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Multiple Regulatory Elements Contribute Differentially to Muscle Creatine Kinase Enhancer Activity in Skeletal and Cardiac Muscle

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We have used transient transfections in MM14 skeletal muscle cells, newborn rat primary ventricular myocardiocytes, and nonmuscle cells to characterize regulatory elements of the mouse muscle creatine kinase (MCK) gene. Deletion analysis of MCK 5′-flanking sequence reveals a striated muscle-specific, positive regulatory region between −1256 and −1020. A 206-bp fragment from this region acts as a skeletal muscle enhancer and confers orientation-dependent activity in myocardiocytes. A 110-bp enhancer subfragment confers high-level expression in skeletal myocytes but is inactive in myocardiocytes, indicating that skeletal and cardiac muscle MCK regulatory sites are distinguishable. To further delineate muscle regulatory sequences, we tested six sites within the MCK enhancer for their functional importance. Mutations at five sites decrease expression in skeletal muscle, cardiac muscle, and nonmuscle cells. Mutations at two of these sites, Left E box and MEF2, cause similar decreases in all three cell types. Mutations at three sites have larger effects in muscle than nonmuscle cells; an A/T-rich site mutation has a pronounced effect in both striated muscle types, mutations at the MEF1 (Right E-box) site are relatively specific to expression in skeletal muscle, and mutations at the CArG site are relatively specific to expression in cardiac muscle. Changes at the AP2 site tend to increase expression in muscle cells but decrease it in nonmuscle cells. In contrast to reports involving cotransfection of 10T1/2 cells with plasmids expressing the myogenic determination factor MyoD, we show that the skeletal myocyte activity of multimerized MEF1 sites is 30-fold lower than that of the 206-bp enhancer. Thus, MyoD binding sites alone are not sufficient for high-level expression in skeletal myocytes containing endogenous levels of MyoD and other myogenic determination factors.

The mammalian muscle creatine kinase (MCK) gene is expressed primarily in skeletal and cardiac muscle. The mouse MCK gene contains an enhancer located approximately 1,100 bp 5′ of the transcription start site. A 206-bp enhancer-containing fragment of the mouse gene and analogous regions of other mammalian MCK genes confer muscle-specific expression in cultured cells and transgenic mice (30, 36–38, 76, 81, 92). In vitro and in vivo footprinting have identified a variety of binding sites within the rat and mouse MCK enhancers (10, 28, 30, 31, 57).

MEF1, a skeletal myocyte-specific nuclear factor, was identified by gel shift assays and DNase footprinting using the MCK enhancer (10). The MEF1 or Right site contains the E-box sequence, CAnnTG (16, 23), characteristic of the recognition sites of the helix-loop-helix (HLH) family of DNA-binding proteins (58). The MEF1 complex contains the HLH myogenic determination factor, MyoD (41). MyoD, as well as the skeletal muscle determination factors myogenin, Myf5, and MRF4/herculin/Myf6, can bind the MEF1 site in vitro with various affinities, either alone or in combination with other HLH partners (2, 6, 9, 11, 12, 43, 59, 90). Mutational analysis has shown that the MCK MEF1 site and a nearby site of similar sequence (the Left site) contribute to skeletal myocyte MCK enhancer activity (10, 41). E boxes in the regulatory regions of several other muscle-specific genes also have been shown to be functionally important in skeletal muscle (for example, references 68, 73, and 89). Since skeletal muscle determination factors are not detected in heart (3, 44, 65, 74), it is of interest to determine what role E boxes play in cardiac gene regulation. An E box in the regulatory region of the human cardiac actin promoter is important for myocardiocyte expression (72); however, E-box mutations or deletions in several other genes have little or no effect on cardiac expression (32, 61, 62, 80).

The CArG site in the MCK enhancer was recognized by its near match to the consensus CC(A or T)$_6$GG sequence, which was first identified within highly conserved regions of avian and mammalian striated muscle actin genes (49). CArG boxes are found in muscle-specific control regions of a variety of muscle genes and have been implicated as important for regulation in both skeletal and cardiac muscle (15, 24, 39, 52, 55, 66, 72, 87, 95). They also appear in regulatory regions of genes whose expression is not muscle specific, including the β-actin gene (63), which is down-regulated in differentiating skeletal muscle, and the γ-actin gene (53), which is constitutively expressed. In addition, CArG sequences constitute a part of the serum response elements found in immediate-early genes such as *fos* (53, 82, 83).

The MCK enhancer contains two sites rich in adenine and thymine residues, the MEF2 and A/T-rich sites. The MEF2 site binds the muscle-specific factor MEF2 as well as ubiquitous factors (10, 19, 28, 30, 31, 93). MEF2 and related sites have been implicated in the regulation of a variety of muscle genes (7, 61, 62, 78, 89, 95). The MCK A/T-rich site was shown recently to be important for enhancer function in skeletal myocytes and binds the mesoderm-specific homeodomain protein, MHox (18).

The AP2 site in the MCK enhancer is a good match to two consensus sequences reported for the nuclear factor AP2 (33, 51). In vivo footprinting showed myocyte-specific occu-

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pancy of this site (57), and authentic AP2 binds this site in vitro (49a).

To ascertain the contribution that each of these elements makes to MCK enhancer function in skeletal and cardiac muscle, we have made two different mutations at each site mentioned above and tested each mutation in three enhancer-containing MCK-chloramphenicol acetyltransferase (CAT) constructs. Our transient transfection results suggest that the AP2 site is a negative element and that all other sites contribute to various extents to positive activity of the 5′ MCK enhancer. In myocardiocytes, the most drastic effects are seen with A/T-rich and CArG site mutations. The most deleterious mutations in skeletal myocytes are those at the MEF1 site; however, we show that multiple MEF1 sites do not substitute for the full enhancer.

MATERIALS AND METHODS

Plasmids. MCK-CAT deletion constructs shown in Fig. 2 have been described previously (36). Plasmids TKCAT (48), 4R-TKCAT (88), (+enh206)TKCAT (previously called en2+TKCAT) (36), (+enh110)80MCKCAT (10), and (−110 enh)80MCKCAT (10) are as described previously. Plasmids (+enh206)80MCKCAT and (−enh206)80MCKCAT and their mutated derivatives were constructed by inserting the *Hin*dIII-to-*Bam*HI enhancer fragment from wild-type or mutated pUC-E (10) or from −1256MCKCAT into the *Sal*I site of −80MCKCAT. All MCK-CAT constructs are derivatives of the promoterless CAT plasmid p118CAT (36). Mutated −1256MCKCAT plasmids were made by replacing the enhancer-containing *Hin*dIII-to-*Nsi*I fragment or *Sph*I fragment of wild-type −1256MCKCAT with the corresponding fragment from a mutated −1256MCKCAT plasmid. Mutations were performed by standard oligomer-mediated site-directed mutagenesis (40, 96), and DNA sequences of mutants were verified by the dideoxynucleotide method (70). Oligonucleotides were synthesized by the Howard Hughes Medical Institute Chemical Synthesis Facility, University of Washington. Reference plasmid pSV2Apap (29) was kindly provided by T. Kadesch. In some experiments, a pSV2Apap derivative, pUCSV2pap, was used. pUCSV2pap was constructed in two steps. First, the *Hin*dIII-to-*Xba*I fragment from pSV2Apap containing the human placental alkaline phosphatase (PAP) structural gene and simian virus 40 (SV40) small-t intron and SV40 early poly(A) signals was ligated into the polylinker of *Hin*dIII-*Xba*I-cut pUC118 (84). Then, the *Acc*I-to-*Hin*dIII fragment from pSV2CAT (27) containing the SV40 early promoter/enhancer was ligated upstream of the PAP structural gene at the unique *Hin*dIII site.

Cell culture. Mouse MM14 skeletal myoblasts were propagated as described previously (17). Primary newborn rat ventricular myocardiocytes were prepared essentially as described by Iwaki et al. (34), a protocol that utilizes a discontinuous Percoll gradient to purify cardiac myocytes from nonmuscle cells. Cells were plated at a density of 2.7×10^6 cells per 100-mm-diameter or 0.9×10^6 cells per 60-mm-diameter collagen-coated tissue culture dish in plating medium (Dulbecco's modified Eagle's medium-medium M199 [4:1] containing 5% fetal bovine serum, 10% horse serum, 250 ng of amphotericin B per ml, 100 U of penicillin G per ml, and 0.1 mg of streptomycin per ml). Typically, the cultures contained 90 to 95% myocardiocytes, as assessed by immunocytochemistry with myosin antibody MF-20 (1). Cardiac nonmuscle cells were removed from the discontinuous Percoll gradient and cultured in Ham F10C medium with 15% horse serum, 2 to 4 ng of human recombinant basic fibroblast growth factor (bFGF) per ml, 250 ng of amphotericin B per ml, and 0.06 mg of gentamicin per ml. Cells were passaged twice and stored frozen in liquid nitrogen until use. Parallel plates stained with myosin antibody MF-20 indicated that 99% of these cells are myosin negative. Primary kidney cells from 2-day-old newborn Sprague-Dawley rats were prepared as for primary myocardiocytes, omitting the Percoll purification step, and cultured in plating medium until harvest.

Transfection procedure. All cell types were transfected via a standard calcium phosphate technique (10) with slight modifications. DNA precipitates contained 8 μg of MCK-CAT test plasmid and 2 μg of reference plasmid, pSV2Apap or pUCSV2pap (described above). After the 20-min incubation with DNA, MM14 cultures were fed with 10 ml of Ham F10C medium plus 15% horse serum and without added bFGF; 4 h later, cells were glycerol shocked and switched to low-serum (1.5 or 5%), bFGF-free Ham F10C medium plus 1 μM insulin. In myocardiocyte and cardiac nonmuscle cell transfections, DNA was added directly to the medium on each plate. Four hours later, cells were glycerol shocked and switched to serum-free Dulbecco's modified Eagle's medium-M199 (4:1) containing 1 μM insulin and 250 ng of amphotericin B per ml.

Transfection analysis. Cultures were harvested 30 h (MM14 myocytes) or 48 h (myocardiocytes and cardiac nonmuscle cells) after glycerol shock. Cells were scraped from the plates with a rubber policeman, collected by centrifugation, and resuspended in 0.25 M Tris (pH 7.8) containing 0.5% (vol/vol) Triton X-100. After a 10-min room temperature incubation, extracts were centrifuged at 15,000 × *g* for 2 to 5 min; supernatants were then transferred to new tubes and frozen for subsequent analysis. Assays for the test gene (CAT) and reference gene (PAP) were performed as previously described (10, 29). CAT activity values were obtained by comparing CAT activity in test extracts with a standard curve. CAT activity was corrected for transfection efficiency by dividing by reference gene activity, scaled to the activity of the parental construct as described in the figure legends, and is presented as the mean value ± standard deviation. The value for the lowest non-zero standard on the curve determined the assay sensitivity for each set of transfection samples. Because of differences in activity of wild-type parental constructs to which values are scaled and differences in transfection efficiency, which varies from experiment to experiment, values preceded by < symbols differ (see legends to Fig. 3 and 5). The number of observations appears in figures or legends. For each construct tested, at least two plasmid preparations were each used in at least two transfections. Certain values were compared for statistical differences, using Student's *t* test (77).

RNA isolation and Northern (RNA) analysis. RNA was extracted from tissue (newborn rat heart and adult rat brain) and tissue culture cells (primary myocardiocytes and primary kidney cells) as described by Chomczynski and Sacchi (14). Ten micrograms of total RNA was fractionated on 1.2% agarose gels in the presence of 18% (vol/vol) formaldehyde. RNA was transferred to nylon membrane, and MCK transcripts were detected with the random prime-labeled mouse MCK-m3′ cDNA probe, which is mostly 3′ untranslated sequence (13). Blots were reprobed with a ~1,300-bp random prime-labeled chicken glyceraldehyde 3-phosphate dehydrogenase (GADPH) *Pst*I fragment (21) to assess RNA loading.

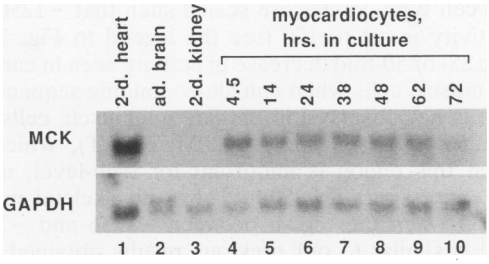


FIG. 1. Expression of endogenous MCK mRNA in ventricular myocardiocytes. Ten micrograms of RNA from newborn rat heart (lane 1), adult rat brain (lane 2), newborn rat kidney cells cultured for 62 h (lane 3), and newborn rat ventricular myocardiocytes cultured for the times indicated (lanes 4 to 10) was fractionated on a 1.2% agarose gel in the presence of 18% formaldehyde. Northern analysis for MCK and GADPH transcripts was performed as described in Materials and Methods. Myocardiocyte cultures were grown as described for transfection; cells were plated in medium containing 15% serum and, approximately 20 h later, switched to serum-free medium for the duration of the experiment.

RESULTS

Expression of endogenous MCK in ventricular myocardiocytes. A Northern blot was performed to detect steady-state MCK transcript levels in intact rat tissue and during rat primary cell culture. As shown in Fig. 1, MCK transcripts are detected in intact newborn rat heart (lane 1) but not in adult rat brain (lane 2) or newborn rat primary kidney cell culture (lane 3). MCK levels remain fairly constant during a 3-day culture period (lanes 4 to 10), although the ratio of MCK transcripts to GAPDH transcripts drops slightly during the first day of culture (compare lanes 1, 4, and 5). For this reason, myocardiocyte transfection analysis was done at times during the culture period when MCK mRNA levels are stable; DNA was introduced after about 20 h in culture, and cells were harvested after about 70 to 72 h in culture. Thus, myocardiocyte transfections identify regulatory elements important for maintenance of MCK gene expression. Previous studies with MM14 skeletal muscle cells showed that MCK transcripts first appear as myoblasts differentiate into myocytes (13); thus, transfection analysis in these cells identifies elements important for initial activation as well as maintenance of MCK gene expression.

MCK 5′-deletion analysis in myocardiocytes. To identify MCK cardiac regulatory regions, a reporter construct, −3300MCKCAT, which contains bp −3300 to +7 of the mouse MCK gene fused to CAT, and several 5′-deletion derivatives of −3300MCKCAT were tested in myocardiocyte transfections (Fig. 2). The activity of each construct is expressed relative to that of the most active construct, −1256MCKCAT, whose activity is set to 100. CAT activity remains constant as sequences between −3300 and −1409 are removed but increases about fivefold with a deletion to −1256. A comparable increase in activity was not seen in MM14 skeletal myocytes (36); this finding may indicate that a cardiac-specific negative regulatory region lies between −1409 and −1256. Removing 236 bp from −1256MCKCAT results in a 25-fold decrease in cardiac CAT activity (see activity of −1020MCKCAT), suggesting that this region confers strong positive regulation. A similar decrease is seen in MM14 skeletal myocytes (36) (Fig. 3). The activity of −776MCKCAT is low (Fig. 2), indicating that consensus CArG, AP2, and E-box sequences located between −160 and −330 of the MCK proximal promoter (35) are not

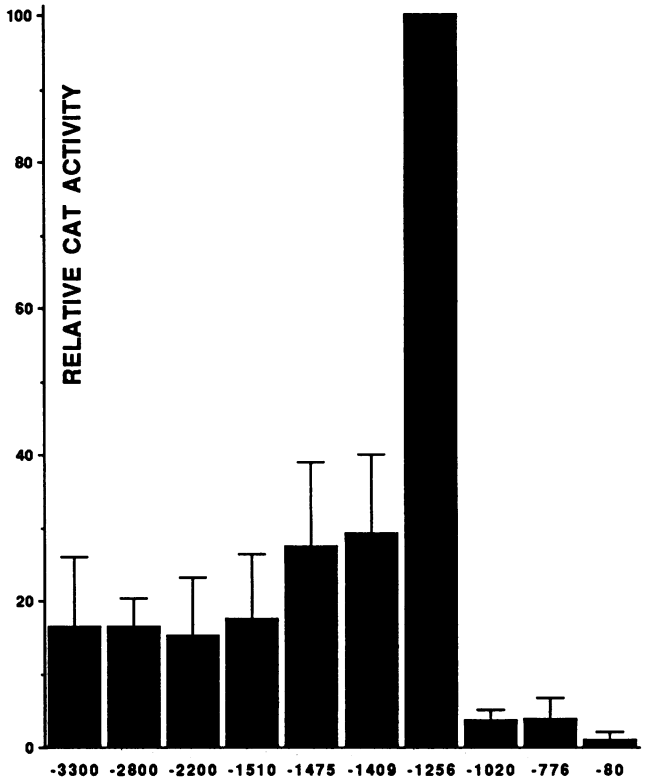
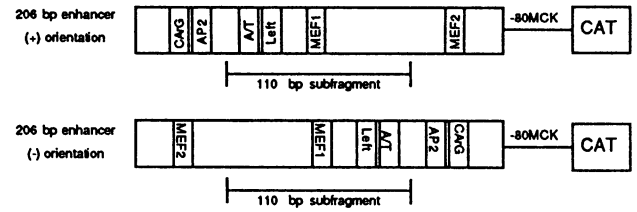


FIG. 2. MCK 5′-deletion analysis in myocardiocytes. Deletion constructs were as described previously (36) and contain MCK sequences from the upstream position indicated in the construct name through +7 relative to the transcription start site. Transfections were performed as described in Materials and Methods. CAT activity is corrected for transfection efficiency by using a cotransfected reference plasmid, scaled such that the activity of −1256MCKCAT is set to 100 in each experiment, and presented as mean value ± standard deviation. Numbers of individual transfections are 20 for −80MCKCAT, 9 for −3300MCKCAT, 8 for −1475MCKCAT, −1409MCKCAT, and −1020MCKCAT, 6 for −2200MCKCAT, −1510MCKCAT, and −776MCKCAT, and 5 for −2800MCKCAT.

sufficient for high-level myocardial expression. Low-level expression of a similar construct, −723MCKCAT, was previously observed in the cardiac muscle of transgenic mice (38).

Expression of MCK-CAT constructs in muscle and nonmuscle cells. To examine the relative transcriptional strength of MCK sequences among the cell types used in this study, we compared the absolute CAT activity of −1256MCKCAT with absolute reference gene activity (driven by the SV40 enhancer/promoter). Comparisons were also made between the ubiquitously expressed TKCAT and −1256MCKCAT activities in each cell type. Assuming similar expression among cell types from the constitutive promoters, these comparisons indicate that −1256MCK sequences confer 5- to 12-fold more activity in skeletal myocytes than in myocardiocytes and 4- to 10-fold more activity in myocardiocytes than in cardiac nonmuscle cells (data not shown). This finding is consistent with the muscle specificity that we and others have previously characterized (30, 36–38, 76, 81, 92). The observed 5- to 12-fold difference in skeletal myocyte versus myocardiocyte −1256MCKCAT expression may be



Construct	Relative CAT activity: Skeletal myocytes	Myocardiocytes	Non-myocytes
-1256MCKCAT	=100 ±24 (12)	=100	=100
-1020MCKCAT	1.8 ± 0.2 (6)	3.8 ± 1.5 (8)	100 ±60 (4)
-80MCKCAT	0.14± 0.11(22)	<1 (20)	43 ±32 (4)
(+enh206)80MCKCAT	100	9.6 ± 3.4 (16)	<10 (4)
(-enh206)80MCKCAT	160 ±50 (13)	73 ±37 (18)	<10 (4)
(+enh110)80MCKCAT	78 ±41 (12)	<1 (4)	<10 (4)
(-enh110)80MCKCAT	130 ±80 (12)	<2 (4)	<10 (4)

FIG. 3. Importance of the MCK upstream enhancer. Test constructs with MCK regions driving the CAT reporter gene were transiently transfected into MM14 cells, myocardiocytes, and cardiac nonmuscle cells, and results were analyzed as described in Materials and Methods. Plasmids −1256MCKCAT, −1020MCKCAT, and −80MCKCAT contain MCK upstream sequences from the number in the plasmid name to +7 with respect to the transcription start site. The enhancer (enh)-containing plasmids are shown schematically with positions of potential regulatory sites in boxes and have the 206- or 110-bp enhancer-containing fragments in either orientation as indicated by the plasmid names. CAT activity is corrected for transfection efficiency by using a cotransfected reference plasmid (see Materials and Methods). In skeletal myocyte transfections, values were scaled such that the activity of (+enh206)80MCKCAT was set to 100 in each experiment; means and standard deviations were then scaled up by a factor of 1.01 to set the mean value for −1256MCKCAT equal to 100. In myocardiocyte and cardiac nonmuscle cell transfections, values were scaled such that the activity of −1256MCKCAT was set to 100 in each experiment. Means and standard deviations are shown, with the number of observations given in parentheses. The absolute activity of −1256MCKCAT is 5- to 12-fold higher in skeletal myocytes than in myocardiocytes and 4- to 10-fold higher in myocardiocytes than in cardiac nonmuscle cells (discussed in the text). Mean values preceded by a < symbol indicate that one or more observations were below the sensitivity of the assay (see Materials and Methods); observations falling below assay sensitivity but above the zero CAT standard were assigned values equal to the assay sensitivity and averaged into the mean. Observations lower than the zero CAT standard were averaged as zero.

due to differences between continuous and primary cells. However, the expression ratio is similar to that seen in endogenous steady-state MCK transcript levels in the two tissues, and it is consistent with CAT levels observed in skeletal and cardiac muscle of transgenic mice carrying −1256MCKCAT as a transgene (38).

Importance of the MCK upstream enhancer. Previous analysis of the mouse MCK gene indicated that the −1256 to −1050 fragment, enh206, confers muscle-specific expression in a variety of configurations and cell types (36–38). In addition, enh110, a 110-bp internal fragment of enh206, confers high activity in skeletal myocytes (10). Figure 3 compares values of several different MCK-CAT constructs which were tested in MM14 skeletal myocyte, primary rat ventricular myocardiocyte, and cardiac nonmuscle cell transfections. For ease of comparison between constructs within a cell type, values are scaled such that −1256MCKCAT activity is set to 100 (see the legend to Fig. 3). The dramatic 25- or 50-fold decrease in activity seen in cardiac or skeletal muscle cells when enh206-containing sequences are removed is not observed in cardiac nonmuscle cells (compare −1256MCKCAT and −1020MCKCAT), which indicates that this region is important for high-level, muscle-specific expression. The 50-fold drop in skeletal myocyte expression when the region between −1256 and −1020 is removed is similar to our previous results obtained with a slightly different protocol (36).

To analyze the activities of enh206 and enh110 in isolation, fusions were made between these enhancer fragments and a basal promoter construct, −80MCKCAT, and tested in transfections (Fig. 3). In skeletal myocytes, enh206 in combination with −80MCKCAT is nearly identical in activity to −1256MCKCAT, and enh110 confers more than half the activity of enh206. The activity varies slightly with orientation, with the activity of the minus orientation higher than that of the normal (plus) orientation. However, the enhancer-containing constructs, whether enh206 or enh110 and regardless of orientation, have approximately 1,000 times the activity of the basal MCK promoter −80MCKCAT construct. In ventricular myocardiocytes, enh206 combined with −80MCKCAT confers orientation-dependent activity which is at least 10- or 70-fold higher than that of −80MCKCAT, with the minus orientation having almost 8-fold higher activity than the plus orientation. The levels of activity conferred by (−enh206)80MCKCAT approach the levels seen with −1256MCKCAT. In contrast to its activity in skeletal myocytes, enh110 confers virtually no activity in myocardiocytes, suggesting that sequences outside that region are required for myocardial enh206 activity. The activities of all enhancer fusion constructs in cardiac nonmuscle cells are low, and addition of either enh206 or enh110 actually decreases the activity of −80MCKCAT.

Mutational analysis of the enhancer. Six elements within enh206 were chosen for mutational analysis; all but one of these sites are perfectly conserved between mouse and rat MCK genes (see the legend to Fig. 4). Because any sequence alteration could artificially create new regulatory elements, each site studied was changed to two different mutant sequences, as shown in Fig. 4. The sequence located immediately under the wild-type sequence shows bases changed in mutation 1, and the lower sequence represents mutation 2. Each mutation was tested in three configurations (Fig. 5A): the plus and minus orientations of enh206 in combination with the −80 to +7 MCK basal promoter [represented as (+enh206)80MCKCAT and (−enh206)80MCKCAT, respectively], as well as in the entire −1256 to +7 MCK region (represented as −1256MCKCAT). The latter configuration has the enhancer in the same position relative to the transcription start site as in the endogenous gene, while the enh206 fusion constructs have nearly 1 kb of sequence deleted. Although the level of cardiac CAT activity conferred by (+enh206)80MCKCAT is approximately 10-fold lower than that of −1256MCKCAT (Fig. 3), it reproducibly is well above the limit of sensitivity of the CAT assay (see Materials and Methods). Mutations at several sites in this construct cause activity to fall below assay sensitivity (Fig. 5C), as discussed below. MEF1 mutation 1 and Left site mutation 1 have previously been tested in a construct containing the 110-bp enhancer subfragment (10, 41).

Effects of E-box mutations. Figure 5B shows that of all mutations tested in skeletal myocytes, the MEF1 site mutations have the most dramatic effect. This is most pronounced

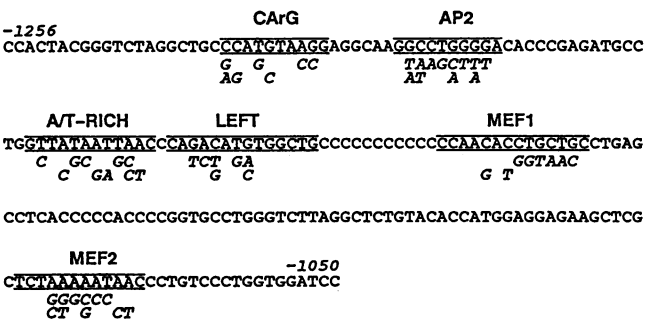


FIG. 4. Mutations introduced into the mouse MCK upstream enhancer. The sequence of the MCK upstream enhancer from −1256 to −1050 is shown. The six elements studied are boxed above and below by lines, with our name for each site shown in bold above. All of these elements are perfectly conserved between mouse and rat MCK genes, with the exception of the Left site, which differs at 4 of the 14 positions (the rat sequence is TGGACACGTGGTTG) (30). Changes made in the two mutations at each site are shown below the wild-type sequence. The upper and lower mutations shown are referred to as mutations 1 and 2 in Fig. 5 and 6 and in the text. Mutation 2 at the MEF2 site in the −1256MCKCAT configuration differs from that shown here; it lacks the changes at the two 3′-most positions. CArG mutation 2 in the (+enh206)80MCKCAT and (−enh206)80MCKCAT constructs has a 4-bp deletion from −1251 to −1248 in addition to the mutation indicated.

(30- to 100-fold decrease) in the (+enh206)80MCKCAT construct. Somewhat less effect (8- to 15-fold) is seen with (−enh206)80MCKCAT. Even in the context of −1256MCK-CAT, the 2-bp modification of mutation 2 lowers activity 8-fold and the 6-bp change of mutation 1 decreases activity 30-fold. In contrast, the same MEF1 mutations have much less effect in ventricular myocardiocytes, with the largest drop in activity being sevenfold (Fig. 5C). Most MEF1 mutations decrease activity by less than 50% in cardiac cells, and one actually increases activity.

We reported earlier (41) that Left site mutation 1 has less of an effect on enhancer function in skeletal myocyte transfections than does MEF1 site mutation 1 when tested in the smaller enh110 enhancer subfragment. Similar results are obtained with enh206 (Fig. 5B). The Left site mutations, although less dramatic than MEF1 site mutations, still cause a significant two- to fivefold decrease in activity. In myocardiocytes, similar moderate decreases of two- to sevenfold are seen (Fig. 5C). However, in contrast to results in skeletal myocytes, Left site mutations are more potent than MEF1 site mutations in cardiac muscle cells. Since a differential effect is observed in skeletal and cardiac muscle cells upon MEF1 site mutation (a site which is identical in mouse and rat MCK genes) and similar effects are seen in these two cell types upon Left site mutation (a site less well conserved; see the legend to Fig. 4), we believe that the reported differences reflect true tissue differences in element function and not species differences between mouse MM14 skeletal myocytes and rat myocardiocytes.

A double-mutant construct with mutation 1 sequences at the MEF1 and Left sites was also tested in the enh206 fusion constructs. In skeletal myocytes, the double mutant has significantly less activity than either single mutant in both orientations (Fig. 5B). Although the double mutation in (+enh206)80MCKCAT has a mean activity greater than that of the basal −80MCK promoter alone, the difference is not statistically significant ($P > 0.10$). In myocardiocytes, the activity of the double mutant is similar to that of the Left site mutant 1 alone (Fig. 5C).

Effects of A/T-rich and MEF2 mutations. Mutation of the A/T-rich site has a dramatic effect on enhancer function, causing a 3- to 20-fold decrease in skeletal myocyte activity (Fig. 5B) and a 6- to 50-fold drop in cardiac activity (Fig. 5C). In myocardiocytes, mutation 2 is more potent when the enhancer is fused in the negative orientation; otherwise, mutations at this site seem relatively orientation independent in both cell types.

The three sites mentioned above lie within enh110, which confers more than half the activity of enh206 in skeletal myocytes. Thus, in theory, one might expect that mutation of sites outside enh110 would not have more than a twofold effect unless spacing differences between enh110 in the two configurations (i.e., alone upstream of the promoter or within enh206) play a role in its activity. Conversely, since enh110 confers virtually no activity in myocardiocytes, we hypothesized that sequences outside of enh110 were essential for enh206 activity in cardiac cells.

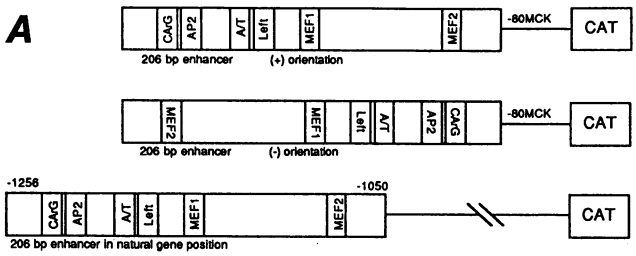
The MEF2 site is one of three sites tested in enh206 which lie outside of enh110. In the configuration placing the MEF2 site closest to the transcription start site, (+enh206) 80MCKCAT, both mutations decrease skeletal myocyte activity five- to eightfold, whereas in the (−enh206)80MCK CAT and −1256MCKCAT configurations, which place the MEF2 site further from the transcription start site, an approximately twofold effect is noted (Fig. 5B). In contrast, myocardial activity of MEF2 mutants in the enh206 fusion constructs is relatively orientation independent, with mutation 1 decreasing activity 13- to 20-fold and mutation 2 decreasing activity 2- to 3-fold (Fig. 5C). In the −1256MCK CAT configuration, the mutations reduce cardiac activity two- to fourfold.

Effects of CArG mutations. The CArG site is also outside of enh110. In both cell types, mutation of CArG sequences has a greater effect in the (−enh206)80MCKCAT configuration, which places the site closest to the transcription start site. Up to a 4-fold decrease is noted in skeletal myocytes (Fig. 5B), whereas a 12- to 50-fold drop is seen in myocardiocytes (Fig. 5C). In the other configurations, in which the mutated CArG is farther from the transcription start site, the effect is 2-fold or less in skeletal myocytes and 4- to 20-fold in cardiac muscle cells (Fig. 5B and C). The CArG mutations are among the most drastic seen in myocardiocytes yet are relatively mild in skeletal myocytes; the CArG mutations have 3- to 10-fold more effect in cardiac than in skeletal muscle cells (Fig. 5B and C).

Effects of AP2 mutations. Unlike the five sites discussed above, which appear to contribute positively to enhancer function, the AP2 site appears to act as a negative element in the MCK enh206 fragment. In skeletal muscle cells, mutations at the AP2 site cause increases of 30 to 50% in enhancer activity. This effect is consistent in all configurations tested (Fig. 5B). AP2 mutations also cause activity increases in myocardiocytes, although the fold increases are much less consistent, and in one case, a slight drop in activity is noted (Fig. 5C). Although AP2 mutation 2 causes a dramatic 6.5- to 12-fold increase in cardiac reporter activity when tested in the enh206 fusion constructs, this mutation increases activity only 2-fold when tested in the −1256MCKCAT construct, in which enh206 lies in its natural gene position.

Effects of enhancer mutations in nonmuscle cells. To examine the muscle specificity of each site, one representative −1256MCKCAT mutant at each of the six sites was tested for activity in cardiac nonmuscle cells. Since activities of the

A



B

Construct	Relative CAT activity in skeletal myocytes (+enh206)80MCKCAT	(-enh206)80MCKCAT	-1256MCKCAT
wild-type enh206	=100	=100	=100
-80MCKCAT	0.14 ± 0.11 (22)	0.09± 0.07 (22)	0.11± 0.04(4)
CArG mut1	50 ±15 (6)	23 ± 7 (6)	55 ±24 (6)
mut2	71 ±11 (6)	46 ±18 (6)	81 ±22 (6)
AP2 mut1	130 ±30 (6)	130 ±60 (6)	140 ±40 (6)
mut2	150 ±60 (6)	150 ±70 (6)	140 ±40 (6)
A/T mut1	8.5 ± 2.5 (6)	9.6 ± 3.4 (6)	31 ± 7 (6)
mut2	6.7 ± 2.7 (6)	5.3 ± 1.1 (6)	8.8 ± 2.0 (6)
Left mut1	20 ± 7 (6)	21 ± 7 (7)	48 ±18 (6)
mut2	28 ±12 (6)	50 ±15 (6)	44 ±12 (6)
MEF1 mut1	1.1 ± 0.4 (6)	6.7 ± 1.6 (7)	3.5 ± 0.8 (7)
mut2	3.3 ± 1.3 (6)	12 ± 4 (6)	12 ± 3 (6)
MEF1+Left mut	0.24 ± 0.15 (7)	0.57± 0.27 (8)	N.D.
MEF2 mut1	21 ± 3 (6)	57 ±13 (6)	51 ±16 (6)
mut2	12 ± 5 (6)	46 ±12 (6)	70 ±15 (6)

C

Construct	Relative CAT activity in myocardiocytes (+enh206)80MCKCAT	(-enh206)80MCKCAT	-1256MCKCAT
wild-type enh206	=100	=100	=100
-80MCKCAT	<15 (20)	<2 (20)	<1 (20)
CArG mut1	<5 (4)	<2 (5)	18 ± 4 (4)
mut2	27 ± 9 (4)	7.7 ± 4.4 (6)	25 ± 13 (6)
AP2 mut1	84 ± 14 (4)	120 ± 50 (6)	180 ± 80 (5)
mut2	1200 ±600 (4)	650 ±190 (4)	220 ±110 (4)
A/T mut1	<5 (4)	<2 (4)	16 ± 6 (4)
mut2	16 ± 7 (4)	<2 (5)	14 ± 6 (7)
Left mut1	<15 (6)	13 ± 9 (8)	37 ± 21 (6)
mut2	46 ± 24 (4)	58 ± 33 (4)	54 ± 20 (4)
MEF1 mut1	<15 (6)	53 ± 25 (6)	62 ± 32 (4)
mut2	95 ± 37 (5)	280 ±140 (4)	81 ± 26 (5)
MEF1+Left mut	<15 (4)	8.3 ± 3.0 (4)	N.D.
MEF2 mut1	<5 (4)	7.7 ± 4.4 (5)	54 ± 30 (8)
mut2	36 ± 16 (4)	57 ± 24 (4)	27 ± 16 (5)

FIG. 5. (A) Schematic diagram of constructs used in mutational analysis. Each of the mutations shown in Fig. 4 was tested in three enhancer configurations: (+enh206)80MCKCAT (top), (−enh206) 80MCKCAT (middle), and −1256MCKCAT (bottom). (B) Effects of MCK enhancer mutations in skeletal myocytes. Transfections were performed and data were analyzed as described in Materials and Methods. For the (+enh206)80MCKCAT and (−enh206)80MCK CAT configurations, values were scaled such that the value for (+enh206)80MCKCAT was set to 100 in each experiment; means and standard deviations for the negative orientation constructs were then scaled down by a factor of 1.63 to set the mean value for (−enh206)80MCKCAT equal to 100 (see Fig. 3). For the mutant −1256MCKCAT constructs, values were scaled such that the mean wild-type −1256MCKCAT value was set to 100 in each experiment. The number of observations appears in parentheses. (C) Effects of MCK enhancer mutations in myocardiocytes. Transfections were performed and data were analyzed as described in Materials and Methods. For all construct configurations, values were scaled such that the value for −1256MCKCAT was set to 100 in each experiment. Means and standard deviations for (+enh206)80MCKCAT constructs were then scaled up by 10.4 to set the mean value for (+enh206)80MCKCAT equal to 100, and means and standard deviations for (−enh206)80MCKCAT constructs were scaled up by a factor of 1.36 to set the mean value of (−enh206)80MCKCAT equal to 100. The number of observations appears in parentheses. Mean values preceded by a < symbol indicate that one or more observations were below the sensitivity of the assay (see Methods); these values were scaled as described above. Observations falling below assay sensitivity were averaged into the mean as described in the Fig. 3 legend. N.D., not determined.

wild-type enh206 fusion constructs (+enh206)80MCKCAT and (−enh206)80MCKCAT in nonmuscle cells were typically below assay sensitivity (Fig. 3), mutated derivatives of these constructs were not tested in this cell type. The nonmuscle cell results for −1256MCKCAT and mutated derivatives are compared graphically in Fig. 6 with results obtained for identical constructs in skeletal and cardiac muscle cell transfections (Fig. 5). All six mutations decrease nonmuscle cell activity, but only Left site mutation 1 has over a twofold effect, reducing activity about threefold.

To facilitate the comparison of each mutation between cell types, the activity of wild-type −1256MCKCAT is scaled to 100 within each cell type in Fig. 6. However, as described earlier, the absolute CAT activity of wild-type −1256MCKCAT is much lower in nonmuscle cells than in muscle cells. Two mutations, Left site mutation 1 and MEF2 mutation 1, cause similar moderate decreases in activity in all three cell types (Fig. 6). However, cell type differences are noted with mutated AP2, A/T-rich, CArG, and MEF1 constructs. The AP2 site seems to play a negative role in muscle and a positive role in nonmuscle enh206 regulation. The decrease in nonmuscle cell activity seen with AP2 mutant 2 is statistically different ($P < 0.05$) from the increase observed in skeletal myocytes and, to a lesser extent ($P < 0.10$), from the increase seen in myocardiocytes. An intact A/T-rich site appears more important for high-level activity in striated muscle cells than in nonmuscle cells; there is a four- to sevenfold greater decrease in A/T-rich mutant 2 activity relative to the wild-type construct in skeletal and cardiac myocytes than in nonmuscle cells ($P < 0.01$ for both pairwise comparisons.)

As mentioned earlier, mutations at CArG and MEF1 sites differentially affect enh206 activity in striated muscle cell types (Fig. 5). We now extend this observation to include comparisons of these mutations in nonmuscle cells (Fig. 6). CArG mutation 1 differentially affects cardiac muscle; it reduces activity to a threefold greater extent (relative to the wild type) in myocardiocytes than in skeletal myocytes ($P < 0.01$) or nonmuscle cells ($P < 0.05$). In contrast, MEF1 mutation 1 is approximately 17-fold more potent relative to wild-type −1256MCKCAT in skeletal myocytes than in myocardiocytes ($P < 0.05$) or nonmuscle cells ($P < 0.001$), suggesting a skeletal myocyte-specific role.

Comparison of multiple MEF1 sites with the whole enhancer. After discovering the muscle-specific binding factor MEF1 and finding that mutation of its binding site led to drastically reduced enhancer activity in skeletal myocytes (10), we tested whether one or several MEF1 binding sites would constitute a positive regulatory region. MEF1 binding sites were placed upstream of the MCK −80 to +7 basal promoter or the herpes simplex virus thymidine kinase (TK) promoter as well as downstream of the CAT reporter gene in a −776MCKCAT construct. When tested in MM14 mouse skeletal muscle myocytes, such constructs yielded reporter gene activities above those of the parental constructs but far lower than those of constructs containing enh206 (10a). However, when other investigators tested analogous con-

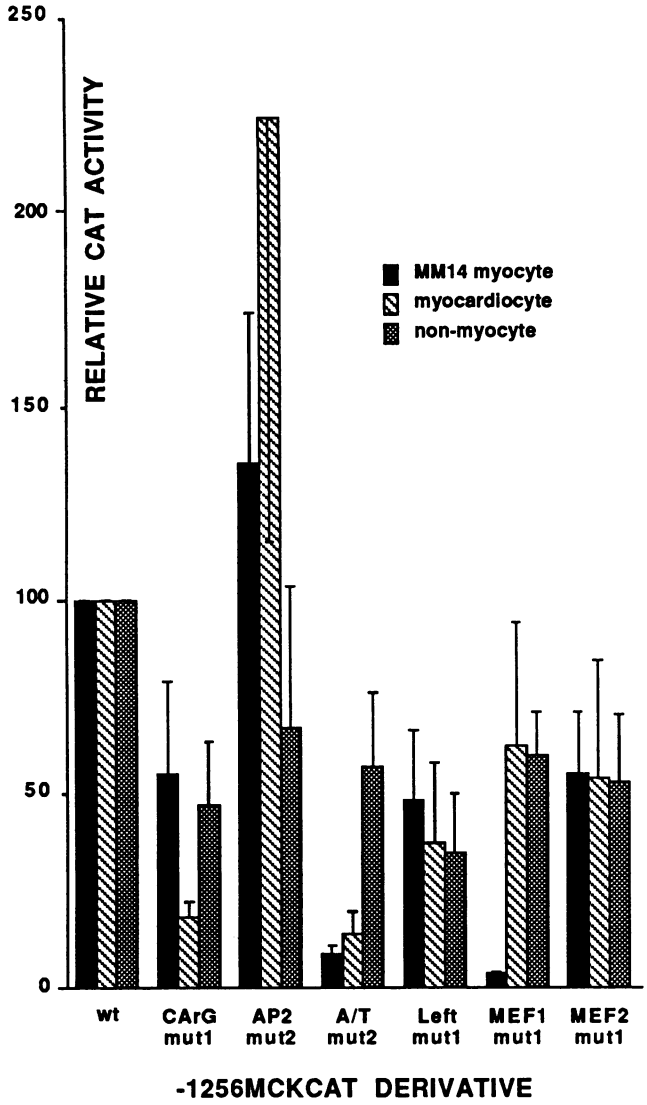
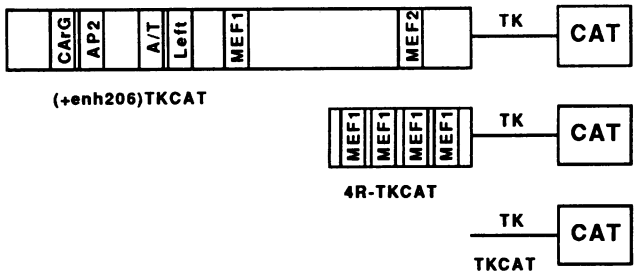


FIG. 6. Effects of MCK upstream enhancer mutations in cardiac nonmuscle cells. One representative −1256MCKCAT mutant at each of the six sites was tested for activity in cardiac nonmuscle cell transfections and compared with data obtained for the same mutated constructs in skeletal myocytes and myocardiocytes (see Fig. 5B and C). Cells were transfected and data were analyzed as described in Materials and Methods. Values were scaled such that the value for −1256MCKCAT was set to 100 in each experiment and cell type in order to facilitate comparison of the effects of mutations between cell types. The absolute activity of −1256MCKCAT is 5- to 12-fold higher in skeletal myocytes than in myocardiocytes and 4- to 10-fold higher in myocardiocytes than in cardiac nonmuscle cells (discussed in the text). The number of observations for each construct in striated muscle cells is indicated in Fig. 5; the number in nonmuscle cells is four. Error bars indicate the standard deviation for each value.



Construct	Relative CAT Activity
(+enh206)TKCAT	=100
4R-TKCAT	2.8 ± 1.1 (6)
TKCAT	1.0 ± 0.3 (6)

FIG. 7. Comparison of multiple MEF1 binding sites with the entire MCK upstream enhancer in skeletal myocytes. All plasmids contain the herpes simplex virus TK promoter driving the CAT reporter gene. 4R-TKCAT contains four copies of the MCK enhancer MEF1 site inserted upstream of the TK promoter (88), and (+enh206)TKCAT contains the entire 206-bp enhancer fragment upstream of the TK promoter (36). MM14 transfections were performed and data were analyzed as described in Materials and Methods. Values were scaled such that the value for (+enh206) TKCAT was set to 100 in each experiment. Relative to (+enh206)80MCKCAT, means and standard deviations for the three constructs are 180 ± 38, 5.0 ± 2.0, and 1.9 ± 0.6.

structs in cells expressing high levels of myogenic determination factors, several MEF1 sites appeared to have activity as great as that of the whole enhancer. For example, in the presence of exogenously produced MyoD, a construct with four MEF1 sites and the TK promoter (4R-TKCAT) has activity in 10T1/2 cells similar to that of the enhancer-containing −3300MCKCAT construct (88).

We speculated that the apparent discrepancy between our results and those of Weintraub et al. (88) is related to differences between the two test systems. In particular, MyoD levels might be abnormally high in transfected 10T1/2 cells containing unknown quantities of the MyoD cDNA plasmid driven by a strong constitutive promoter compared with the situation in MM14 myocytes, in which MyoD is produced solely from the endogenous gene. This could account for differences in expression of a target gene containing multiple MyoD binding sites. Alternatively, the discrepancy could be due to differences in the exact sequences and positions of MyoD binding sites in the constructs tested. We examined the latter possibility by using the 4R-TKCAT construct (graciously provided by H. Weintraub). When tested in MM14 myocytes, 4R-TKCAT gave about three times higher activity than did the parental construct, TK-CAT ($P < 0.05$), but its activity was still 30-fold lower than that of the construct containing enh206 upstream of TKCAT (Fig. 7). Thus, as indicated by our previous (unpublished) results, multiple MyoD binding sites do not substitute for the entire MCK enhancer in MM14 skeletal myocytes.

DISCUSSION

Complexity of enhancer function. We have tested mutations at six sites within the 206-bp MCK enhancer fragment, and all affect enhancer function in skeletal and cardiac myocytes (Fig. 5 and 6). At least five positive-acting elements contribute to full enhancer activity. These include the two MyoD-binding E-box elements, the MEF1 (or Right) site and the Left site; the two adenine/thymine-rich sites, MEF2 and A/T-rich; and a CArG site. An AP2 site appears to serve as a negative element in striated muscle.

The discovery that the MyoD family of myogenic regulatory factors are DNA-binding proteins and the prevalence of

recognition sites for these factors in regulatory regions of muscle-specific genes led to speculation that one or some of these factors cause the correlated expression of muscle genes. However, this hypothesis is not broad enough to explain the role of the numerous sites and factors unrelated to the myogenic regulatory factors which play a role in skeletal muscle-specific gene expression (see below and references 25, 46, 47, 61, and 80). Some links between the MyoD family and other transcription factors have been suggested. For example, exogenous expression of myogenin induces MEF2 binding activity (19, 93), an intact A/T-rich site in the MCK enhancer is required for transactivation of a reporter gene by myogenic HLH proteins (18), and myosin heavy-chain IIB regions that contain MEF1 and A/T-rich sites synergistically increase reporter expression when placed together upstream of a basal promoter (78). Since MyoD family members are not expressed in the heart at any time during development (3, 44, 65, 74), the role of E boxes and their putative binding factors in cardiac gene regulation is yet to be established. An E-box sequence in the human cardiac α-actin promoter is important for cardiac expression (72); however, E-box mutations in rat myosin light-chain 2 (62), β-myosin heavy-chain (80), and human phosphoglycerate mutase (61) gene promoters as well as an E-box deletion in the chicken cardiac troponin T promoter region (32) have little or no effect on promoter strength when tested in cardiac muscle cultures. In addition, an array of sites other than E boxes have been implicated in cardiac-specific expression (see below and references 26, 32, 45, 61, 62, 66, 67, 80, and 91).

E boxes and enhancer function. Other investigators have tested one or several E boxes placed upstream of minimal promoters driving reporter genes in cells expressing high levels of exogenous myogenic determination factors (5, 8, 22, 43, 88, 94). In this situation, the E boxes do yield levels of expression comparable to those of more diverse muscle enhancer regions. This contrasts to the results of the present study, which shows low expression of multiple E-box constructs in myocyte cultures expressing normal (endogenous gene) amounts of determination factors (Fig. 7). Our results suggest that skeletal muscle enhancer function involves more complexity than just E boxes. This also seems to be the case for cardiac MCK gene regulation, since a 110-bp enhancer subfragment that contains two E boxes (MEF1 and Left sites) confers virtually no activity to a basal MCK promoter construct (Fig. 3).

Configuration and apparent effects of mutations in skeletal muscle. In MM14 skeletal myocytes, a construct containing the muscle-specific 206-bp MCK enhancer, enh206, in combination with the MCK −80 to +7 minimal promoter is about as active in driving a reporter gene as is the much larger −1256 to +7 region of the gene. A 110-bp fragment from the center of enh206 confers greater than half as much activity (Fig. 3). Despite the high activity of the smaller fragment, mutations outside of the 110-bp region can reduce activity by more than twofold. For example, in (+enh206)80MCKCAT, mutation of the MEF2 site yields a 5- to 10-fold decrease in activity. How is this effect consistent with the less than twofold decrease caused by entirely removing the MEF2 site as well as additional sites in the (+enh110)80MCKCAT construct? One explanation is that such a deletion also removes negative-acting elements such as the AP2 site, whereas the site-specific mutation retains the negative element(s). Another possibility is that the positions of regulatory elements (with respect to one another and/or to the transcription start site) are important.

The latter possibility is supported by the orientation dependence of mutation effects. In the cases of both the MEF2 and the CArG sites, the effect of mutation is more pronounced in the orientation with the site closer to the transcription start (Fig. 5B). The 5- to 10-fold loss of activity in (+enh206)80MCKCAT when the MEF2 site is mutated suggests that the MEF2 site performs an important function when the enhancer is in the positive orientation. Yet both mutant MEF2 (−enh206)80MCKCAT constructs have activities similar to that of wild-type (+enh206)80MCKCAT (Fig. 3 and 5A). This finding suggests that some other position-dependent site in (−enh206)80MCKCAT is playing the role that the MEF2 site plays in (+enh206)80MCKCAT.

A pronounced effect of orientation is also seen when mutated MEF1 site constructs are examined, although the MEF1 site itself is in nearly identical positions in the plus- and minus-orientation constructs (Fig. 5). The explanation would therefore seem to lie in the position or orientation dependence of the element(s) which remains intact in these mutants.

Most of the enhancer mutations have similar effects in the enh206 fusion constructs and in the larger −1256MCKCAT constructs, indicating that the additional −1050 to −80 region of the MCK gene does not contain regulatory information which can compensate for the mutated sites. The most notable exception is the A/T-rich site mutation 1, which has a threefold greater effect in the enh206 fusion constructs. This difference is not seen with mutation 2 at the A/T-rich site, even though both mutations result in sequences unlike the wild-type sequence. An overlapping element unrelated to the element destroyed by both mutations may be responsible for the difference in the mutated −1256MCKCAT constructs.

Configuration and apparent effects of mutations in myocardiocytes. The activity of the enh206 region in combination with the basal MCK promoter is dependent on orientation in myocardiocytes (Fig. 3). The minus-orientation construct, (−enh206)80MCKCAT, has high levels of activity which approach that of the much larger −1256MCKCAT; however, the levels of (+enh206)80MCKCAT are 7- to 10-fold lower than the levels of the other two constructs. Thus, although the enh206 region is required for high-level activity of −1256MCKCAT in both striated muscle cell types, it does not confer orientation-independent expression in myocardiocytes when fused to −80MCKCAT. This contrasts to the situation in skeletal myocytes, in which enh206 exhibits relatively orientation-independent expression in combination both with −80MCKCAT and with heterologous promoters (36) (Fig. 3).

Orientation dependence of mutation effects is also seen in cardiac myocytes. As the wild-type enh206 fusion constructs exhibit orientation-dependent activity in myocardiocytes (see above), one might expect that mutations which lessen this difference would identify sites involved in establishing the orientation effect. Both CArG mutation 2 and A/T-rich mutation 2 fit this criterion, since they decrease activity to a greater extent when in the minus orientation than when in the plus orientation. However, since the effect of each mutation is greater in the orientation which places the site closer to the transcription start, the effect may be one of absolute position, not orientation. As seen in skeletal myocytes, MEF1 mutation 1 is more potent in the (+enh206)80MCKCAT configuration, even though the position of this site relative to the transcription start changes little between the two orientations.

We also tested the effect of each mutation in the context of

the larger −1256 to +7 MCK region (−1256MCKCAT). In almost every case, the effect of mutation is less drastic in this larger construct, suggesting that sequences between −1050 and −80 can partially compensate for the mutated site (Fig. 5C).

Tissue differences in regulatory region and individual element function. Transient transfection analysis of MCK 5′-deletion constructs revealed the presence of two regulatory regions: a striated muscle-specific positive element between −1256 and −1020 (Fig. 3) and a cardiac-specific negative regulatory region farther upstream between −1409 and −1256 (36) (Fig. 2). Previous transgenic analysis also had identified a striated muscle-specific positive element between −1256 and −723 (38). However, the average ratio of cardiac to skeletal CAT activity in each transgenic individual was higher for −3300MCKCAT than for −1256MCKCAT, suggesting that the region between −3300 and −1256 contains a cardiac-specific positive regulatory element (38). The apparent discrepancy observed in the two assay systems regarding the region upstream of −1256 suggests that the −3300 to −1256 region may modulate transcription in response to signals from the environment, instead of being strictly a negative or positive element. The environmental and physical stimuli are presumably very different between the transgenic whole animal system and the serum-free environment of the transfected myocardiocytes.

The positive regulatory region which contains enh206 was examined by comparing mutant activity in skeletal myocytes, myocardiocytes, and cardiac nonmuscle cells (Fig. 6). This analysis indicated tissue-specific differences of mutation at the AP2 site, the MEF1 (or Right) site, the CArG site, and the A/T-rich site.

AP2 mutation decreases activity in nonmuscle cells but increases activity in skeletal and cardiac myocytes. The high levels of AP2 transcripts in cultured fibroblasts (50) is consistent with the hypothesis that AP2 contributes positively to the low activity of −1256MCKCAT in cardiac nonmuscle cells, since a majority of these cells are probably fibroblasts. However, the lack of AP2 transcripts in mouse somites, embryonic heart, and adult heart and the very low levels in adult skeletal muscle (50) suggest that a different factor binds the AP2 site in striated muscle cells and negatively regulates MCK transcription. Since a comparison of the human and mouse MCK promoters reveals a high degree of sequence conservation around the core AP2 site in addition to the site per se, negative regulation may operate through a site which overlaps the AP2 consensus-like sequence.

A MEF1 site mutation is much more potent relative to the wild type in skeletal myocytes than in myocardiocytes or nonmuscle cells. Thus, as discussed earlier, although concatenated MEF1 sites do not substitute for the full MCK enhancer, an intact MEF1 site is critical for high-level enhancer activity in MM14 myocytes. In contrast, the relatively mild effect of MEF1 mutations in myocardiocytes and nonmuscle cells may reflect the fact that the MyoD family of myogenic regulators, which bind MEF1 sites in vitro, are not detected in these cell types.

A mutation at the CArG site causes a threefold greater decrease in activity relative to the wild type in myocardiocytes than that seen in skeletal myocytes or nonmuscle cells (Fig. 6). The apparent tissue specificity of the CArG site and its absence from the MCK enh110 enhancer subfragment may explain why enh110 fusion constructs are virtually inactive in myocardiocyte transfections yet are very active in skeletal myocytes (Fig. 3). The CArG box sequence is similar to that of serum response elements (82), sites which bind the cloned serum response factor (SRF) (64). Some studies have indicated that CArGs and serum response elements can be exchanged and still confer appropriate regulation (79, 83), while others indicate that these sites perform distinct functions (71, 85). CArG boxes bind SRF (4, 54), and a variety of muscle-specific and ubiquitous factors (42, 53, 60, 86) have been identified by in vitro binding methods. In addition, accessory factors that bind SRF to form ternary complexes with DNA have been identified (20, 56, 75). Cardiac-specific CArG-binding factors have not yet been reported, raising the question of how the apparent tissue specificity of the MCK CArG element is achieved. Possible explanations include cardiac-specific modifications of ubiquitous CArG-binding factors, cardiac-specific accessory factors which bind SRF or other CArG-binding factors, or interaction of CArG-binding factors with cardiac-specific factors bound elsewhere. In addition, the slight difference between the MCK enh206 CArG sequence and that proposed as the consensus CArG sequence (49) may be important for cardiac MCK expression.

Skeletal and cardiac myocytes share a requirement for an intact A/T-rich site (Fig. 5 and 6). Others have shown that an MCK enhancer A/T-rich mutation decreases enhancer activity in skeletal myocytes and prevents transactivation by HLH myogenic regulators in nonmuscle cells (18). A cloned novel homeodomain protein, MHox, that binds this site in vitro is expressed in mesodermal tissues of the mouse embryo and at high levels in adult mouse skeletal muscle, heart, and uterus (18). Since MEF1 and A/T-rich site mutations were the most deleterious of those tested in skeletal myocytes in this study, it is intriguing to postulate that MHox and the MyoD family of determination factors may act together to regulate MCK transcription in skeletal muscle. If MHox is also involved in cardiac MCK regulation, it most likely is dependent upon factors which bind at sites other than E boxes, since the 110-bp MCK enhancer subfragment containing an A/T-rich site and two E boxes confers virtually no activity in myocardiocytes (Fig. 3). Other cloned factors which bind adenine/thymine-rich consensus sequences are the SRF-related proteins isolated from nonmuscle cDNA libraries (69) and MEF2 isolated from muscle cDNA libraries (93). The transcripts of these related factors appear to be alternatively spliced isoforms of two genes, one of which accumulates preferentially in muscle and brain (93). While SRF binds a consensus CArG sequence, CC(A or T)$_6$GG, these SRF-related factors bind a different consensus that contains a longer stretch of internal adenine and thymine residues (69, 93). Interestingly, the MCK enhancer mutations that decrease myocardial activity most drastically were those at CArG and adenine/thymine-rich sites, suggesting that SRF and related factors may be involved in cardiac MCK regulation.

Although the MCK enhancer is critical for high-level expression in both striated muscle types, individual sites within the enhancer contribute differently to full enhancer activity in skeletal and cardiac muscle. The relative importance of each of the six sites analyzed in this study to tissue-specific MCK gene regulation may depend upon the presence of distinct DNA-binding or accessory factors as well as different concentrations or modifications of identical factors in skeletal and cardiac muscle cells.

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