tested. Thus, activity of the MLC enhancer in vivo appears to require binding of MEF2 family transcription factors as well as myogenic factors.

(iii) hox site. The MLC enhancer contains two juxtaposed core homeodomain binding sites (i.e., a hox site [consensus, ATTA]; MLC sequence, 5′-TTATTAAATTACC-3′), but the ability of this site to bind homeodomain-containing proteins has not been reported. To address this point, we assayed the binding of two purified, recombinant hox proteins, B4 and C8, to the enhancer in a DNase I footprinting assay. As shown in Fig. 4, lanes 3 and 4, both proteins bound to the enhancer and protected the hox recognition sequence (GGTAATTTAAT AA on the opposite strand) from digestion by DNase I. To show that the binding activity was sequence specific, mutations were introduced into both core hox elements and the footprinting assay was repeated. As shown in Fig. 4, lanes 7 and 8, this mutation abolished binding of both proteins. Thus, the MLC enhancer can bind hox proteins.

On the basis of these results, we tested two mutations of the hox element in tissue culture and in transgenic mice. The core sequence ATTAAATTA was changed to TCGCGATTA and GCGCAGGCG in MCATEmH1 and MCATEmH2, respectively, thereby mutating the first core element in MCATEmH1 and both core elements in MCATEmH2. Both mutants decreased activity in Sol 8 cells to ~30% of wild-type levels (Fig. 3a). Likewise, both mutants behaved similarly in transgenic mice, resulting in average activities of 4 to 5% of wild-type levels (Fig. 3b). Thus, full activity of the MLC enhancer appears to require binding of homeodomain proteins.

Deletion analysis of the MLC enhancer. Studies presented so far demonstrate that two E boxes, a MEF2-binding site, and a hox site are all necessary for full enhancer activity in vivo (Fig. 3). All of the sites lie within a 173-bp core region that has been shown to retain most of the activity of the 900-bp enhancer in the C2 muscle cell line (90). To determine whether these sites were sufficient for full activity, we constructed a transgene lacking sequences flanking the core (MCATEcore [Fig. 5a]). The core retained ~40% of the activity of the 900-bp enhancer when tested by transfection in Sol 8 cells (data not shown). It was, however, nearly inactive in transgenic mice: significant CAT activity was detected in only 6 of 19 transgene-positive mice tested, and in 5 of these 6 mice, levels of activity were <0.02% of those measured in mice bearing the complete transgene (Fig. 5b). Thus, the elements clustered in the core are sufficient to direct high levels of transgene activity in two different mouse myogenic cell lines, but they are insufficient to maintain high levels of activity in vivo.

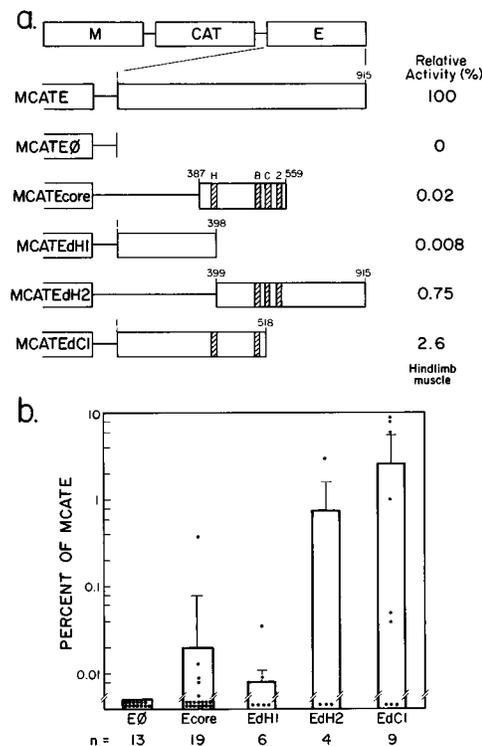A second construct in this series, MCATEdC1, had the dis-

FIG. 5. Truncations of the MLC enhancer. (a) Diagrams of transgenes and specific activities of CAT in hindlimb muscles of transgenic mice, expressed as percentages of the specific activity of MCATE. H, hox site; B, E box B; C, E box C; 2, MEF2 site. (b) Transgene activities in hindlimb muscles of individual mice, plotted as in Fig. 3b. Open bars and vertical lines show means plus standard deviations.

tal 400 bp of the enhancer deleted, removing the MEF2 site and disrupting E box C. Interestingly, this construct was more active in transgenic mice than the core enhancer was, with three of nine mice tested having CAT activities that were >5% of the wild-type level (Fig. 5b). This result suggests that some sequences 5′ of the core enhancer are important for high-level activity in vivo even though they are dispensable in cultured cells.

Additional constructs were made to test the two halves of the enhancer separately: MCATEdH1 contained nt 1 to 398 of the enhancer and MCATEdH2 contained nt 399 to 915. Both constructs were expressed poorly in transgenic mice, i.e., at <0.01% of wild-type levels in 7 of 10 animals tested (Fig. 5b). This result is consistent with those obtained with the mutations described above, in that the MEF2 site and E boxes B and C were missing from MCATEdH1 and the hox site was disrupted in both constructs. On the other hand, significant activity was detectable in 8 of the 10 mice. This residual activity was enhancer dependent, as no activity above background levels was detected in any of 13 mice bearing a transgene from which the entire enhancer had been deleted (MCAT0) (Fig. 5).

Deletion analysis of the MLC1 promoter. To seek functionally important regions of the MLC1 promoter fragment contained in MCATE, we generated and analyzed a series of 5′ truncations. Deletion of 323 or 452 bp from the 5′ end of the promoter fragment led to a modest decrease in activity as assayed by transfection in Sol 8 cells (M835CATE and M676CATE [Fig. 6a]). Deletion of 560 bp resulted in a further loss of activity, to 19% of wild-type values (M598CATE [Fig. 6a]). These results suggest the existence of multiple functional elements in the promoter region between nt −1158 and −598.

Truncation of the promoter to nt −835 led to a moderate reduction of activity in transgenic mice (12% of the control level [Fig. 6a]), as it did in Sol 8 cells. Further truncations, however, had considerably more dramatic effects in vivo than they did in cultured cells: transgenic mice bearing transgene M676CATE, which generated 64% of wild-type activity in cultured cells, had an average of ~1% of control levels of CAT activity in their hindlimb muscles, with values of <0.1% for six of eight animals tested. Likewise, the level of CAT expression from M598CATE was <0.1% of the wild-type level in 21 of 23 animals tested. Further truncation, to nt −391, led to expression in only 2 of 11 animals. In one of these two animals muscles had ~0.01% of control levels of CAT activity, and the other animal had high-level activity (12%), possibly reflecting an unusual site of transgene insertion. Together, these results support the conclusion that the MLC1 promoter bears at least two functional sites, one 5′ and one 3′ of nt 835. In addition, the comparison of assays in vivo and in vitro indicates that sequences between nt −835 and −598, although inessential for high-level activity in transfected cells, are critically important in vivo.

To further characterize the function of sequences 5′ to the MLC1 basal promoter, we fused sequences between nt −1158 and −377 to a basal promoter from the hsp68 gene. The hsp68 promoter alone (hspCATE) was more active than the wild-type MLC1 promoter in Sol 8 cells but was >100-fold less active in transgenic mice (Fig. 6). Addition of the MLC1 fragment to this promoter increased activity 2-fold in Sol 8 cells and >10-fold in transgenic mice (MΔhspCATE [Fig. 6a]). Although the fragment containing sequences between nt −1158 and −377 did not completely restore wild-type activity, its effect was highly significant: 9 of 14 mice bearing the MΔhspCATE transgene exhibited activity levels higher than those exhibited by any of 5 mice bearing hspCATE (Fig. 6b). These results provide a second line of evidence for functionally
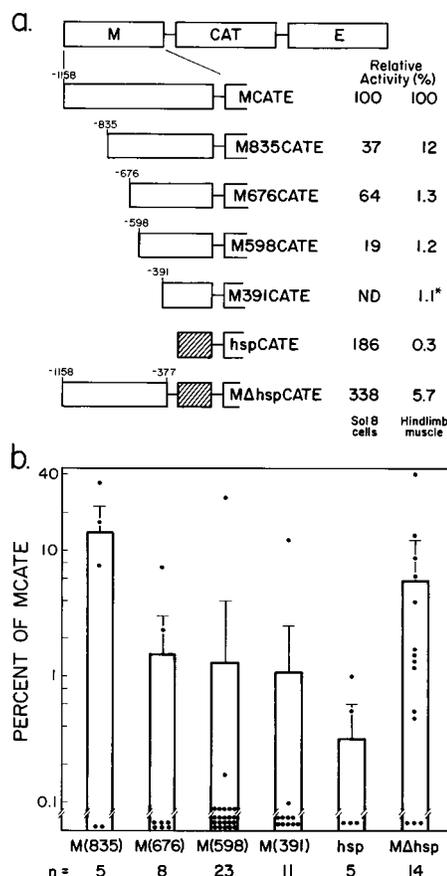

FIG. 6. Truncation and replacement of the MLC1 promoter. (a) Diagrams of transgenes and specific activities of CAT in Sol 8 cells and hindlimb muscles of transgenic mice, expressed as percentages of the specific activity of MCATE. The asterisk indicates that the value would be <<0.1% if the single highest point were excluded. ND, not determined. (b) Transgene activities in hindlimb muscles of individual mice, plotted as in Fig. 3b. Open bars and vertical lines show means plus standard deviations. hsp, promoter from the heat shock protein 68 gene.

important sites in the 5′ region of the MLC1 promoter fragment.

Effect of mutations on muscle-specific expression. Recent studies of genes specifically expressed in neurons have identified negatively acting regulatory elements that prevent expression in nonneuronal cells (85). Likewise, muscle-specific gene expression may arise not only from activation by factors specifically expressed in muscle but also from repression by "silencer" factors expressed in nonmuscle cells (9). Results presented above mapped sites in the MCATE transgene responsible for activation in muscle. To test the possibility that tissue-specific expression of MCATE also reflects silencing in nonmuscle cells, we asked whether any mutations of the transgene led to an increase in its expression in either liver or spleen tissue. Results of this survey are shown in Table 3. For no construct was activity in liver or spleen tissue >2% of that observed in hindlimb muscle. In some cases, levels of activity in muscle were so low that background levels in liver or spleen tissue approached the total level of activity measured in muscle; in these cases, the calculation of ratios was not meaningful. For those animals with CAT levels in muscle high enough to avoid this problem (levels >1% of those seen with the unmutated transgene), there were no individuals in which the specific activity in liver or spleen tissue was >5% that seen in

TABLE 3. Tissue-specific expression directed by
mutant MCATE transgenes

Transgene	n	Sp act in[a]:	
		Liver tissue	Spleen tissue
MCATE	3	0.7	0.6
MCATEmB1	7	1	0.4
MCATEmC1	3	0.2	<0.1
MCATEmF1	7	1	0.7
MCATEmH1	9	0.1	0.9
MCATEmH2	4	0.2	0.3
MCATEcore	6	0.5	0.2
MCATEdC	6	0.7	0.1
MCATEdH1	2	2	2
MCATEdH2	1	2	1.3
M835CATE	3	1.6	1.3
M676CATE	8	1.7	1.2
M598CATE	7	1.7	<0.1
M391CATE	1	0.5	ND[b]
hspCATE	2	0.9	1.6
MΔhspCATE	3	<0.1	<0.1

[a] Specific activities in liver and spleen tissues are expressed as percentages of the activity in hindlimb muscle, measured in the same animal.

[b] ND, not determined.

muscle. Thus, we obtained no evidence that muscle-specific expression of MCATE reflects silencing of MCATE expression in nonmuscle tissues.

Effect of mutations on differential expression in fast and slow muscles. We previously used a histochemical stain for CAT to demonstrate that expression of the MCATE transgene is fiber type dependent, in the order IIB > IIX > IIA > I (21). Histochemical staining was infeasible in the present study because absolute levels of transgene activity were often low. As an alternative, we compared transgene activity in the soleus, which contains only type I and IIA fibers, with that in the neighboring muscles of the hindlimb, all of which contain a preponderance of type IIX and IIB fibers (21, 76). This method has been used to assess the fiber type specificities of several muscle-specific genes and transgenes (4, 34, 44, 81). Ratios of transgene activity in the soleus to that in four fast hindlimb muscles are presented in Fig. 7. For the control MCATE transgene, the ratio was ~0.1, and for nearly all mutants tested the ratio was <0.15. For two transgenes with 3′ truncations of the enhancer, however, the ratios were at least threefold higher than that for MCATE (0.33 and 0.31 for MCATEdH1 and MCATEdC1, respectively). These constructs both lack sequences from nt 518 to 915 of the enhancer. In contrast, MCATEcore, which lacks sequences from nt 560 to 915, had a ratio of 0.12. Together, these results suggest that sites between nt 519 and 559 either selectively promote expression in fast muscles or selectively inhibit expression in slow muscles. These sites lie within the core enhancer but apparently do not overlap E box C or the MEF2 site, disruption of either of which is without effect on the slow/fast ratio (MCATEmC1 and MCATEmF1 [Fig. 7]).

Effect of mutations on position-dependent transgene expression. The most striking feature of the MCATE transgene is that it is expressed in a rostrocaudal gradient. We showed previously that position-dependent expression does not require a high transgene copy number (82) and does not reflect a unique site of integration into the host genome (23). To seek regulatory elements responsible for the generation or maintenance of the gradient, we assayed CAT activity in a set of eight head, neck, and trunk muscles from transgenic mice bearing

the mutated MCATE transgenes illustrated in Fig. 3, 5, and 6. To facilitate analysis of the data, we set the CAT activity of the T10 intercostal at 100% for each individual mouse and then expressed activities in other muscles relative to this value. This procedure allows us (i) to compare expression patterns of many transgenes even though absolute levels of activity varied by >1,000-fold among transgenes and (ii) to obtain a single average value from all mice generated from each construct even though absolute values of expression frequently varied manyfold among animals bearing a single construct. Results of this analysis are presented in Fig. 8.

In brief, neither subtle mutations nor deletions of the MLC enhancer had drastic effects on the rostrocaudal gradient of transgene expression (Fig. 8a to h and data not shown). In most cases, activity in hindlimb muscle was at least 20-fold greater than that in head muscles and activity in the T10 intercostal was at least 5-fold greater than that in the T2 intercostal. Particularly noteworthy were hox binding site mutants (MCATEmH1 and MCATEmH2), which might have been expected to affect position-dependent expression but did not (Fig. 7e and data not shown). Moreover, the two halves of the enhancer tested separately (MCATEdH1 and MCATEdH2) both generated graded expression patterns (Fig. 8g and data not shown). Strikingly, graded expression persisted even when overall activity levels were decreased by over 3 orders of magnitude with respect to those of MCATE. (Activity levels that were <0.1% of the MCATE level were readily detectable because of the very high level of expression of the wild-type transgene [Table 1].) Unfortunately, however, attempts to remove the MLC enhancer completely were ineffective: neither MCATE0 (Fig. 5) nor a transgene in which a simian virus 40 viral enhancer replaced the MLC enhancer (data not shown; construct illustrated in Fig. 6c of reference 22) was expressed at detectable levels in transgenic mice. Nonetheless, our data are all consistent with the notion that sequences outside of the enhancer are responsible for graded expression.

On the basis of these results and the demonstration that graded expression is not attributable to sequences in the reporter gene (Fig. 1 and 2), we assayed patterns of transgene expression in the promoter truncations. Qualitatively, trunca-
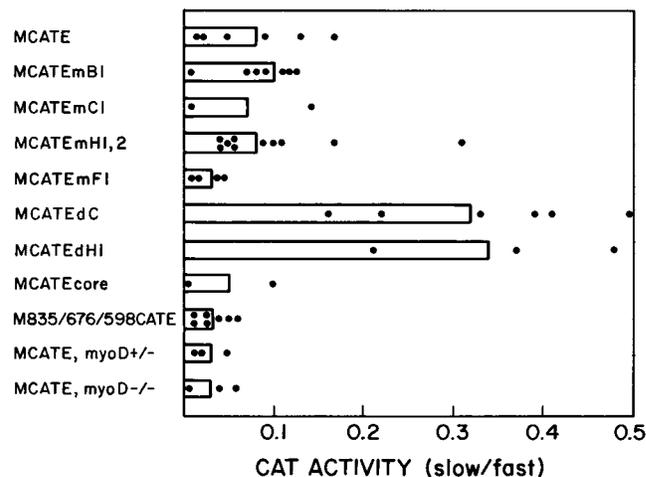

FIG. 7. Fiber-type-selective expression directed by MCATE transgenes. Values are ratios of specific activity in the soleus to that in a set of four neighboring, predominantly fast crural (lower hindlimb) muscles—the tibialis anterior, the extensor digitorum longus, the gastrocnemius, and the plantaris. Bars show means, and points show values from individual animals. MCATEdC differs significantly from MCATE (P < 0.01 by Student's t test).
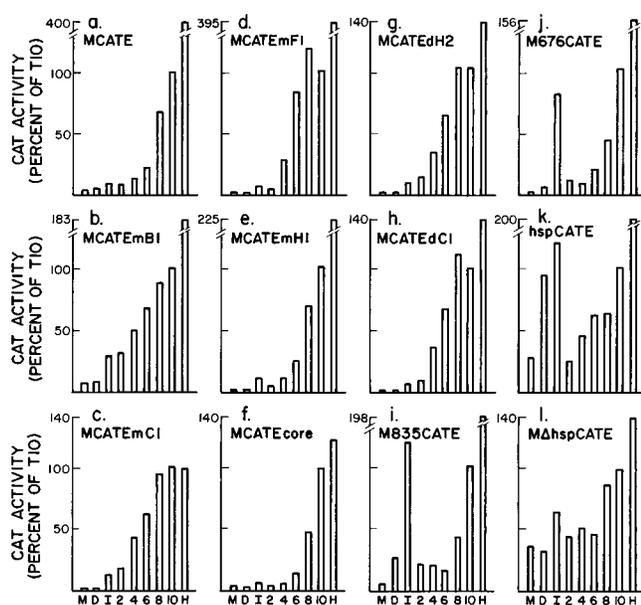
FIG. 8. Position-dependent expression of mutant transgenes. The specific activity of CAT was measured in individual mice. The activity in the T10 intercostal was set at 100% for each mouse, and activities of other muscles were adjusted to that value. This procedure permitted determination of an overall pattern for each construct and facilitated comparison among constructs. Numbers of lines or founders analyzed for the constructs were as follows: MCATE, 3; MCATEmB1, 7; MCATEmC1, 2; MCATEmF1, 4; MCATEmH1, 6; MCATEcore, 4; MCATEdH2, 1; MCATEdC1, 5; M835CATE, 3; M676CATE, 4; hspCATE, 5; and MΔhspCATE, 4. Abbreviations of muscles are defined in the legend to Fig. 1.

tion of the 1,158-bp promoter to nt −835, −676, or −598 had little effect on the pattern of transgene expression in most muscles: in all three cases, activity levels remained high in hindlimb muscles, low in the masseter muscle, and graded in intercostals. All three truncations, however, led to a striking relative increase in transgene expression in the infrahyoid muscles of the neck (Fig. 8i and j and data not shown). This result suggests that sequences between nt −1158 and −835, in the 5′ end of the promoter fragment, selectively inhibit expression in the neck. Moreover, a shorter promoter fragment (M598CATE) led to increased relative expression in the digastricus as well as in the infrahyoid muscles in some but not all animals tested (data not shown). This result suggested that additional sequences between nt −676 and −598 selectively inhibit expression in the digastricus.

Because further truncation of the promoter abolished activity completely (M391CATE [Fig. 6]), we next examined patterns of expression in mice in which a promoter fragment from the hsp68 gene was substituted for the MLC1 promoter. In this case, relative expression was increased in the digastricus and the infrahyoid muscles (Fig. 8k), supporting the conclusion that sequences in the MLC1 promoter selectively inhibit expression in these muscles. Surprisingly, expression remained graded in intercostal muscles, but the gradient was less systematic and severalfold less steep than that seen with the complete transgene or the enhancer mutants. Thus, the use of a heterologous promoter clearly supports the conclusion that the 5′ portion of the MLC1 promoter contains sequences that inhibit expression in neck muscles and is consistent with the possibility that sequences in the 3′ portion of the promoter direct graded expression in intercostal muscles.

As a final test of this model, we examined transgene expression in mice containing transgene MΔhspCATE, in which the

5′ portion of the MLC1 promoter was fused to the hsp promoter. Our expectation was that mice bearing this transgene would have poorly graded expression in intercostals, because they lacked the 3′ region of the MLC1 promoter, and that they would have similar levels of expression in head, neck, and rostral intercostal muscles, because they contained sequences that inhibited expression in neck muscles. In fact, as shown in Fig. 8l, both of these predictions were confirmed: the difference in activity between masseter and hindlimb muscles was <4-fold and that between intercostals T2 and T10 was only 2-fold, compared with differences of ~100-fold and >10-fold, respectively, for MCATE.

Influence of myoD on MLC1-CAT expression. myoD and its homologs are crucial regulators of gene expression in skeletal muscle (67) and can activate the MLC1-CAT transgene in vitro (90). We showed previously that levels of myogenic factors do not vary greatly among muscles (82), but subtle intramuscular differences have been suggested to affect fiber type (41) and might underlie position-dependent transgene expression. We tested this possibility in two ways. First, we assayed expression of MCATE in mice lacking myoD. Mice lacking the *myoD* gene have no obvious phenotype (80), presumably because other myogenic factors substitute for it (67). Nonetheless, compensation is likely to be imperfect, and myoD may have unique roles in subtle processes such as determination of fiber type (41) or intermuscular differences in gene expression. We therefore mated MLC1-CAT transgenic mice to myoD mutants and then crossed the offspring to obtain $myoD^{-/-}$ and $myoD^{+/-}$ mice, all of which carried the MLC1-CAT transgene. MLC1-CAT expression did not differ significantly between $myoD^{-/-}$ and $myoD^{+/-}$ mice, either in absolute levels (not shown) or in pattern (Fig. 9a and b). Likewise, the selective expression of MCATE in fast-twitch fibers was unaltered by the absence of myoD (Fig. 7).

Second, we assayed expression of MCATE in mice that overexpressed myoD. To this end, we mated a mouse homozygous for MCATE to a mouse chimeric for a transgene by which myoD was expressed in skeletal and cardiac muscles under the control of a promoter from the mouse creatine kinase gene (60). myoD-overexpressing mice die prenatally of cardiac defects, and so the litter was taken at embryonic day 16 and individual fetuses were genotyped by PCR. CAT activity in neck, chest, and hindlimb muscles from the single myoD-overexpressing fetus and four littermates was assayed. As shown in Fig. 9c, position-dependent expression persisted in the presence of excess myoD. Together, these results support our earlier evidence (85) that subtle intermuscular differences in myogenic factor expression do not underlie intermuscular differences in MLC1-CAT expression.

DISCUSSION

Like the endogenous MLC1f/3f gene from which it is derived, the MCATE transgene displays high levels of expression in skeletal muscle, negligible expression in other tissues, appropriate developmental regulation, and selective expression in appropriate fiber types (21, 79). In addition, unlike the endogenous gene, the transgene is expressed in a striking rostrocaudal gradient (23), which provides a molecular correlate of nerve-muscle affinities deduced from electrophysiological studies (47, 48, 82, 91, 92). To map elements within the MCATE transgene responsible for these patterns of expression, we generated transgenes in which the MLC1f promoter, the MLC enhancer, or the reporter had been mutated, truncated, or replaced. Transgenes were tested in a muscle cell line (Sol 8) and in transgenic mice. Five aspects of transgene ex-
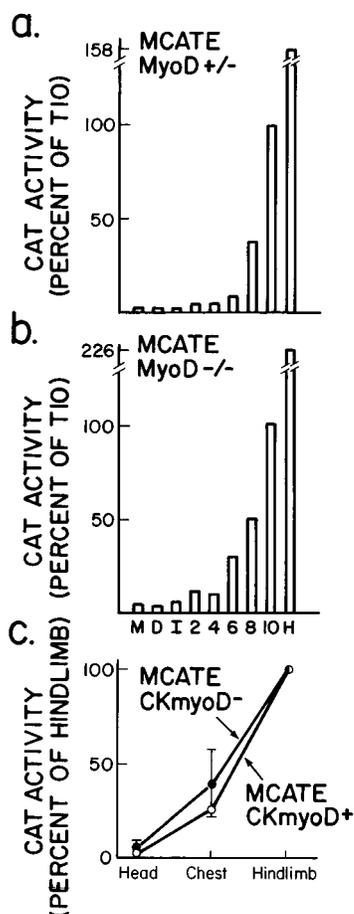
FIG. 9. Position-dependent expression of MCATE in mice with altered levels of myoD. (a and b) MCATE and $myoD^{-/-}$ mice were crossed, and muscles from the F_2 generation were analyzed. Data are expressed as in Fig. 7. Abbreviations of muscles are defined in the legend to Fig. 1. (a) MCATE$^{+/-}$ $myoD^{+/-}$ ($n = 3$); (b) MCATE$^{+/-}$ $myoD^{-/-}$ ($n = 3$). An MCATE homozygote was crossed with a chimeric mouse that overexpressed myoD under the control of the creatine kinase promoter (CKmyoD). On embryonic day 16, neck, chest, and hindlimb muscles were removed from the four MCATE$^+$ CKmyoD$^-$ fetuses and one MCATE$^+$ CKmyoD$^+$ fetus in the litter and CAT activities were assayed.

pression were then assayed: (i) activity in cultured myotubes; (ii) activity in adult hindlimb muscle; (iii) the ratio of activity in nonmuscle tissues to that in hindlimb muscle; (iv) the ratio of activity in a slow hindlimb muscle (the soleus) to that in adjacent, fast muscles; and (v) patterns of activity in muscles that span the rostrocaudal axis. From analysis of these data, we conclude that patterns of MCATE expression are reporter independent but are regulated by a minimum of nine distinct sites within the promoter and enhancer. The regions implicated are diagrammed in Fig. 10 and discussed in the following paragraphs.

E boxes B and C. The MLC enhancer contains several copies of a sequence, CANNTG, which is called an E box and has been shown to serve as the DNA recognition motif for transcriptional regulators of the helix-loop-helix family (11, 49, 62). E boxes have been shown to be crucial for the transcriptional activation of many muscle-specific genes, presumably by virtue of their ability to bind the myogenic factors myoD, myogenin, myf5, and MRF4/herculin (67). Three E boxes, A to C, are clustered in a 90-bp stretch within the core of the MLC enhancer. All three of these sites have been shown to bind myoD.

However, mutation of E box A or C decreases expression of the MCATE transgene in cultured myotubes significantly more than does mutation of E box B (90). Our studies extend these results to transgenic mice: mutation of E box B decreases transgene activity ~5-fold in hindlimb muscle, whereas mutation of E box C decreases activity ~100-fold. Interestingly, however, the effects of both mutations are severalfold more severe in vivo than they are in vitro (Fig. 3). On the other hand, neither E box is necessary for fiber-type- or position-related intermuscular differences in transgene activity. Moreover, neither inactivation nor overexpression of the *myoD* gene had detectable effects on intermuscular differences in transgene activity. These results suggest that myogenic factors are required for transcriptional activation of MCAT but not for its complex pattern of expression.

MEF2 site. The MEF2 motif, YTA(A/T)$_4$TAR, was identified as a common element in numerous muscle-specific genes recognized by nuclear proteins from muscle cells (11, 16, 31). Subsequently, MEF2 factors were demonstrated to be transcriptional activators of the MADS family (68). The MLC enhancer contains a single site within its core, 5′-TTTAAAAA TAA-3′, that corresponds to the MEF2 consensus and is capable of binding MEF2 (31). Mutation of this site reduces transgene activity in hindlimb muscle by >100-fold. Mutations of the MEF2 site were similar to mutations of the E boxes in several respects: both had more-drastic effects in vivo than they did in vitro and neither affected fiber-type- or position-dependent expression. In its requirement for both E box and MEF2 binding activities, MCATE resembles some other muscle-specific genes, including *MRF4* (6, 64). On the other hand, MCATE behaves differently from myogenin and desmin transgenes, in which E box and MEF2 mutations have mild effects individually and drastic effects only when combined (13, 52).

hox binding site. The MLC enhancer contains the sequence ATTAAATTA, which comprises two copies of the core binding site for homeodomain proteins of the hox class (29, 37, 39). We used DNase footprinting analysis to show that the MLC enhancer can bind purified, recombinant hox proteins at its hox motif and that the binding is sequence specific. Moreover, mutation of the hox binding site led to a decrease in transgene activity both in Sol 8 cells (~3-fold) and in hindlimbs of transgenic mice (~20-fold). Although we have not identified the binding proteins that interact with this site in vivo, we have shown that nuclear extracts (20) from myotubes contain factors that bind the hox site (76a). Moreover, myogenic cells have previously been shown to express hox genes (14, 33, 50), and we have recently detected hox RNAs in adult muscle, albeit at low levels (76a). In contrast, MEF2, which recognizes related AT-rich sites (16), does not bind to the sequence (66a). Together, these results raise the possibility that transcription of muscle-specific genes may be modulated by hox proteins. In
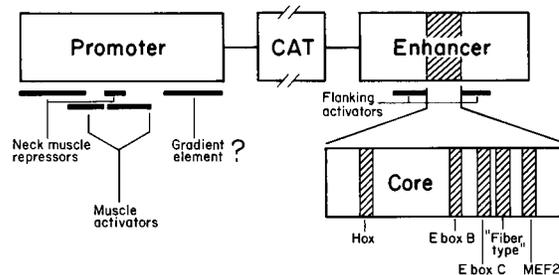

FIG. 10. Summary of functionally important sites in MCATE identified by analysis in transgenic mice. See Discussion for details.

light of evidence that *hox* genes specify axial position in vertebrates and directly affect muscle identity in *Drosophila melanogaster* (59), we suspected that the *hox* element would regulate the axial gradient of MCATE expression as well as overall transgene activity. This was not, however, the case: mutation of the *hox* element reduced activity in all muscles to similar extents, thereby maintaining fiber-type- and position-dependent intermuscular differences in expression.

Sequences flanking the core enhancer. Several key binding sites in the MLC enhancer are clustered in an ~150-bp stretch. These include the MEF2 site, the hox binding site, and three E boxes discussed above, as well as an additional variant E box and a "CArG" box (90). In cultured cells, a 173-bp core enhancer that contains these sites is about half as active as the full 0.9-kb enhancer contained in MCATE. It was therefore surprising to find that this core (MCATEcore) was <1% as active as MCATE in hindlimb muscles of transgenic mice. This result suggests that sequences flanking the core are required for activity of the transgene once it is stably integrated into chromatin. Enhancer-flanking elements required for activity in transgenic animals but not in transfected cells have been reported for several other genes, including those for adenosine deaminase (2) and immunoglobulin μ (27); such elements have been termed facilitators, matrix attachment regions, and locus control regions. The precise location of these elements in MCATE remains to be determined, but it is noteworthy that Kelly et al. (43) obtained high levels of expression of a mouse MLC3f transgene containing a 260-bp enhancer that included the 173-bp core plus ~25 bp 5′ of the core and ~60 bp 3′ of the core. This result suggests that facilitatory elements may be located directly adjacent to the core.

Activators in the MLC1 promoter. MCATE contains a 1.2-kb MLC1 promoter fragment which includes the transcriptional initiation site at its 3′ end. Deletion of the 323 bp from the 5′ end of this fragment (to nt −835) led to a modest (<5-fold) decrease in activity in transgenic mice, whereas deletion of an additional 159 bp (to nt −676) resulted in an ~100-fold loss of activity and deletion to nt −391 produced a transgene with no detectable activity. Likewise, truncations led to a progressive decrease in the activity of the transgene in cultured myotubes. Together, these results indicate the presence of at least two sites in the promoter that are crucial for activity of MCATE, one 5′ and one 3′ of nt 835.

A fiber-type-selective element. MCATE was the first transgene shown to reproduce the fiber-type-selective expression pattern of the endogenous gene from which it was derived: activity levels are highest in type IIB fibers and successively lower in type IIX, type IIA, and type I fibers (21). Several other transgenes that are preferentially expressed in fast-twitch (type II) (34, 35, 81) or slow-twitch (type I) (4, 15, 44, 51) fibers have been described. In no case, however, have the sequences responsible for fiber type expression been defined. In our studies, no mutations of defined binding elements affected the preferential expression of the transgene in fast-twitch fibers. However, deletion analysis indicated that sequences within the distal 40 bp of the core enhancer reduced fiber type selectivity. Within this region are three sequences that are completely conserved between the rat and human (78) genes: E box C, the MEF2 site, and the sequence 5′-CCTAATTCCTCA-3′. In that directed mutations of E box C and the MEF2 site had no detectable effect on fiber type specificity, it may be useful to test the third region of conservation in future studies.

A neck-specific repressor. Truncation of the 1,158-bp MLC1 promoter to nt −835 led to a striking increase in transgene expression in the infrahyoid muscles of the neck, as measured relative to expression in neighboring rostral muscles (the mas-

seter, the digastricus, and rostral intercostals). Fiber-type-dependent expression cannot account for the pattern, because the promoter truncations had no effect on relative activity in the soleus and because the infrahyoid muscles are typical fast-twitch muscles (reference 21 and data not shown). We therefore conclude that elements between nt −1158 and −835 repress expression of the transgene in muscles of the neck.

Results obtained with several other transgenes provided support for this conclusion. First, increased relative expression in neck muscles was seen with truncations to nt −676 or −598, both of which also lacked the fragment containing nt −1158 to −835, indicating that the pattern did not arise from fortuitous integration sites or generation of a novel site at the boundary of a transgene. Second, relative levels of CAT expression in the neck muscles of hspCATE mice, in which the MLC1 promoter was replaced by a heterologous promoter from heat shock protein 68, were high. Activity levels were also higher in infrahyoid muscles than in other rostral muscles in mice in which a thymidine kinase promoter replaced the MLC1 promoter in MCATE (data not shown; transgene described in reference 58). Finally, fusion of the 5′ portion of the MLC1 promoter, including the sequence from nt −1158 to −835, to the hsp68 promoter restored a pattern in which the transgene was expressed at similar levels in all rostral muscles tested.

Our results also provide some evidence for a second element that selectively affects transgene activity in rostral muscles. When the MLC1 promoter was truncated to nt −598, transgene activity was dramatically increased in the digastricus muscle as well as in the infrahyoid muscles of one of the three mice characterized. Although this may represent an integration site-dependent effect, it is noteworthy that replacement of the MLC1 promoter by hsp68 also increased relative expression in the digastricus as well as the infrahyoid muscles. In contrast, truncation of the MLC1 promoter to nt −835 or −676 increased relative expression in the infrahyoid muscles but not in the digastricus. Thus, sequences between nt −676 and −598 of the MLC1 promoter may repress expression in the digastricus, a muscle of the jaw that is rostral in position and source of innervation to the infrahyoid complex (23). Together, these results suggest that distinct sites within the MLC1 promoter regulate transgene activity in distinct muscles.

Elements responsible for an axial gradient. The axial gradient of MCATE expression is most evident in the intercostals, a set of similar muscles in the thorax, each of which arises from a single somite and is innervated from a single spinal segment (discussed in reference 92). Activity of the MCAT transgene increases by approximately 2-fold per two segments or by about 15-fold from T2 to T10 (23). There are systematic differences in fiber type composition among intercostals, but these are insufficient in magnitude to account for the graded expression (17, 21). Moreover, we have shown directly that transgene activity varies with position among fibers of a single type (21).

What sequences account for this graded expression? Two lines of evidence implicate sequences in the MLC1 promoter rather than those in the MLC enhancer. First, no mutations or deletions of the MLC enhancer exerted marked effects on the gradient. Second, Kelly et al. (43) have generated and characterized transgenic mice in which the MLC enhancer is paired with the MLC3 promoter instead of the MLC1 promoter. They report equivalent levels of activity in all intercostal muscles of these mice. Consistent with their observations, we find that the thoracic gradient of activity is decreased from >10-fold to 2-fold by replacement of the 3′ portion of the MLC1 promoter with the heterologous hsp68 promoter. This result suggests that elements near the transcriptional start site of the MLC1

promoter are critical for graded expression of the transgene. Inconsistent with this conclusion, however, is the retention of some graded expression by hspCATE. This anomaly remains an obstacle to concluding that the MLC1 promoter is the source of elements that account for graded transgene expression.

Conclusions. Expression of the MCATE transgene is muscle specific, developmentally regulated, fiber type dependent, and axially graded. An understanding of how these modes of expression are mediated and integrated will provide insight into the processes that control the differentiation and diversification of muscle in general. As a first step, we have attempted to map and characterize functionally important sites within the transgene. Our main results are the following. (i) Maximal activity depends on the presence of an E box, a MEF2 site, and a hox binding site within a 173-bp core enhancer, presumably reflecting a requirement for simultaneous activation by transcriptional regulators of the myoD, MADS, and homeodomain families. (ii) Neither E boxes nor the MEF2 site is necessary for fiber-type-selective or position-dependent expression of the transgene, and patterns of expression are unaffected in mice that lack or overexpress myoD. Likewise, intermuscular differences in reporter activity persist in three distinct hox site mutants, even though several *hox* genes are expressed in position-dependent patterns in adult muscle. (iii) Sequences that flank the core enhancer are necessary for full activity of the transgene in vivo but not in transfected cells, suggesting that the enhancer shares properties with locus control or matrix attachment regions. (iv) Many mutations reduced activity in muscle, but none increased activity in nonmuscle cells. Thus, we obtained no evidence that muscle-specific expression depends on a silencing mechanism. (v) A sequence in the core enhancer that is distinct from previously identified motifs contributes to preferential expression of the transgene in fast-twitch muscle fibers. (vi) Axially graded expression of the transgene appears to result from multiple, independent sites in the MLC1 promoter, including one that selectively represses expression in neck muscles. Thus, the whole-body gradient of expression directed by the whole transgene may be controlled by the integrated effects of several discrete sites, each of which is maximally active in subsets of muscles. Such a system of combinational control might be similar to ones that regulate expression of genes such as *hairy* and *ftz* in *D. melanogaster*: there, too, patterns of expression that span the anterior-posterior axis appear to result from a multiplicity of enhancers, each of which activates transcription in only a few segments (71).

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