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E-Box Sites and a Proximal Regulatory Region of the Muscle Creatine Kinase Gene Differentially Regulate Expression in Diverse Skeletal Muscles and Cardiac Muscle of Transgenic Mice

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Previous analysis of the muscle creatine kinase (MCK) gene indicated that control elements required for transcription in adult mouse muscle differed from those required in cell culture, suggesting that distinct modes of muscle gene regulation occur in vivo. To examine this further, we measured the activity of MCK transgenes containing E-box and promoter deletions in a variety of striated muscles. Simultaneous mutation of three E boxes in the 1,256-bp MCK 5′ region, which abolished transcription in muscle cultures, had strikingly different effects in mice. The mutations abolished transgene expression in cardiac and tongue muscle and caused a reduction in expression in the soleus muscle (a muscle with many slow fibers) but did not affect expression in predominantly fast muscles: quadriceps, abdominals, and extensor digitorum longus. Other regulatory sequences with muscle-type-specific activities were found within the 358-bp 5′-flanking region. This proximal region conferred relatively strong expression in limb and abdominal skeletal muscles but was inactive in cardiac and tongue muscles. However, when the 206-bp 5′ enhancer was ligated to the 358-bp region, high levels of tissue-specific expression were restored in all muscle types. These results indicate that E boxes and a proximal regulatory region are differentially required for maximal MCK transgene expression in different striated muscles. The overall results also imply that within skeletal muscles, the steady-state expression of the MCK gene and possibly other muscle genes depends on transcriptional mechanisms that differ between fast and slow fibers as well as between the anatomical and physiological attributes of each specific muscle.

Creatine kinase catalyzes the regeneration of ATP from creatine phosphate and provides energy for contraction in all striated muscle types. The muscle creatine kinase (MCK) gene is transcriptionally activated during differentiation of myoblasts to myocytes (8, 31) and encodes the predominant creatine kinase isoform expressed in mammalian skeletal and cardiac muscle. While it seems likely that intrinsic differences in striated muscle anatomy and physiology would affect MCK gene expression, little is known concerning the steady-state mechanisms of MCK transcription in diverse muscle types. This transgenic study seeks to identify regulatory regions and elements within the MCK gene that are involved in controlling muscle-type-specific expression.

Several regions within the mouse MCK gene 5′-flanking sequence are required for muscle-specific expression in cultured myocytes and cardiomyocytes (1, 32, 64). Of particular interest is a 206-bp enhancer located approximately 1 kb upstream of the transcription start site. This region functions in an orientation-independent manner and drives the expression of heterologous promoters in skeletal myocytes (32). The 5′ MCK enhancer contains a number of sequence motifs—including the E box, CArG, and AT-rich sites—which are recognized by tissue-specific and ubiquitous transcription factors. Mutational analysis of these sites showed that several are critical for activity in cultured skeletal and cardiac muscle (1, 7, 21, 27); however, the mutations had different effects when tested in transgenic mice (16). Mutation of the Right E box, which is a target for basic-helix-loop-helix (bHLH) DNA-binding factors (17, 48, 69) such as the myogenic factors MyoD and myogenin and the ubiquitous bHLH factors E12/E47 and E2-2 (2, 40, 45), caused a nearly 100-fold reduction in expression in transfected skeletal muscle cells and a 6-fold decrease in expression in transiently transfected cardiomyocytes (1, 7). However, when the identical mutation was introduced into transgenic mice, it had no detectable effect on expression in adult mouse hind limb muscle or cardiac muscle (16). A nearby E-box site, called the Left E box, is less well understood and exhibits transcriptional activities and binding properties that are distinct from that of the Right E box (1, 2, 40). Interestingly, concomitant mutation of both E boxes in the 206-bp enhancer and a conserved E box at position −249 also had no effect on transgene expression in hind limb muscle (16).

The relative importance of other control elements within the 206-bp MCK enhancer has also been examined by using cultured muscle cells and transgenic mice. Distinct differences were again observed between the two experimental systems. The CArG site mutation resulted in a nearly 50-fold loss of activity in cultured cardiomyocytes and a severalfold loss of activity in cultured skeletal myocytes (1). However, the same mutation produced no significant effect on transgene expression in either adult cardiac or hind limb muscle of transgenic mice (16). In contrast, mutation of the MCK enhancer AT-rich element, which reduced activity about 15-fold in cultured skeletal and cardiac myocytes (1), eliminated nearly all transgene

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expression in the hind limb muscles of transgenic mice but had no effect on expression in cardiac muscle (16).

The 1-kb region (bp −1020 to +1) immediately 3′ of the 206-bp enhancer also exhibits differential activity when tested in cell culture and in transgenic mice. Transgenic analysis suggested that this region plays a more important role in the expression of the MCK gene than expected from analysis in cultured skeletal and cardiac muscle cells, in which it exhibited little activity (1, 16, 33). The 1-kb region is transcriptionally active in transgenic hind limb muscle but not in transgenic cardiac muscle (16, 33). Also in contrast to results obtained with cultured cells, combination of the 1-kb region with the 5′ enhancer resulted in greater activity than that of either region alone in both skeletal and cardiac muscle (16). Sequences in the 1-kb region contain a number of potential control elements, but those that are important for muscle-specific expression had not been delineated in the earlier transgenic studies (16, 33).

The studies described above suggest that transcriptional control of the MCK gene is accomplished by distinct mechanisms under different physiological conditions. To examine this hypothesis in greater detail, we assessed the activity of transgenes containing E-box mutations and promoter deletions in a variety of striated muscles: quadriceps, abdominal, extensor digitorum longus (EDL), soleus, and tongue skeletal muscles, as well as cardiac muscle. Our results indicate that MCK E-box elements and a 358-bp proximal regulatory region play different roles in cardiac and skeletal muscle. Furthermore, MCK regulatory elements exhibit distinct properties in anatomically different skeletal muscles and may play a role in determining the relative level of expression of the MCK gene in fast and slow muscles.

MATERIALS AND METHODS

MCK-CAT constructs. All MCK-CAT constructs (Fig. 1A) consist of mouse MCK 5′ sequences to bp +7 (GenBank accession number M21390) fused to the 1.6-kb chloramphenicol acetyltransferase (CAT) structural gene-simian virus 40 small-t intron–poly(A) cassette (20). 1256MCK-CAT and 1256[3E mut]MCK-CAT were constructed as previously described (16, 32). A construct containing the 358-bp proximal regulatory region (bp −358 to +7) of MCK fused to CAT was made by exonuclease III digestion of a 776MCK-CAT construct (32) after digestion at the *Pst*I and *Acc*I sites in the pUC polylinker. A *Hin*dIII-*Bam*HI fragment containing the 206-bp enhancer (bp −1256 to −1050) was blunt-end ligated 5′ of MCK sequences in the 358MCK-CAT construct to create enh358MCK-CAT, which includes 12 bp of polylinker DNA (CCCTGCATGC CC) between MCK nucleotides (nt) −1050 and −358. The enh358(Δ1)MCK-CAT construct was generated by a sticky/blunt ligation of a *Hin*dIII-*Bgl*I fragment of enh358MCK-CAT, containing the MCK enhancer and sequences from bp −358 to −189, to a *Hin*dIII-*Apa*I-cut 177MCK-CAT construct, resulting in juxtaposition of bp −189 and −80 of the MCK proximal regulatory region. enh358(Δ2)MCK-CAT was generated by blunt-end ligation of a *Hin*dIII-*Bsa*AI fragment of enh358MCK-CAT containing the MCK enhancer and sequences from bp −358 to −299 to a *Hin*dIII-cut 177MCK-CAT construct, creating a junction equivalent to the juxtaposition of bp −299 and −180 of the MCK proximal regulatory region but separated by an additional T in the coding strand resulting from polylinker DNA. Sequences were confirmed by dideoxynucleotide sequencing. Correct initiation of transcription was previously confirmed for the 1256MCK-CAT construct and a construct in which the 206-bp enhancer was linked to the MCK basal promoter (bp −80 to +7) (34).

Production of transgenic mice. MCK-CAT transgenes were prepared such that only minimal polylinker sequences remained. Transgenes were then separated from vector sequences by agarose gel electrophoresis, isolated on NA45 (Schleicher & Schuell) paper, and purified on an Elutip-D column (Schleicher & Schuell) prior to microinjection. Transgenic mice were derived from the eggs of $(C57BL6 \times C3H)F_1$ matings by standard techniques (25). Founder animals were identified by hybridization of tail DNA to an *Eco*RI fragment within the CAT gene. Transgenic animals were analyzed between 6 weeks and 6 months of age, and no age-related differences in transgene expression were detected.

Gene copy numbers were estimated by comparison of the relative intensity of transgene and endogenous MCK gene bands on Southern blots or by comparison of duplicate DNA dots probed with an *Eco*RI fragment within the CAT gene and an endogenous MCK cDNA fragment. Autoradiographic analysis was carried out by the PhosphorImager Facility of the Markey Molecular Medicine Center (PhosphorImager model 400S; Molecular Dynamics). Most copy numbers were determined from spleen DNA, and a few were determined from abdominal muscle DNA. Transgenic animals exhibiting extremely low levels of CAT activity in all tissues were shown to contain an intact transgene by Southern analysis.

Mouse dissection. Mice were sacrificed by cervical dislocation. Atria were removed from cardiac tissue to reduce contamination by associated fibroblasts. Quadriceps are the anterior thigh muscles including the rectus femoris, vastus lateralis, vastus intermedius, and vastus medialis. Abdominals are the muscles taken from the distal aspect of the rib cage to the pelvic region, which include the internal and external obliques, transversus, and rectus abdominis. EDL and soleus are the anterior and posterior lower hind limb muscles respectively. Tongue muscle was obtained from the distal half of the tongue. Tissues were immediately frozen in liquid nitrogen and stored at −70°C.

Protein extraction and CAT assays. Tissue samples were homogenized with a Kontes motorized pestle in 250 mM Tris (pH 7.8)–5 mM EDTA, heated at 65°C for 10 min, and centrifuged, and the supernatants were stored at −20°C. Protein concentrations were determined by the method of Bradford (5). CAT assays were performed on each extract within 2 weeks as previously described (7), except that the incubation times were typically 60 to 100 min. CAT enzyme standards were analyzed in parallel to ensure that activities of diluted extracts were within the linear range of the assay. Counts per minute (cpm) from [*acetyl*-^{14}C]chloramphenicol were converted to microunits of CAT activity, where 1 U of CAT activity is defined as the amount sufficient to acetylate 1 μmol of chloramphenicol per min at 37°C (61). Samples which exhibited less than twice the CAT assay background counts per minute (40 to 60 cpm) are indicated as 0 μU of CAT per mg of protein.

Statistical analysis. Statistical comparisons were carried out with the Wilcoxon rank sum test, a nonparametric analog of the *t* test for two independent samples (56), which is appropriate for analysis of data sets in which large variances prohibit a useful comparison of mean values. To carry out this analysis, data sets from two transgenes whose expression is being compared are intermingled to form a single rank-ordered data set from highest to lowest activity. The sum of ranks for one of the data sets is compared with a table of critical values (56) that is based on the number of observations in each data set. This table specifies the *P* value for the null hypothesis. A *P* value less than 0.05 was used as a criterion to reject the null hypothesis and to indicate, for example, that the mutated transgene data represent a statistically different distribution from that of the wild-type transgene. In some instances, the ranked data were displayed in bar graphs to illustrate relative activities of the two transgenes (see Fig. 3 and 5). When comparisons were made among many different transgenes, the data sets were analyzed by the Wilcoxon rank sum procedure and then displayed as individual points on a log-scale graph (see Fig. 4). An unpaired *t* test was used to determine whether ratios of CAT expression between different muscles within individual founder mice were statistically different.

RESULTS

In previous studies, we observed a broad range of MCK transgene activity (>100-fold) among founder mice and mouse lines carrying the same transgene (16, 33). This phenomenon has been observed for many mammalian transgenes and is attributed to genomic effects at different transgene integration sites (30, 50). To circumvent problems of data variability caused by this phenomenon, we examined at least 12 independently generated founder animals carrying each transgene and evaluated the data by a statistical test (Wilcoxon rank sum) which is appropriate for assessing significant differences between data sets containing large internal variations (56). Transgene copy numbers were determined for all mice examined; no correlation between copy number and CAT activity level was evident for any construct (Tables 1 and 2).

The skeletal muscles chosen for this analysis represent different anatomical locations and different compositions of muscle fiber types (24, 53, 60). Comparison of transgene activities in the EDL and the soleus is of interest because both are lower hind limb muscles that are similar in innervation, size, and function but have dramatically different fiber type compositions. The EDL is composed of only fast myosin heavy chains (MyHC) (72% MyHC IIb, 25% IIx, and 3% IIa) whereas the soleus is 42% β/slow MyHC (type I), 45% fast IIa, and 13% fast IIx (38, 71). Abdominal and quadriceps muscles are composed of groups of fast fibers, expressing predominantly MyHC IIb but also all other isoforms to a lesser extent (24, 39, 53). Tongue muscle was analyzed because it is a primarily fast muscle type (75% MyHC IIb and 25% IIx [13]) with highly

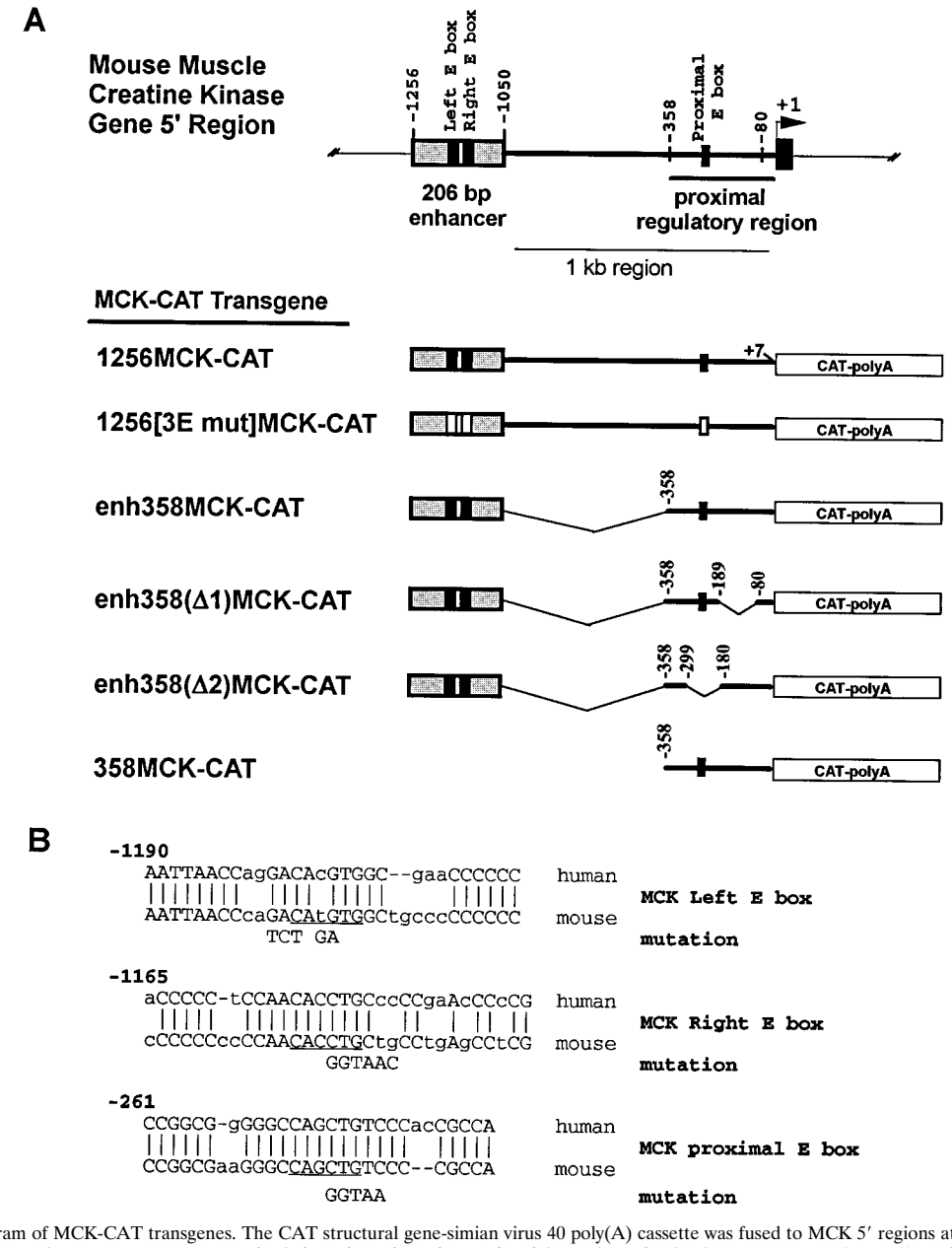

FIG. 1. (A) Diagram of MCK-CAT transgenes. The CAT structural gene-simian virus 40 poly(A) cassette was fused to MCK 5′ regions at nt +7 in all constructs. Mouse MCK enhancer and promoter sequences are in their native orientation. Left, Right, and proximal E boxes at nt −1178, −1153, and −249, respectively, are indicated by solid boxes. 1256MCK-CAT and 1256[3E mut]MCK-CAT contain 1,256 bp of MCK upstream sequences, with the latter construct harboring mutations in the Left, Right, and proximal E boxes (open boxes). Three other transgenes contain the MCK 206-bp enhancer (bp −1256 to −1050) fused to MCK sequences which consist of 358 bp of upstream sequences (enh358MCK-CAT) or 358 bp of upstream sequences containing internal deletions of the proximal regulatory region [enh358(Δ1)MCK-CAT and enh358(Δ2)MCK-CAT]. The Δ1 deletion removes sequences between bp −188 and −81, and the Δ2 deletion removes sequences between bp −298 and −181. 358MCK-CAT contains 358 bp of upstream sequences. (B) Sequences of mutated E box sites in the 1256[3E mut]MCK-CAT transgene. The human MCK sequence is shown above to demonstrate conservation of E-box sequences. These sequences are also conserved in the rabbit (73) and rat (27) MCK genes. Core CANNTG sequences are underlined, and mutated nucleotides are indicated below. The 5′ nucleotide positions of the mouse sequences are indicated. Left and Right E-box mutations are the same as Left mut1 and MEF1 mut1 described in reference 1.

different functional demands from those of the fast leg muscles.

Muscle-specific expression of the wild-type MCK 1,256-bp 5′-flanking region. Previous transgene studies had shown that the 1256MCK-CAT gene (Fig. 1A) was expressed at high levels in hind limb (primarily quadriceps) and cardiac muscle but was inactive in nonmuscle tissues (16, 33). To determine whether 1256MCK-CAT transgene activity in quadriceps muscle reflects its behavior in other skeletal muscles, we analyzed expression in abdominal, EDL, soleus, and tongue muscles (Table 1). The range and median CAT activity normalized to protein concentration were similar in quadriceps, abdominals, and EDL (Fig. 2). The activity in these skeletal muscles was 4 to 5 orders of magnitude greater than that in any nonmuscle tissues assayed (liver and kidney [Table 1]). Interestingly, the activity of the 1256MCK-CAT transgene in tongue muscle was 2 to 3 orders of magnitude below that of all other skeletal muscles examined (Fig. 2). As in previous transgene studies

TABLE 1. CAT reporter activity in skeletal muscles and nonmuscle tissues for MCK-CAT transgenic mice

Transgene and mouse no.	Copy no.	CAT activity (μU of CAT/mg of protein) in[a]:							
		Quads	Abs	EDL	Soleus	Tongue	Cardiac	Liver	Kidney
1256MCK-CAT[b]									
14247	33	7.6×10^6	1.1×10^7	4.8×10^8	3.2×10^6	1.2×10^5	6.5×10^4	9.5×10^2	1.7×10^3
5	3	6.3×10^6					7.6×10^3	19	
14266	3	1.6×10^6	9.0×10^6	1.1×10^6	3.3×10^5	3.3×10^3	8.0×10^2	27	70
14193	3	1.2×10^6	2.7×10^6	1.4×10^6	2.5×10^5	2.5×10^4	9.5×10^3	32	15
2	5	1.2×10^6					1.5×10^3	78	
14333	23	8.1×10^5	4.0×10^5	1.5×10^6	9.6×10^5	7.2×10^2	1.7×10^3	1	2
14233	7	4.8×10^6	4.6×10^5	6.1×10^5	1.7×10^5	4.9×10^2	1.3×10^4	1	3
1	12	1.7×10^5					20	9	
14331	2	1.5×10^5	2.7×10^5	1.2×10^5	1.6×10^4	31	15	<1	<1
14227	0.2	1.3×10^5	5.5×10^4	1.6×10^5	2.4×10^4	7.5×10^2	9.6×10^2	<1	2
14349	12	1.2×10^5	2.9×10^5	8.5×10^4	3.8×10^4	3.8×10^2	1.4×10^4	1	3
14338	1	1.1×10^5	8.2×10^4	3.5×10^4	1.5×10^4	90	45	<1	1
3	1	7.0×10^4					5	0[c]	
14258	21	6.6×10^4	1.2×10^5	1.7×10^5	7.5×10^4	3.9×10^2	1.1×10^4	<1	1
4	1	6.0×10^4					8	0	
14324	1	5.9×10^4	1.0×10^5	5.1×10^4	1.2×10^4	2	10	<1	<1
14281	0.3	2.8×10^4	4.7×10^3	12	89	0	<1	<1	0
1256[3E mut]MCK-CAT									
12063	6	8.9×10^6	5.4×10^6	2.5×10^6	1.2×10^5	60	1.3×10^2	10	54
12055	4	4.2×10^6	3.6×10^6	1.3×10^6	1.4×10^4	3	27	25	3
12024	0.5	2.8×10^6	1.0×10^6	6.8×10^5	8.7×10^3	0	17	8	3
11995	3	1.6×10^6	2.4×10^6	1.6×10^6	1.5×10^5	5	8	10	6
12073	3	1.3×10^6	1.8×10^6	1.4×10^6	1.4×10^5	7.9×10^2	38	24	11
12075	1	6.3×10^5	4.2×10^5	1.9×10^5	9.4×10^4	9	62	5	4
12070	1	4.7×10^5	2.6×10^5	5.9×10^5	1.9×10^4	0	4	1	1
12010	2	1.7×10^5	4.7×10^4	2.3×10^3	3.8×10^2	0	3	<1	<1
30073	1	1.4×10^5	2.9×10^5	9.1×10^4	1.9×10^4	0	4	<1	1
12083	1	1.1×10^5	4.6×10^4	2.9×10^4	1.6×10^3	2	6	<1	1
12023	2	6.8×10^4	5.7×10^4	1.5×10^4	1.5×10^3	0	1	<1	<1
12005	3	3.3×10^3	1.1×10^3	2.0×10^2	4	0	8	<1	0
12090	0.5	1.2×10^3	2.0×10^2	0	7	0	0	<1	<1
12056	5	3.1×10^2	2.7×10^2	89	8	0	2	0	0
enh358MCK-CAT									
10235	11	5.3×10^6	2.6×10^6	4.4×10^6	5.4×10^5	6.5×10^4	1.2×10^5	14	17
10177	12	4.3×10^6		3.2×10^6	2.8×10^5		2.5×10^3	23	14
10130	5	2.6×10^6	1.9×10^6			2.8×10^4	3.7×10^2	7	7
10227	10	2.5×10^6		2.5×10^6	1.6×10^5	8.8×10^3	5.8×10^3	4	6
10195	3	2.3×10^6		1.7×10^6	7.1×10^4		1.4×10^3	30	8
10214	49	2.3×10^6		2.2×10^6	1.6×10^5		2.9×10^3	3	6
10251	36	1.8×10^6	4.2×10^6			1.6×10^4	4.6×10^3	51	69
10158	5	8.5×10^5	2.6×10^6			2.4×10^3	1.2×10^3	17	11
10141	3	6.8×10^5	6.6×10^5			2.9×10^3	65	1	4
10172	1	6.0×10^5	9.4×10^5			6.0×10^2	21	13	14
10213	3	5.2×10^5	2.5×10^5			3.4×10^2	8	2	2
10144	5	5.2×10^5	8.0×10^5			80	80	9	<1
10217	3	4.8×10^5		4.9×10^5	3.4×10^4	74	13	<1	3
10209	8	4.2×10^5		1.5×10^5	8.4×10^3		11	2	4
10232	2	2.5×10^5	1.4×10^5			78	10	<1	2
10132	1	2.0×10^5	2.1×10^5			1.1×10^3	7.6×10^2	<1	<1
10237	4	1.8×10^5	2.4×10^5	1.6×10^5	6.6×10^3	28	92	<1	<1
10201	1	1.7×10^5	3.2×10^5			21	33	<1	<1
10208	2	1.1×10^5	2.0×10^4			0	<1	<1	2
10188	0.5	1.0×10^5		4.0×10^4	2.2×10^4	3.4×10^3	1.1×10^2	<1	1
10176	2	9.2×10^4		5.0×10^4	1.3×10^4		3	<1	<1
10233	1	5.2×10^4	8.8×10^3	3.9×10^3	1.3×10^2	0	<1	<1	0
10220	1	3.8×10^3		21	8	0	<1	<1	<1
10245	2	1.5×10^2		75	2		1	0	0
10173	1	51	94			2	0	0	0

[a] Quads and Abs signify quadriceps and abdominal skeletal muscles, respectively. A blank space indicates that a sample was not obtained or measured.

[b] CAT activities from nine transgenic animals described in an earlier study (16) were included for the 1256MCK-CAT transgene, including five animals for which only quadriceps and cardiac muscle was analyzed (founders 1 to 5). Addition of these data gives a more complete indication of the range of 1256MCK-CAT activities encountered but does not affect any of the statistical comparisons described.

[c] A value of 0 indicates that CAT activity was less than twice background.

TABLE 2. CAT reporter activity in skeletal muscles and nonmuscle tissues for MCK-CAT transgenic mice

Transgene and mouse no.	Copy no.	CAT activity (μU of CAT/mg of protein) in[a]:							
		Quads	Abs	EDL	Soleus	Tongue	Cardiac	Liver	Kidney
enh358(Δ1)MCK-CAT									
13209	2	7.3×10^5				5.4×10^3	71	1	
13328	4	4.7×10^5				38	23	3.9×10^2	
13306	2	3.5×10^5	3.5×10^5	3.1×10^5	2.1×10^4	1.8×10^2	1.3×10^2	30	22
13207	2	3.2×10^5		3.8×10^5	1.8×10^4	15	4	2	5
13289	0.5	2.4×10^5		5.8×10^4	8.4×10^3	2.6×10^3	15	2	
13213	1	1.5×10^5		4.0×10^4	6.0×10^2	10	<1	<1	
13299	1	4.8×10^4	5.1×10^4	1.8×10^4	6.8×10^2	0[b]	<1	<1	<1
13171	4	2.5×10^4	3.6×10^3	7.0×10^2	1.1×10^3	0	<1	<1	0
13188	1	1.4×10^4	1.8×10^4	5.2×10^3	2.2×10^3	0	<1	<1	<1
13265	1	2.0×10^3	1.6×10^3	1.4×10^4	1.8×10^2	0	0	0	0
13301	4	1.4×10^3		2.5×10^3	83	3	1	<1	
13250	1	1.0×10^2	2			0	0	0	0
enh358(Δ2)MCK-CAT									
13667	4	1.4×10^6				4.5×10^3	4.2×10^3	5	
13493	2	5.6×10^5	8.4×10^5	2.5×10^5	3.9×10^4	52	1.2×10^3	3	4
13646	6	4.1×10^5	8.6×10^5	2.4×10^5	1.7×10^5	82	3	5	8
13628	1	2.8×10^5		1.1×10^5	8.6×10^3	8.1×10^2	2.1×10^2	2	5
13684	1	1.8×10^5				13	2.7×10^2	<1	
13615	15	9.4×10^4	4.8×10^5	1.9×10^5	9.8×10^4	5	17	1	7
31984	2	8.3×10^4				6	48	<1	
13614	1	6.7×10^4	1.1×10^5	1.9×10^4	4.9×10^3	<1	36	<1	
13620	1	4.9×10^3	2.4×10^3	6.3×10^2	67	0	0	0	0
13609	14	1.4×10^3	2.2×10^2	7	2	2	6	0	0
13521	1	2.5×10^2		31	3	0	1	0	
13636	3	63		21	0	0	<1	0	
13603	1	10	2.7×10^2			0	<1	0	0
13671	2	0				0	<1	0	
358MCK-CAT									
10262	8	1.9×10^6	9.3×10^5			<1	19	5	4
10272	3	4.4×10^5	1.5×10^5			0	0	<1	1
10389	5	3.8×10^5	2.2×10^5			0	1	2	4
10376	9	2.3×10^5		5.9×10^4	3.3×10^3	20	7	3	2
10264	2	3.6×10^4	3.9×10^3			0	<1	1.1×10^2	0
10345	2	3.6×10^3	1.3×10^4			0	0	0	0
10395	2	1.7×10^3		76	7.5×10^2	0	<1	<1	<1
10363	1	1.4×10^3		9.8×10^2	6.7×10^2	0	0	<1	0
10357	23	1.1×10^3	2.7×10^2			0	0	0	0
10391	32	1.1×10^3		7.1×10^2	15	0	1	<1	<1
10409	2	1.6×10^2		2	0	0	<1	<1	<1
10388	3	68	79			0	<1	0	0
10353	10	20	44			0	0	0	0
10416	0.5	1		0	0		1	0	<1
10277	2	1	0			0	0	0	0
10455	1	<1		0	0		0	0	2
10370	3	0		0	0		0	<1	0

[a] Quads and Abs signify quadriceps and abdominal skeletal muscles, respectively. A blank space indicates that a sample was not obtained or measured.
[b] A value of 0 indicates that CAT activity was less than twice background.

(16, 33), cardiac muscle expression was also significantly lower than quadriceps expression and was roughly equivalent to that in tongue muscle (see below).

In 11 of 12 founder mice examined, the activity of the 1256MCK-CAT transgene was lower in the soleus than in the quadriceps, abdominals, or EDL (Table 1). This difference is more apparent when the comparisons of muscle activity are based on the ratios of CAT activity between different muscles within each founder mouse. This comparison allows examination of transgene activity without interference from genomic effects as a result of different integration sites. When this was calculated, activity ratios between the fast EDL and the largely slow soleus muscle were significantly higher than the activity ratios between EDL and quadriceps or between abdominals and quadriceps ($P < 0.001$) (Table 3; compare across the first row for the 1256MCK-CAT transgene). This was due to significantly lower relative expression levels in the soleus. In contrast, CAT activity ratios in the fast muscles—EDL/quadriceps and abdominals/quadriceps—were each close to unity for the 1256MCK-CAT transgene (Table 3), suggesting that expression levels in these primarily fast muscle types are very similar.

Muscle-specific differences in expression of an MCK transgene containing multiple E-box mutations. E-box sites in the MCK enhancer are known to be important for transcriptional activity in cultured myocytes and cardiomyocytes (1, 2, 7). However, previous transgene studies indicated that mutation

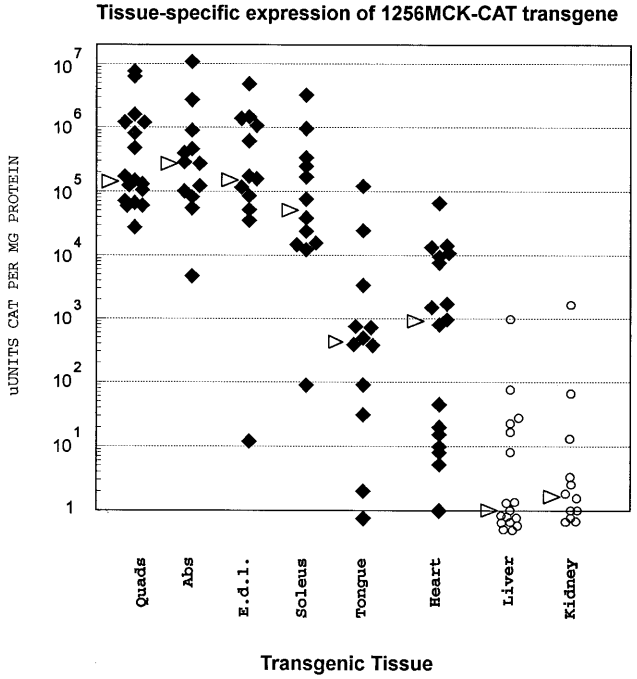

FIG. 2. Expression of 1256MCK-CAT transgene in skeletal muscle, cardiac muscle, and nonmuscle tissues. Each diamond or circle represents the CAT activity in microunits per milligram of protein from each founder mouse for the indicated tissue. Quads and Abs signify quadriceps and abdominal muscle, respectively. Twelve animals were tested for all tissues, except for quadriceps, cardiac and liver muscle, for which data from 17 animals are reported (including 9 animals described in an earlier study [16]). Open triangles indicate the median value of the data for each tissue. Note that the ordinate of the graph is in logarithmic scale.

of three conserved E boxes within the MCK enhancer and the proximal regulatory region [1256(3E mut)MCK-CAT (Fig. 1)] caused no transcriptional decrease in adult quadriceps muscle (16). We therefore examined whether the triple E-box mutation would affect the activity of the transgene in other adult muscles. Statistical analysis confirmed that there was no significant difference between the activities of the wild-type and the triple-E-box-mutated transgenes in abdominal muscle (Fig. 3A) ($P = 0.68$) or in EDL muscle (Table 1) ($P = 0.55$). The activity of the triple-E-box-mutated transgene also appeared similar to that of the wild-type transgene in soleus muscle (Table 1). However, when the ratios of activities between different skeletal muscles were calculated for each mouse, a more subtle effect of the E-box mutations was revealed. CAT activity ratios in the fast muscles—EDL/quadriceps and abdominals/quadriceps—were close to unity for the mutated transgene, as they were for the wild-type 1256MCK-CAT transgene (Table 3; compare down the second and third data columns). However, the activity ratio between the EDL and the soleus was much larger than the ratios between the EDL and other fast muscles (Table 3; compare across the second row). Additionally, the EDL/soleus activity ratio for the triple-E-box-mutated transgene (mean, 27.8) was significantly larger than that for the wild-type 1256MCK-CAT transgene (mean, 3.7) ($P < 0.05$) (Table 3; compare down first data column). This suggests that the triple E-box mutation decreases the expression in the soleus by about sevenfold. An even more dramatic effect of the triple E-box mutation was observed in tongue skeletal muscle, in which the mutations virtually abolished the expression of the transgene ($P = 0.001$) (Fig. 3B).

Effect of the triple E-box mutation on cardiac muscle expression. Mutation of the three conserved E boxes also caused a dramatic loss of CAT activity in cardiac muscle ($P = 0.002$) (Fig. 4). Only five of the mice carrying the mutated transgene had more than 10 μU of CAT per mg of protein in cardiac muscle, whereas the majority of mice carrying the wild-type transgene exhibited at least 100-fold-higher activities (Table 1). Thus, the three conserved MCK E-box sequences play a critical regulatory role in expression of the MCK transgenes in cardiac muscle.

Delineation of MCK gene-proximal regulatory regions which exhibit muscle-type-specific differences. Prior transgenic analysis of MCK gene regulation indicated that the 1-kb region immediately 5′ of the transcription start site directs tissue-specific expression in mouse skeletal muscle (16). In addition, the combination of the MCK enhancer and the 1-kb region produces greater activity in both thigh and cardiac muscle than does either region alone (16). To further delineate the active portions of the 1-kb region, we compared mouse, rat, rabbit, and human MCK sequences (27, 31, 65, 73) to determine which portions were most highly conserved. The first 358 bp of the 5′ region exhibited more than 50% sequence similarity, whereas there was little similarity between bp −1049 and −359. We therefore tested a transgene in which the 206-bp MCK enhancer was linked to the highly conserved 358-bp proximal region (enh358MCK-CAT [Fig. 1A]). The activity of this construct was indistinguishable from that of the 1256 MCK-CAT transgene in quadriceps (Fig. 5A), abdominal, EDL, and tongue muscles (Table 1). In cardiac muscle, the enhancer linked to the 358-bp proximal region also had statistically identical activity to that of 1256MCK-CAT ($P = 0.11$) (Fig. 4).

Removal of the region between −1049 and −359 produced a more subtle effect on transcriptional activity in the soleus muscle than on activities in the fast muscles. CAT activity ratios in the fast muscles—EDL/quadriceps and abdominals/quadriceps—were again close to unity for the enh358MCK-CAT transgene (Table 3; compare down the second and third data columns). This transgene exhibited a mean ratio of CAT activity in the fast EDL to the largely slow soleus muscle which was significantly higher than the EDL/quadriceps and abdom-

TABLE 3. Mean ratios of skeletal muscle CAT activities[a]

Transgene	Ratio (no. of mice)[b]		
	EDL/soleus	EDL/quadriceps	Abdominals/quadriceps
1256MCK-CAT	3.7 ± 0.6 ($n = 11$)	1.1 ± 0.2 ($n = 11$)	1.3 ± 0.2 ($n = 11$)
1256[3E mut]MCK-CAT	27.8 ± 9.9 ($n = 10$)	0.6 ± 0.1 ($n = 10$)	0.9 ± 0.2 ($n = 10$)
enh358MCK-CAT	13.4 ± 2.3 ($n = 10$)	0.7 ± 0.1 ($n = 10$)	1.2 ± 0.2 ($n = 13$)

[a] Mice expressing very low levels of CAT activity (<500 μU/mg of protein) in any of these muscles were not included in this activity ratio comparison; mice 14281, 12010, 12005, 12090, 12056, 10233, 10220, 10245, and 10233 (Table 1) were excluded.

[b] Values shown are mean ± standard error of the mean.

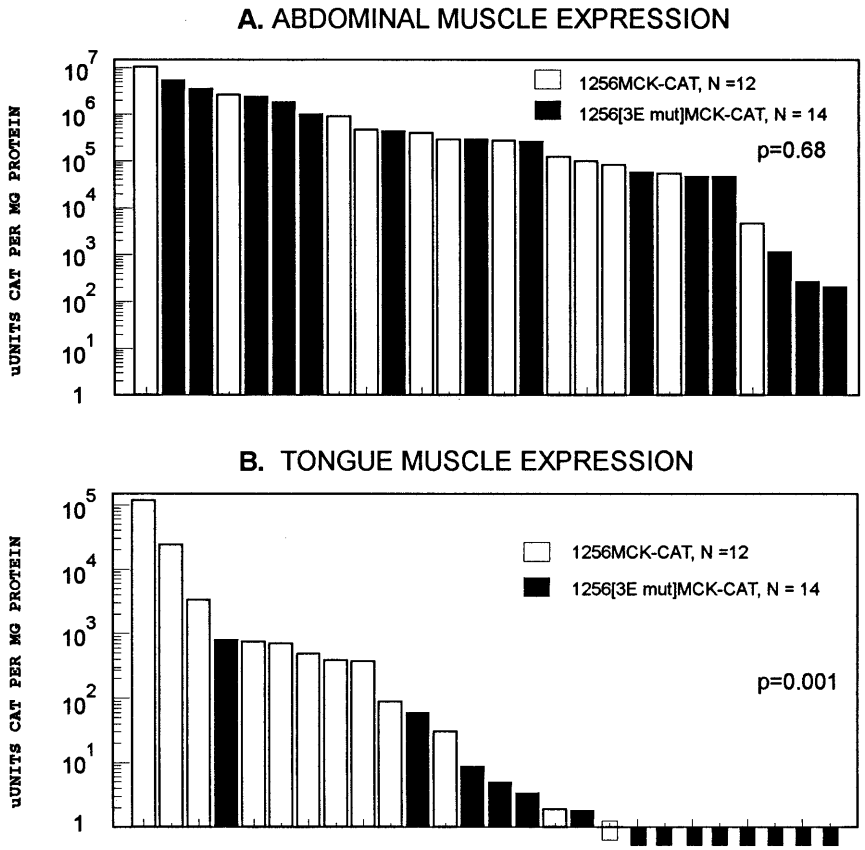

FIG. 3. Comparison of the activity of 1256MCK-CAT and 1256[3E mut]MCK-CAT in abdominal (A) and tongue (B) skeletal muscle. Each bar represents μUnits CAT per mg protein measured for each founder mouse in the tissue indicated. The abdominal muscle comparison is similar to results of a comparison of the expression of these two transgenes in EDL and soleus. In this type of graphical representation, alterations of MCK sequences which significantly reduced transcriptional activity result in data sets that are clustered toward the right-hand side of the graphs.

inal/quadriceps ratios (Table 3; compare across each row). Furthermore, the EDL/soleus CAT activity ratio for the enh358MCK-CAT transgene (mean, 13.4) was significantly higher than that for the wild-type 1256MCK-CAT transgene (mean, 3.7) ($P < 0.001$) (Table 3; compare down the first data column). The overall muscle-specific expression conferred by the enhancer linked to the 358-bp proximal region is very similar to that of the larger, 1,256-bp region. This suggests that the sequences between bp −1049 and −359 are not critical for transcriptional activity in fast skeletal muscles and in cardiac muscle. However, the bp −1049 to −359 region does contribute to MCK gene expression in the soleus muscle, possibly because of its higher proportion of slow fibers.

The 358-bp proximal regulatory region is sufficient for tissue-specific expression in limb and abdominal muscles but not in cardiac and tongue muscles. To determine whether the 358-bp proximal regulatory region was sufficient for controlling tissue-specific expression, we examined the activity of a transgene containing the proximal regulatory region alone (358MCK-CAT [Fig. 1A]). This transgene exhibited skeletal muscle-specific expression in quadriceps, abdominal, EDL, and soleus muscle, whereas no expression was observed in nonmuscle tissues (Table 2). The activity of the 358-bp proximal regulatory region, however, was roughly 2 to 3 orders of magnitude lower than that of the enh358MCK-CAT transgene in quadriceps ($P < 0.001$) and abdominal ($P = 0.01$) muscles. Interestingly, the 358MCK-CAT transgene exhibited essentially no CAT activity in cardiac muscle (Fig. 4) or in tongue muscle (Table 2). Taken together, these results demonstrate that the 358-bp proximal regulatory region functions in a weak but tissue-specific manner in quadriceps, abdominal, EDL and soleus muscles but that the MCK enhancer is required for transgene expression in cardiac and tongue muscle.

Further delineation of muscle specificity within the 358-bp proximal regulatory region. To identify sequences in the proximal regulatory region that produce tissue-specific expression in cooperation with the enhancer, we created two internal deletions in the enh358MCK-CAT transgene (Fig. 1A). The Δ1 deletion removed MCK sequences between bp −188 and −81 (31), including a highly conserved 26-bp region at nt −180 containing a CArG element (CCATACAAGG). The Δ2 deletion removed MCK sequences between bp −298 and −181, including a highly conserved 15-bp region at nt −253 which contains the conserved proximal E box (GGGCCAGCTGTC CCC). Transgenes containing either deletion retained significant expression in all skeletal muscles examined (Table 2). However, the activities of the Δ1 and Δ2 transgenes were both significantly lower than that of enh358MCK-CAT in quadriceps ($P = 0.03$ and $P = 0.009$) (Fig. 5B and C) and in tongue muscle ($P = 0.037$ and $P = 0.016$) (Table 2). The activities of the Δ1 and Δ2 transgenes were also slightly reduced in abdominal, EDL, and soleus muscles (Tables 1 and 2). Furthermore, as was observed with the parental enh358MCK-CAT construct, the activity of the Δ1 or Δ2 transgenes was generally greater in the quadriceps, abdominal, and EDL muscles than in the largely slow soleus muscle.

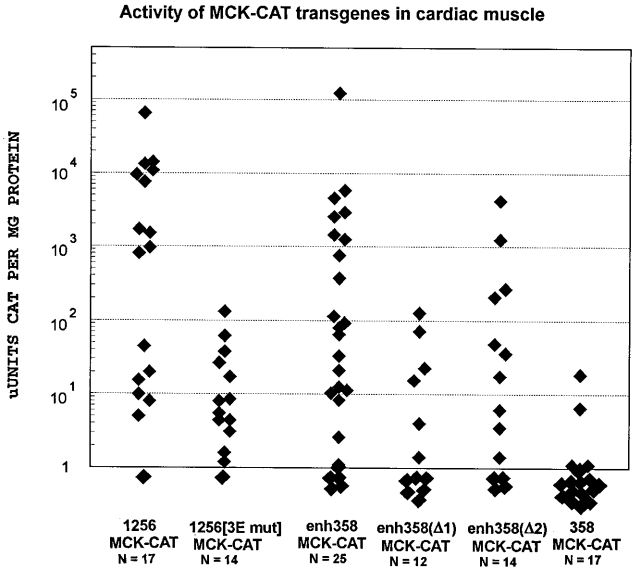

FIG. 4. Activities of MCK-CAT transgenes in cardiac muscle. Each diamond represents CAT activity in microunits per milligram of protein for each founder animal.

The Δ1 and Δ2 internal-deletion transgenes exhibited differential expression in cardiac muscle. The activity of enh358(Δ1) MCK-CAT was significantly lower than that of enh358MCK-CAT in cardiac muscle ($P = 0.011$) (Fig. 4), and half of the mice carrying the Δ1 transgene had no measurable CAT activity in cardiac muscle. In contrast, the Δ2 deletion had no significant effect ($P = 0.20$) (Fig. 4). The differential effect of these deletions suggests that MCK sequences between bp −188 and −81, either by themselves or in combination with enhancer elements, play a more important role in cardiac transcription than do sequences between bp −298 and −181.

DISCUSSION

The transcriptional regulation of muscle-specific genes has been extensively examined by using cultured cells, and numerous *cis*-regulatory elements have been identified. Despite this level of understanding, the in vivo mechanisms of differential gene expression in skeletal and cardiac muscle and in different types of skeletal muscle are only now being delineated. Results from this study show that the enhancer, the conserved E-box sites within the enhancer and proximal region, and the 358-bp proximal region of the MCK gene are required for transgene expression in subsets of different adult muscle types but not in others.

Simultaneous mutation of the Left, Right, and proximal E boxes in the 1,256-bp 5′ region of the MCK gene resulted in a substantial loss of activity in cardiac and tongue muscles (Fig. 3B and 4). However, transgene expression was not dramatically affected in quadriceps, abdominal, or EDL skeletal muscles (Table 1). These potential binding sites for the myogenic bHLH factors (MyoD, myogenin, myf-5, and MRF-4) thus do not appear critical for steady-state transcriptional control of the MCK gene in a variety of adult limb skeletal muscles, but they are critical in cardiac and tongue muscles. A roughly sevenfold relative decrease in expression of the triple-E-box-mutated transgene was also observed in the soleus muscle when ratios of EDL to soleus activity were calculated for individual mice (Table 3) (see below).

On the basis of the present and previous transgenic studies of the MCK gene (16), the MCK enhancer Left E box may be the more critical E box for cardiac muscle expression. This hypothesis is supported by the observation that mutation of the Right E box alone in the context of the 1256MCK-CAT transgene (16) or deletion of the region containing the proximal E box in the enh358(Δ2)MCK-CAT transgene did not decrease cardiac muscle expression levels. Since the triple E-box mutation decreased cardiac muscle expression by at least 100-fold (Fig. 4), the Left E box alone or the combination of the Left E box with either the Right or the conserved proximal E box must be critical. Other studies have indicated that the MCK Left and Right E boxes form distinct complexes with cardiac muscle nuclear extracts (2). It is not yet known which cardiac factors bind these E boxes, but they are not the myogenic bHLH family members, because these are absent from cardiac muscle. Cardiac muscle cells contain alternatively spliced homologs of the bHLH regulatory factor Id (18, 37, 62), and embryonic cardiomyocytes contain a transiently expressed bHLH factor which binds the MCK Right E box in vitro (42), as well as several bHLH factors (Th1/Th2, dHand/eHand) which are involved in early cardiogenesis (26, 63). Indirect evidence also suggests roles for other bHLH factors in cardiac gene regulation (44, 47, 59), but no cardiac muscle-specific Left E-box-binding factor has been identified.

The 358-bp proximal regulatory region of the MCK gene also exhibits different activities in specific muscles. When tested alone, it is sufficient for muscle-specific expression in quadriceps, abdominals, EDL, and soleus, and it appears to have similar activity to the entire 1-kb region 5′ of the transcription start site ($P > 0.10$) (16, 33). However, this region was not active when tested alone in cardiac and tongue muscles (Fig. 4; Table 2). When combined, the enhancer and proximal regulatory region exhibited greater activity in all skeletal muscles than did the 358-bp proximal regulatory region alone or a combination of the enhancer plus a basal MCK promoter (117 or 80 bp) (16, 33). Therefore, both the MCK enhancer and 358-bp proximal region exhibit muscle-specific expression and appear to act cooperatively to produce greater transcriptional activity. The combination of the MCK enhancer and 358-bp proximal region may also have practical application in gene therapy for muscle diseases (12, 35, 54) as a relatively small (564-bp), highly active skeletal and cardiac muscle-specific regulatory cassette.

Additional studies showed that sequences within the 358-bp proximal region are differentially required for expression in skeletal and cardiac muscle. Transgenes containing the enhancer linked to internal deletions of the proximal regulatory region (Δ1 and Δ2) retained tissue specificity but exhibited a 10- to 100-fold loss of activity in skeletal muscles (Fig. 5B and C; Table 2). Their residual activity, however, was still slightly higher than that of the 358-bp proximal regulatory region alone. The loss of activity may result from removal of important *cis* elements or from altered spacing between critical regulatory elements and the TATA box. The Δ1 deletion, but not the Δ2 deletion, resulted in a significant loss of transgene activity in cardiac muscle (Fig. 5C). On the basis of the importance of CArG elements in regulating other cardiac muscle genes (10, 51), the potential control element within the deleted region (bp −188 to −81) may be the CArG site at nt −177. Analysis of rabbit MCK 5′-flanking sequences by direct DNA injection into adult rat hearts also identified the region containing the proximal CArG site as critical for expression (67); however, our transgene data differ in that the MCK 206-bp enhancer plus the proximal promoter region is required for cardiac muscle-specific expression. The Δ1 region also contains

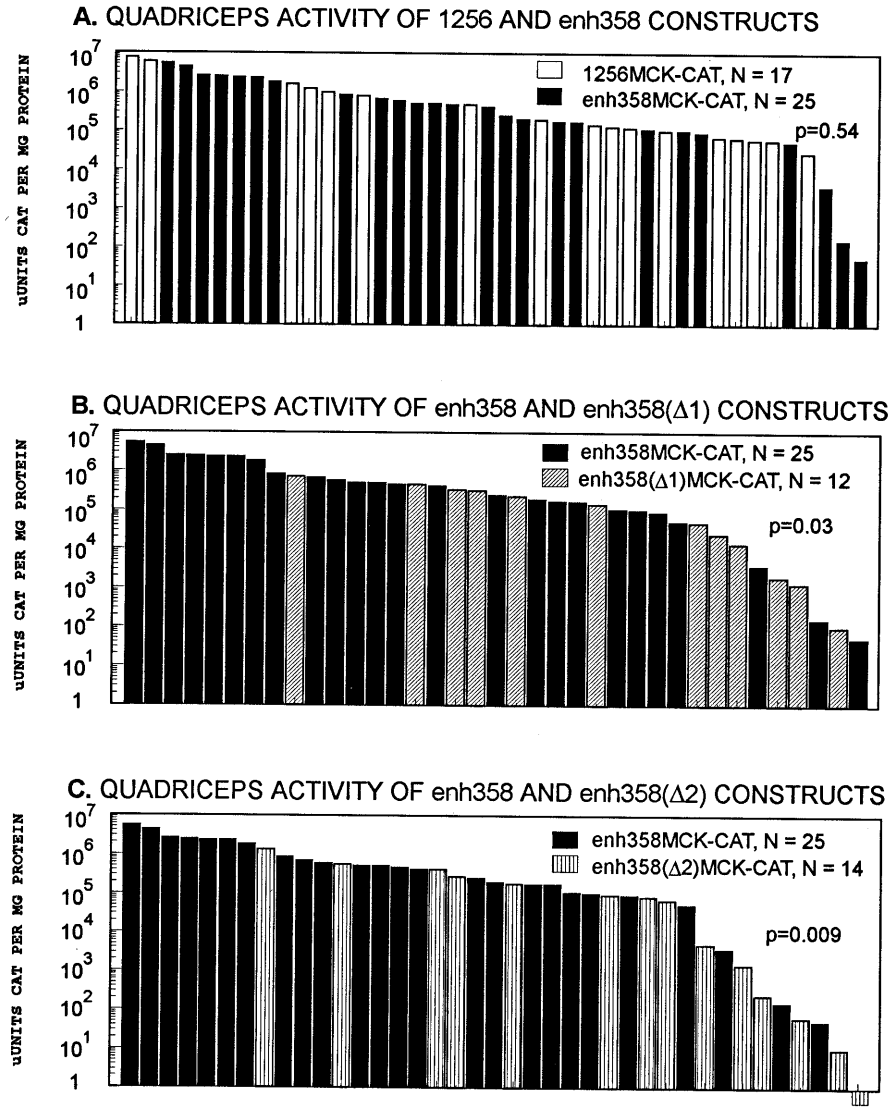

FIG. 5. Comparison of activities of transgenes in quadriceps skeletal muscle. (A) Comparison of the activity of 1256MCK-CAT and enh358MCK-CAT. Quadriceps muscle data were representative of abdominal, EDL, soleus, and tongue muscles, which also exhibit no significant difference between 1256MCK-CAT and enh358MCK-CAT activity. (B and C) Comparison of the activity of enh358MCK-CAT with enh358(Δ1)MCK-CAT (B) and enh358(Δ2)MCK-CAT (C) in quadriceps muscle.

p53 activation sites (29, 74), as well as an unconserved E box at nt −98. It would be surprising if the latter E box were functional, because it is not conserved in the rabbit MCK promoter and because a CAT transgene with 117 bp of the mouse MCK promoter has no activity (16). Taken together, our results suggest that steady-state expression of the MCK gene in adult heart muscle is regulated by both E-box-dependent and E-box-independent pathways and that maximal activity requires both the enhancer and portions of the 358-bp proximal regulatory region.

It is not yet understood how muscle fiber phenotypes develop or are maintained (15, 43, 53, 60). Nevertheless, both developmental cues and extrinsic signals affect muscle fiber type and gene expression (3, 14, 19, 22, 46), and genes, including the myosin and troponin families, human aldolase A, and muscle-specific transcription factors, exhibit differential expression in fast and slow muscles (4, 11, 23, 28, 36, 52, 55, 57, 58, 68). The skeletal muscles we examined contain different compositions of fast-twitch glycolytic fibers (expressing MyHC IIb), fast-twitch oxidative-glycolytic fibers (expressing either MyHC IIa or IIx), and slow-twitch oxidative fibers (expressing β/slow MyHC) (24, 53, 60). The wild-type 1,256-bp MCK 5′ region exhibits greater transcriptional activity in several predominantly fast muscles (quadriceps, abdominals, and EDL) than in the largely slow soleus muscle (Table 1). Although the endogenous MCK gene is expressed in all skeletal muscle types, MCK mRNA and enzyme activity levels differ among fiber types. MCK enzyme activity is about twofold higher in rat fast glycolytic fibers than in slow oxidative fibers and intermediate in fast oxidative-glycolytic fibers (72). MCK mRNA is also more abundant in the predominantly fast plantaris and gastrocnemius mouse muscles than in the slow soleus muscle (66). Preliminary results of CAT histochemistry on transgenic muscle sections indicate that expression of the 1256MCK-CAT transgene in quadriceps muscles is greater in fast fibers than in slow fibers (70).

The triple E-box mutation or removal of sequences between bp −1049 and −359 appeared to decrease transgene expres-

sion by about seven- and fourfold, respectively, in the soleus relative to the fast muscles (Table 3). Unlike the other muscles, 42% of the mouse soleus myofibers exhibit a slow phenotype; therefore, these data suggest that the conserved E boxes and sequences between bp −1049 and −359 are much more important for MCK expression in slow than in fast muscle fibers. This may be related to the relative levels of E-box-binding myogenic regulatory factors in these muscles. Myogenin mRNA is present in fast and slow muscles and is more abundant than MyoD mRNA in muscles which contain predominantly slow fibers, whereas MyoD appears to be restricted to non-IIa fast fibers (28, 68). Thus, factors other than MyoD would seem to mediate transcription via the MCK gene E boxes in mouse soleus muscle, which contains mostly type IIa (fast) and type I (slow) fibers.

Tongue muscle was the only skeletal muscle analyzed in which the three conserved MCK E boxes were absolutely required for expression and in which the 358-bp proximal regulatory region was entirely inactive. Despite the foregoing correlations between MCK expression and muscle fiber type, fiber type composition cannot explain MCK transgene expression in the tongue muscle, because the composition of this muscle is very similar to that of the EDL in which the triple E-box mutation had no effect. Unlike the other skeletal muscles examined, tongue muscle undergoes a constant phasic pattern of contraction in association with respiration (6, 49). It is interesting that cardiac muscle, which also undergoes continuous rhythmic contractions, exhibits both a similar requirement for the three MCK E boxes and a lack of expression from the 358-bp MCK proximal regulatory region when tested alone. Tongue and cardiac muscles are the only muscles we examined which are not of somitic origin. They are also the only muscles in which the transcription factor *Nkx*-2.5 is expressed (41), although it does not bind E-box sites directly (9). Owing to their distinct physiology and development, tongue and cardiac muscles may contain different combinations or concentrations of factors which interact with MCK sequences.

Diversity in muscle phenotypes may arise from developmental origins, hormonal stimulation, patterns of contraction, innervation, energy requirements, and other factors. These parameters almost certainly exert their effects via combinations of DNA-binding and accessory factors whose transcriptional activities depend, in turn, upon the array of control elements within different muscle genes. This study demonstrates that the mouse MCK gene is regulated by more complex mechanisms in adult muscles than was anticipated from previous cell culture and transgene studies. Of particular interest was the finding that the E-box control elements which are critical for steady-state transgene expression in cardiac muscle and in some skeletal muscle types are not critical in other muscle types. Understanding the full regulatory repertoire of the MCK gene presents the experimental challenge of analyzing its transcriptional control in many muscle types under diverse sets of physiological conditions.

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