Differences in the Function of Three Conserved E-boxes of the Muscle Creatine Kinase Gene in Cultured Myocytes and in Transgenic Mouse Skeletal and Cardiac Muscle*

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The 1256-base pair enhancer-promoter of the mouse muscle creatine kinase gene includes three CAnnTG E-boxes that are conserved among mammals and have flanking and middle sequences conforming to consensus muscle regulatory factor binding sites. This study seeks to determine whether these E-boxes are critical for muscle creatine kinase expression in physiologically distinct muscles. Mutations of the “right” and “left” E-boxes in the enhancer decreased expression in cultured skeletal myocytes −10- and 2-fold, respectively, whereas a “promoter” E-box mutation had little effect. In neonatal myocardiocytes, the left E-box mutation decreased expression −3-fold, whereas right or promoter E-box mutations had no effect. Very different effects were seen in transgenic mice, where the promoter E-box mutation decreased expression in quadriceps, extensor digitorum longus, and soleus −10-fold, and −100-fold in distal tongue, diaphragm, and ventricle. The right E-box mutation, tested in the presence of the other two mutations, caused a significant decrease in distal tongue, but not in quadriceps, extensor digitorum longus, soleus, or ventricle. Mutation of the left E-box actually raised expression in soleus, suggesting a possible repressor role for this control element. The discrepancies between mutation effects in differentiating skeletal muscle cultures, neonatal myocardiocytes, and adult mice suggested that the E-boxes might play different roles during muscle development and adult steady-state function. However, transgenic analysis of embryonic and early postnatal mice indicated no positive role for these three E-boxes in early development, implying that differences in E-box function between adult muscle and cultured cells are the result of physiological signals.

How is the transcription of muscle-specific genes differentially regulated in different anatomical muscles? The muscle creatine kinase (MCK) gene encodes the muscle-specific isorm of creatine kinase, which is transcribed at high levels in striated muscle. Fast-type muscle fibers have approximately double the enzyme expression compared with slow-type muscle fibers (1), and heart expression is approximately an order of magnitude lower than skeletal muscle (2). The MCK upstream enhancer contains binding sites for MEF2, Mhox, serum response factor, Six4/TrexBF, AP-2, and Oct1 (Refs. 3–6 and references therein) as well as the prototype E-box binding site for the myogenic regulatory factors (MRFs) MyoD, myogenin, MRF4, and Myf5 (7–11). Transgenic mouse and mouse knockout experiments, along with in situ hybridization developmental studies and transfection experiments, have shown that MyoD and Myf5 play essential roles in muscle determination and that myogenin is critical for terminal differentiation (12–14).

An enhancer-promoter region of the mouse MCK gene, which extends from −1256 to +7 relative to the transcription start site, exhibits tissue-specific expression similar to that of the endogenous MCK gene, as assessed by cell culture and transgenic mouse studies (6, 15–19). The enhancer-promoter contains seven E-box core sequences CAnnTG, but only three of these are conserved among mammals. Two conserved E-boxes occur within the MCK −1256 to −1051 upstream enhancer. The MEF1, or right E-box, is a high affinity binding site for all four MRFs. Mutating the right E-box abolishes MRF binding and reduces expression in cultured skeletal myocytes as much as 100-fold (6, 7, 20). The left E-box is a low affinity binding site for the MRFs, and mutating it decreases skeletal myocyte expression 2−5-fold (6, 7). Mutation of the left E-box has a similar effect (2−6-fold) in cultured neonatal myocardiocytes, but in these cells, mutation of the right E-box has variable effects, including a 6-fold decrease, no effect, and even a 3-fold increase in expression depending on the associated enhancer and promoter regions (6).

Because MRFs are primarily restricted to skeletal muscle, the cell culture and nuclear factor binding results were consistent with high affinity MRF binding sites being important for high expression in skeletal muscle. It was thus surprising to discover that mutation of the high affinity right E-box site did not decrease reporter gene expression in the skeletal muscle of transgenic mice (18). We speculated that other E-boxes might compensate for the loss of the right E-box, and therefore tested a construct containing mutations of both enhancer E-boxes plus a conserved promoter E-box (18, 19). The triply mutated basic-helix-loop-helix; CAT, chloramphenicol acetyltransferase; PAP, placental alkaline phosphatase; ANOVA, analysis of variance; HIF-1, hypoxia-inducible factor 1; ARNT, aryl hydrocarbon receptor nuclear translocator; L, left; R, right; P, promoter.

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× J. E. Johnson and S. D. Hauschka, unpublished results.
1 The abbreviations used are: MCK, muscle creatine kinase; EDL, extensor digitorum longus; MRF, myogenic regulatory factor; bHLH, basic-helix-loop-helix; CAT, chloramphenicol acetyltransferase; PAP, placental alkaline phosphatase; ANOVA, analysis of variance; HIF-1, hypoxia-inducible factor 1; ARNT, aryl hydrocarbon receptor nuclear translocator; L, left; R, right; P, promoter.
2 J. E. Johnson and S. D. Hauschka, unpublished results.
3 C. L. Himeda, J. A. Ranish, J. C. Angello, P. Maire, R. Aebersold, and S. D. Hauschka, unpublished results.
construe exhibited expression similar to the wild-type construct in several fast-type muscles. However, expression in the distal tongue and heart was reduced approximately 100-fold, and the ratio of expression in slow muscle to fast muscle was reduced approximately 10-fold. We have now extended the transgenic analysis by examining three new mutant-E-box constructs in a variety of muscle types, and by comparing the expression of the wild-type and triple E-box mutant constructs during development.

EXPERIMENTAL PROCEDURES

DNA Sequence Analysis—Sequence comparisons were performed using the Wisconsin Package version 10.0 (Genetics Computer Group (GGC), Madison, WI) and the National Center for Biotechnology Information (NCBI) BLAST programs (www.ncbi.nlm.nih.gov/BLAST/).

MCK-CAT Constructs—Test plasmid constructs include wild-type or mutated MCK promoter sequences from −1256 to +7 with respect to the transcription start site (GenBank™ accession no. AF188002) fused to the chloramphenicol acetyltransferase (CAT) structural gene, and the SV40 small t-intron and polyadenylation regions (21) in the pUC118 vector (22). The three mutations used singly or in combination were created by standard oligomer-mediated mutagenesis, and the DNA regions with the mutations were sequenced before being excised and inserted into the appropriate plasmids. The MEF1 (right E-box) and left E-box mutations are those referred to as “mut 1” at these sites by Amacher et al. (6); the promoter E-box is the proximal mutation reported in the 1256[3E mut]MCKCAT construct, which also contains the MEF1 (right E-box) and left E-box mutations (19). These mutated sequences are shown in Fig. 1 above the corresponding locations.

Cell Culture—Ventricular myocardiocytes were isolated from 2–3-day-old Sprague-Dawley rats as described by Iwaki et al. (23), with modifications as previously described (24). These cultures contained >95% myocardiocytes as assessed by immunohistochemistry with the myosin-specific antibody MF-20. Mouse MM14 skeletal myoblasts were grown and induced to differentiate by fibroblast growth factor with- out serum (25). Transfections—Primary myocardiocytes or MM14 skeletal myoblasts were transfected with 8 μg of test plasmid and 2 μg of a reference plasmid (pUC852PAP), included to normalize for transfection ef- ficiency, using a standard calcium phosphate technique (6). Four hours later, cells were glycercolated. Myocardiocytes were switched to glycerol shock to serum-free medium (Dulbecco’s modified Eagle’s medium/M199 (4:1) supplemented with 6 μg/ml insulin and Fungizone). Cells were harvested 48 h after glycerol shock and analyzed for chloramphenicol acetyltransferase (CAT) and placental alkaline phosphatase (PAP) activities (6). MM14 cultures were switched to differentiation medium at the time of addition of DNA, and harvested 34 h later. CAT and PAP were assayed as described (6). For each test construct, at least two plasmid preparations were used.

Transgenic Mouse Analysis—Production of mice with the triple E-box mutation has previously been described (18). For additional constructs, MCK-reporter gene fragments for microinjection were produced by re- striction enzyme excision, agarose gel electrophoresis separation, elec- troelution, and purification using a DEAE Elutip column (Schleicher & Schuell). Transgenic mice were produced by the University of Washing- ton Transgenic Resource Program using eggs from C57BL/6J × C3H crosses. Founders or their offspring from matings with C57BL/6J strain mice were analyzed as indicated. Protein extracts were prepared (19) and analyzed for protein by the Bradford assay (26) using bovine serum albumin (Sigma) as a standard, and for CAT using 14C-acetyl-CoA as previously described (20). CAT standards were run to ensure that all sample values used were in the linear range of the assay, and CAT values were not used unless 14C-acetylcholoramphenicol counts/min (cpm) were at least twice the background level. Cpm were converted to CAT units by dividing the value by the amount that acetylates 1 μmol of chloramphenicol/min at 37 °C (27); CAT values are expressed as micromoles/mg of protein. Values were used from both founder trans- genic mice and their offspring with details indicated in figure legends.

Developmental Analysis—Pregnant mice were sacrificed by cervical dislocation. Embryos were staged according to Ref. 28, and tissues were dissected. To reduce contamination by other cell types, atria were removed as whole tissue, and skin, lung, bone, and cartilage were removed from embryonic and fetal hindlimbs. Tongue muscle was ob- tained from the distal half of the tongue. Tissues were snap-frozen in liquid nitrogen and stored at −80 °C. For early embryos, two or three samples of the same type were pooled together for protein extraction.

Newborn and adult mouse dissection, protein extraction, and CAT and protein assays were as described (19).

Statistical Analysis—Comparisons of transfection or transgenic mouse data sets were performed using GraphPad InStat version 3.02 for Windows 95 or GraphPad Prism trial version 3.03 (GraphPad Soft- ware, San Diego, CA; www.graphpad.com) using the tests indicated under “Results.” No transgenic founder mouse or line was eliminated from the data analysis based on its value(s). A few individual mice were omitted from calculation of the mean values of particular transgenic mouse lines if they had one or more values differing by more than a factor of 10 from several other values for mice in the same line and muscle type. In the 18 lines for which we report new data (see Table I for details), 110 individual mice were analyzed, and data from 4 mice were not used because of one or more aberrant expression values (6).

In our developmental studies, we had no a priori expectation of how expression might vary with time. We tried linear, polynomial, and exponential fits of expression as a function of age, seeking the simplest function that would fit all four sets of data for a given tissue. For each type of line or curve, we employed “runs tests” (using GraphPad Prism; www.graphpad.com/curvefit/systematic_deviation.htm) as a measure of the suitability of the curve type. If the data conform to a particular mathematical function, the individual points will randomly scatter on both sides of the curve, giving many “runs” on one side or another. If the data do not conform well to the model, there will be fewer runs than expected, indicating that the model is a poor choice.

RESULTS

Mammalian MCK Enhancer-Promoter Regions Contain Three Conserved E-boxes—One possible indicator for E-box im- portance, in addition to functional assays such as in vitro binding or transfections, is interspecies conservation. We ex- amined four MCK sequences: rat (accession no. M27092; Ref. 29), mouse (AI888002; Ref. 30), human (M21487; Ref. 31), and rabbit (X55146; Ref. 32). Within the mouse sequence from −1256 to +7, three E-boxes have counterparts in the other three species (Fig. 1). We refer to these, from 5′ to 3′, as the left, MEF1 or right, and promoter E-boxes. In addition, the mouse, rat, human and rabbit have four, six, three, and three other E-boxes, respectively, which are not conserved (Fig. 1).

We compared the sequences surrounding the seven E-box core sequences in the 1263-base pair mouse MCK promoter (Fig. 1) to the (c/g)n(a/g)(a/g)(a/g)CA(g/c)(c/g)(c/g)TG(c/t)(c/t)n(c/g) E-box consensus we had previously proposed for muscle-specific reg- ulatory regions (20). Requiring the core CAnnT and weighing other positions equally, the three best matches to the consen- sus were the E-box sites conserved between species (Fig. 1). These three sites are also good matches, with no or one mis- match, to the consensus sequences proposed for MyoD/E12 heterodimer binding by Blackwell et al. and by Kophengnavong et al. (33, 34) based on in vitro binding assays. Other sequences within the mouse −1256 promoter that are good matches to a proposed MyoD/E12 binding consensus sequence, (a/c/g)CA(c/ g)(c/g)TG(c/t)(c/t)n(c/g) (34), do not have counterparts in the human and rabbit sequences and occur in the −1050 to −359 region, which can be deleted without consequence in transgenic mice (19) or cultured mouse skeletal myocytes (6).

The rat, human, and rabbit MCK promoters all contain an E-box at the position equivalent to −222 in the mouse se- quence, whereas the mouse contains a CACCTA sequence at this position. This suggested a sequencing mistake or a muta- tion in the cloned DNA. However, the identical sequence, with- out an E-box, is found in an independently derived mouse clone, from strain C57BL/6J (accession no. NW_000293), as well as our clone from mouse strain Balb/c (35, 36), confirming the lack of an E-box at that position. In addition, the E-box in our Balb/c clones at −98, with counterparts in rat and human but not rabbit promoters, is not found in the C57BL/6J genomic clone, suggesting that this E-box site is polymorphic in mice. Studies reported below focus on the three E-boxes conserved in all four species.
Distinct Functions of Three E-boxes in Striated Muscles

Figure 1. E-box occurrence in four mammalian MCK promoters. Seven core E-boxes, CAnTG, occur in the mouse upstream sequence from −1256 through the transcription start site. Three additional mammalian MCK promoter sequences were aligned with the −1256 to +1 mouse sequence using the PileUp program of the Wisconsin Genetics Computer Group, with settings GapWeight = 3, Gap Length Weight = 0. From top
Distinct Functions of Three E-boxes in Striated Muscles

E-box-specific Mutation Effects on 1256MCKCAT Expression in Cultured Skeletal Myocytes—We previously tested the effect of mutating the left and right (MEF1) E-boxes in a variety of configurations in cultured MM14 skeletal myocytes and in rat neonatal myocardiocytes (6, 7, 20). These mutant sequences plus a mutant variant of the conserved promoter E-box are shown above the aligned sequences in Fig. 1. We have now extended our transfection studies to skeletal myocytes by testing these three mutations in all combinations.

E-box mutations were explored in the context of the 1256MCKCAT construct, containing MCK sequences from −1256 to +7 driving a linked reporter gene. As shown in Fig. 2a, mutation of the left E-box causes a 2-fold decrease in expression in MM14 skeletal myocytes (note the logarithmic y axis in this graph), whereas mutation of the right E-box has nearly 10 times the effect, with expression decreased to 6% of wild-type. These results concur with previous studies (6). In contrast to the left and right E-box mutations, activity of the 1256MCKCAT construct with the mutant promoter E-box is approximately the same as the wild-type construct.

Constructs with two E-box mutations gave results much as predicted by assuming that each mutation abolishes the same proportion of expression as when it is the only mutation in 1256MCKCAT. For example, the single left E-box mutant construct expression level multiplied by the single right E-box mutant construct expression level (0.48 of wild-type × 0.063 of wild-type) predicts 3.0% of wild-type expression for the double mutant versus the observed 2.5%. Deletion of the entire MCK enhancer in the 1020MCKCAT construct, which removes both left and right E-boxes plus four other positive control elements (5, 6), results in a low expression not significantly different from the 1256MCKCAT construct with left and right E-boxes mutated (p > 0.05).

E-box-specific Mutation Effects on 1256MCKCAT Expression in Cultured Myocardiocytes—The effect of mutating E-boxes on myocardiocyte expression is strikingly different from that in skeletal myocytes (Fig. 2b). The right E-box mutant values are not statistically different from the wild-type. In contrast, the left E-box mutant construct displays significantly lower expression, only one-third that of the wild-type construct (p < 0.01, 2-tailed t test with Welch correction for unequal variation). These results are similar to those reported earlier (6). The promoter E-box mutant yields mean expression 40% lower than the wild-type construct, but the difference is not statistically significant (p > 0.05). The triply mutated construct (left plus right plus promoter E-boxes) exhibits significantly lower expression in myocardiocytes than the wild-type construct, but its expression is no lower than the singly mutated left E-box mutant. All of these statistical conclusions hold true at the 0.05 level of significance when the data are corrected by the Holm method for multiple testing (37), or analyzed by nonparametric analysis of variance (ANOVA, Kruskal-Wallis test with Dunn’s post test) (see Fig. 2b legend for details).

Muscle Type-specific Differences between the Effects of E-box Mutations in Adult Transgenic Mice—Transgenic mouse expression is highly variable depending upon the genomic integration site, so that large numbers of lines or founders are necessary to test for statistically significant differences. As previously discussed (24), no statistical difference was seen between founders and lines, or between recent and older data for the same construct. We have therefore included data from both mouse lines and founders and some previously published data (16, 18, 19, 24) (as indicated in table footnotes and figure legends) for the largest data sets.

An earlier study showed that mutation of the right E-box or of three different E-boxes (left, right, and promoter) had no statistically significant effect when tested in several primarily fast transgenic mouse muscles (18, 19) in stark contrast to the dramatic (over 50-fold) decrease in cultured myocytes (Fig. 2 and Ref. 6). We now report data for three additional constructs: left E-box mutant, promoter E-box-mutant, and double left-plus-promoter E-box mutant in 1256MCKCAT. Data for six different muscles are listed in Table I and shown graphically in Fig. 3. Note that the graphs have a logarithmic y axis, so zero expression values of transgene-positive mice cannot be plotted; however, zero values are used in computing median values shown on the graphs, and in all statistical tests.

All of these 1256MCKCAT constructs have high overlapping expression levels in quadriceps and EDL (Fig. 3). As with the constructs previously tested, the expression of the three new constructs is tissue-specific. Expression in liver, a representative non-muscle tissue, was typically 5 orders of magnitude lower than in quadriceps or EDL (data not shown), indicating that the mutations do not abolish muscle specificity.

As shown in Table I and Fig. 3, individual values for transgenic lines and founders bearing a given construct vary over many orders of magnitude in the same muscle. It is thus inappropriate to use means and standard deviations to represent the data, and many standard statistical tests cannot be used. Data for each muscle type were therefore analyzed using the nonparametric two-tailed Mann-Whitney test for pairwise comparisons of transgenic constructs. Fig. 4 shows the p values for these comparisons. Because 65 pairwise comparisons were made, we would expect approximately 3 comparisons to yield p values less than 0.05 if there were no real differences between expression from different constructs; in fact, 25 values were less than 0.05, and 9 values were less than 0.01, indicating significantly different expression between some constructs. Nonparametric ANOVA (Kruskal-Wallis test with Dunn’s post test) performed for simultaneous comparison of expression by all constructs for a particular muscle type or correction for multiple comparisons by either the Holm or Bonferroni method (37) gave similar results (see Fig. 4 legend for details).

In soleus, a muscle reported to contain 34–58% slow fibers in mouse (38–41), mutation of the promoter E-box leads to significantly lower expression (compare promoter E-box mutant construct to wild-type), and the doubly mutated left-plus-promoter E-box mutant construct is also significantly lower than the construct with the single left E-box mutation (Figs. 3 and 4). In contrast, mutation of the left E-box leads to a significant increase in expression suggesting that this element plays a repressor role in the soleus (Figs. 3 and 4). Mutation of the right E-box does not seem to have a detectable effect in soleus, at least in the context of constructs lacking the other two

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to bottom, the promoter sequences are mouse strain Balb/c (mice; accession no. AF188002), rat (accession no. M27092), human (hum; accession no. M21487), and rabbit (rab; accession no. X55146). Asterisks indicate bases that are identical to the base in the mouse sequence, and hyphens indicate gaps introduced to optimize alignment. Numbering is from the mouse sequence, and because of introduced gaps does not correspond to the other sequences. All of the E-box core sequences CAnTG are shown in reverse coloring (white on black). The three E-boxes that are conserved in all four species, “left,” “right,” and “promoter,” are the positions of mutations tested in this study, and the bases changed in the mutants are shown above these three mouse E-boxes. A mouse polymorphism is also shown; the Balb/c strain has a core E-box including a G at −93 in the sequence CAnTG, but the C57BL/6J strain (accession number NW_000260) has an A at that position, and thus no E-box. E-boxes in the Balb/c mouse strain occur at −1178, −1153, −776, −707, −434, −249, and −98, with respect to the transcription start site, with the position of the upstream C in the core sequence used as the location. In rat, the E-boxes are at −1150, −1130, −757, −719, −660, −409, −250, −223, and −99; in human −838, −816, −568, −265, −236, and −98; in rabbit −550, −531, −358, −236, −209, and −137.
Distinct Functions of Three E-boxes in Striated Muscles

Two predominantly fast limb skeletal muscles were examined: quadriceps and EDL. In these muscles, only mutation of the promoter E-box has been shown to have a significant effect; see *p* values for comparison of the wild-type and promoter E-box-mutated constructs (Fig. 4). Another comparison reinforces the conclusion that the promoter E-box is important; in the quadriceps, a significant difference is seen between the left-plus-promoter E-box double mutant construct and the left E-box single mutant construct. In quadriceps, the median expression for the left E-box-mutated construct is higher than the expression for the wild-type construct, and in both of these muscles, the left-plus-promoter E-box double mutant has higher median expression than does the promoter E-box mutant construct (Fig. 3). Although examination of the median values hints at a repressor role for the left E-box in fast muscle, none of the differences is statistically significant (Fig. 4).

The comparisons above demonstrate a positive role for the promoter E-box in predominantly fast quadriceps and EDL as well as in the slower soleus muscle, with significant differences revealed by several statistical comparisons, whereas the left E-box plays a negative role in soleus. (See below for consideration of corrections for multiple statistical comparisons.)

In previous transgenic studies, we found it helpful to compare ratios of expression in two different muscles within individual transgenic mice (19, 24). Because the transgenes are integrated into the same genomic location, the effect of flanking DNA may be partially or wholly abolished by taking such ratios. Soleus over EDL transgene expression ratios are shown in Fig. 5. Comparison of the expression ratios to either soleus or EDL expression in Fig. 3 shows that the variability of the ratios is considerably lower in nearly every case. Differences in soleus/EDL expression ratios for the 5 different constructs are qualitatively similar to those for soleus alone (Fig. 4), but the lower *p* values provide greater confidence in the significance of the differences in the ratios.

The comparisons in Fig. 5 confirm a greater effect of mutating the left and promoter E-boxes on soleus expression than on EDL expression. Interestingly, no significant effect of mutating the right E-box on the soleus/EDL ratio is seen, but this comparison could only be made against the background of left-plus-promoter mutations with and without the right E-box mutation. In ventricles, the promoter E-box mutation has a dramatic three orders of magnitude effect on expression (Figs. 3 and 4). This is true whether the mutation is alone, or combined with a mutation in the left E-box. None of the comparisons testing left or right E-box function indicates a significant effect of mutating either or both of these sites on ventricle expression.

In distal tongue, the promoter E-box is important for high level expression; its mutation decreases median transgene expression levels 100-fold (Figs. 3 and 4). In contrast, mutation of the left E-box site actually raises the median expression level in tongue as seen by comparing the left E-box mutant and wild-type constructs, or the left-plus-promoter E-box mutant and the promoter E-box mutant, although neither comparison reveals a significant difference. The right E-box mutation appears to be important for MCK transgene expression in tongue muscle as seen by comparing the left-plus-right-plus-promoter mutant construct to the left-plus-promoter mutant construct. As a result of the large number of “zero expression” levels which cannot be plotted in Fig. 3, the difference in expression is more easily observed in Table I. These data suggest that the right and promoter E-boxes each contribute to MCK transgene
expression in tongue muscle, and that both are necessary for highest expression.

The relative effects of different E-box mutations on MCK transgene expression in the diaphragm appear to be similar to tongue (Figs. 3 and 4). There is no significant effect of mutating the left E-box, although the median is higher with the left E-box mutated in two comparisons. The promoter E-box mutation causes a significant decrease in transgene expression in the diaphragm (compare promoter E-box-mutated construct to wild-type). Unfortunately, no values for right E-box mutant constructs are available for diaphragm.

As explained in the figure legends for Figs. 4 and 5, corrections for multiple statistical comparisons by either the Holm or Bonferroni methods (37) yield fewer apparently significant differences between pairs of data sets. In particular, the effect of the promoter E-box mutation is only shown to be significant in ventricle and in soleus (via the comparison of left E-box mutant versus left-plus-promoter E-box mutant in the latter case) if the

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**TABLE I**

| construct, founder or line | quadriceps (quads.) | ventricle (vent.) | distal tongue (dis. tong.) | diaphragm (diaphr.) | EDL | soleus |
|---------------------------|---------------------|------------------|---------------------------|---------------------|-----|--------|
| W.T.                      |                     |                  |                           |                     |     |        |
| f14247                    | 7.6E+6              | 6.5E+4           | 1.2E+5                    | 4.8E+6              | 3.2E+6|
| f5                       | 6.3E+6              | 7.6E+3           |                           |                     |     |        |
| 6059                      | 3.5E+6              | 3.1E+4           | 1.3E+5                    | 1.1E+6              | 3.0E+6| 9.9E+5 |
| f66865                    | 1.9E+6              | 5.4E+4           | 8.8E+4                    | 7.1E+6              | 3.6E+5|
| f14266                    | 1.6E+6              | 8.0E+2           | 3.3E+3                    | 1.1E+6              | 3.3E+5|
| f14493                    | 1.2E+6              | 9.5E+3           | 2.5E+4                    | 1.4E+6              | 2.5E+5|
| 1.2E+6                    | 1.5E+3             |                   |                           |                     |     |        |
| 1.0E+6                    | 5.6E+1             | 5.1E+2           | 8.9E+4                    | 1.2E+6              | 5.1E+5|
| 1.3E+5                    | 1.7E+3             | 7.2E+2           | 1.5E+6                    | 9.6E+5              |     |        |
| f14333                    | 8.1E+5              | 3.0E+1           |                           |                     |     |        |
| f14333                    | 6.0E+5              | 2.0E+1           |                           |                     |     |        |
| f14333                    | 7.0E+4              | 5.0E+0           |                           |                     |     |        |
| 14258                    | 6.6E+4              | 1.1E+4           |                           |                     |     |        |
| 6879                      | 6.4E+4              | 2.6E+3           |                           |                     |     |        |
| 6904                      | 6.0E+4              | 6.0E+0           |                           |                     |     |        |
| 6978                      | 6.0E+4              | 2.4E+3           |                           |                     |     |        |
| 14324                    | 5.9E+4              | 1.0E+1           |                           |                     |     |        |
| f14261                    | 2.8E+4              | 0.0E+0           |                           |                     |     |        |
| 6880                      | 2.4E+1              |                   |                           |                     |     |        |

**L mut**

| construct, founder or line | quadriceps (quads.) | ventricle (vent.) | distal tongue (dis. tong.) | diaphragm (diaphr.) | EDL | soleus |
|---------------------------|---------------------|------------------|---------------------------|---------------------|-----|--------|
| 5873                      | 4.9E+6              | 2.7E+3           | 8.5E+4                    | 9.0E+5              | 1.1E+6| 4.9E+5 |
| 5969                      | 3.7E+6              | 1.6E+3           | 6.7E+4                    | 7.7E+5              | 6.1E+5| 3.3E+6 |
| 5805                      | 3.5E+6              | 1.1E+3           | 1.9E+5                    | 7.8E+5              | 8.0E+5| 3.3E+6 |
| f5694                      | 3.0E+6              | 4.6E+3           | 8.4E+4                    | 9.4E+5              | 2.9E+5|
| 5695                      | 2.9E+6              | 1.2E+4           | 2.7E+4                    | 3.2E+5              | 1.7E+6| 3.0E+6 |
| 5616                      | 2.7E+6              | 2.3E+4           | 1.4E+4                    | 6.1E+5              | 6.0E+5| 3.5E+6 |
| 5682                      | 1.3E+5              | 8.7E+2           | 3.6E+3                    | 2.7E+5              | 2.5E+5|
| 6158                      | 4.9E+5              | 6.7E+1           | 3.4E+2                    | 2.6E+4              | 7.2E+5| 1.4E+6|
| 5700                      | 4.2E+5              | 6.3E+0           | 7.8E+2                    | 1.8E+4              | 6.2E+4| 5.0E+5|
| 5872                      | 2.5E+5              | 4.3E+2           | 2.1E+2                    | 1.8E+4              | 4.0E+4| 2.4E+5|
| 6161                      | 2.3E+5              | 4.2E+1           | 7.2E+0                    | 2.0E+3              | 7.5E+3| 2.6E+5|
| 5701                      | 8.9E+1              | 0.0E+0           |                           |                     |     |        |
| 5692                      | 0.0E+0              | 0.0E+0           |                           |                     |     |        |

**R mut**

| construct, founder or line | quadriceps (quads.) | ventricle (vent.) | distal tongue (dis. tong.) | diaphragm (diaphr.) | EDL | soleus |
|---------------------------|---------------------|------------------|---------------------------|---------------------|-----|--------|
| 18dd                      | 3.0E+7              | 1.0E+3           |                           |                     |     |        |
| 17dd                      | 7.0E+6              | 8.0E+3           |                           |                     |     |        |
| 20dd                      | 4.0E+6              | 4.0E+3           |                           |                     |     |        |
| 21dd                      | 7.0E+6              | 6.0E+2           |                           |                     |     |        |
| 22dd                      | 3.0E+3              | 2.0E+1           |                           |                     |     |        |

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**Expression as assessed by CAT enzymatic activity (micro units/mg protein) measured in quadriceps (quads.), ventricle (vent.), distal tongue (dis. tong.), diaphragm (diaphr.), extensor digitorum longus (EDL), and soleus. See "Experimental Procedures" for details of constructs and assays.

b Founder indicated by f at the start of the identifying numbers. All others are lines, with mean values from muscles of 2 or more mice.

c Wild-type and R-mutant values have been reported previously (16, 18, 24).

' Except for f12007, 12032, and 12046, L+s+P mut values have been reported previously (18, 19).
FIG. 3. Role of MCK E-boxes in transgenic mouse muscles. Expression of transgenes in quadriceps, extensor digitorum longus (EDL), soleus, ventricle, distal tongue, and diaphragm. Data include previously published results as available, including for wild-type mice, data from Refs. 16, 18, and 24; triple E-box mutant, new data plus data from Refs. 18 and 19; right E-box mutant, all data from Ref. 18; other constructs, all new data. Each data point represents either a founder mouse, or the mean value within a line derived from a single founder, which has been outbred. Constructs are indicated on the bottom of each graph. All constructs are variants of 1256MCKCAT, either wild-type (w.t.) or with E-boxes mutated as indicated by L, R, or P corresponding to the left, right, and promoter E-boxes indicated in Fig. 1. Note that the values are plotted on a logarithmic scale, so that zero values cannot be plotted. Medians, shown by arrows, are calculated from all data including zero values. (See Table I for zero values, and note the greater frequency of these in the L+P and L+R+P constructs; no median value can be plotted for L+R+P expression in distal tongue because the value is zero.)
corrected values are taken at face value. The goal of the corrections is to apply more stringent criteria for accepting a difference such that low \( p \) values appearing by chance are less likely to be accepted as proof of actual differences. However, there are 12 \( p \) values in Fig. 4 from comparisons directly testing the effect of the promoter mutation, either the wild-type versus promoter E-box mutant, or the left E-box mutant versus left-plus-promoter E-box. Of these, 11 values are under 0.05 or just barely above this value (0.051 or 0.052), justifying the conclusion that the low \( p \) values are not merely chance occurrences among a large number of comparisons.

**Effect of Triple E-box Mutation on Transgene Expression in Developing Mice**—Transfection experiments indicated a positive role for both the left and right E-boxes in a permanent skeletal muscle cell line originally derived from adult mouse leg muscle (6, 7, 20). This positive role is not, however, reflected in the effects of these mutations on transgene expression in adult quadriceps, EDL, or soleus muscle (Fig. 6). Even the

###Fig. 4. Statistical tests of MCK E-box mutations in transgenic mice.

Results of statistical comparisons between different transgene constructs are shown for the data displayed in Fig. 3 (including zero values not plotted in Fig. 3). Pairs of constructs were compared using the two-tailed nonparametric Mann-Whitney test for unpaired values; comparisons for each of six muscle types are shown: quadriceps (quad), extensor digitorum longus (EDL), soleus (sol), ventricle (vent), distal tongue (tong), and diaphragm (diaph). \( p \) values are shown, and values indicating statistically significant differences \((p < 0.01)\) are reverse colors (white on black). \( p \) values between 0.01 and 0.05 are shown in shaded boxes. ANOVA (nonparametric Kruskal-Wallis test) was also performed for each of the muscles examined, and the pairs shown to be significantly different by ANOVA \((p < 0.05)\) corresponded exactly to the pairs with \( p \) values \(< 0.01\) in the Mann-Whitney comparison, except for the one value marked with an asterisk (w.t. versus L+R+P in soleus) for which ANOVA revealed no significant difference \((p > 0.05)\). In addition, either the Holm or Bonferroni correction for multiple testing (37) yields \( p \) values \(< 0.05\) for exactly those pairs with \( p \) values \(< 0.01\) in the Mann-Whitney comparison, and for no others.
triply mutated construct exhibits transgene expression levels similar to those of the wild-type construct in quadriceps and EDL (Figs. 3 and 4). We thus hypothesized that the E-boxes might play a role during early muscle development that is not reflected in adult steady-state expression. This was tested by comparing expression of the wild-type and triple E-box mutant 1256MCKCAT constructs in embryos and young mice.

Because there is such wide variation in expression of the same MCK construct between different transgenic lines or founders (Table I and Fig. 3), we normalized early expression to the average adult values for the same anatomical muscle in each transgenic line, using quadriceps to normalize embryonic hindlimb values (Fig. 7). The three wild-type lines indicate the variability of expression, both within a line and between lines. If the hypothesis were correct that the three E-boxes contribute positively to early expression more than they contribute to adult steady-state levels, we would expect the triple mutant construct to show lower early expression than that of the wild-type construct after the normalization. In fact, the values obtained for the triple mutant in developing hindlimb are not reduced (Fig. 7, upper panel), but instead fall mostly within the mid-range to highest levels.

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Fig. 5. Comparison of MCK E-box mutations in slow and fast muscle. Ratios of expression in soleus over EDL were taken for each transgenic founder or line as displayed in the graph at the left. Results of statistical comparisons between the transgene constructs are shown on the right. Pairs of constructs were compared using the two-tailed nonparametric Mann-Whitney test for unpaired values, and p values for each comparison are shown. Pairs yielding highly significant differences are shown in the black boxes, and the shaded box reveals a comparison that is significant at the 0.05 level. Either the Holm or Bonferroni correction for multiple testing (37) yields p values < 0.05 for exactly those pairs with p values < 0.01 in the Mann-Whitney comparison and for no others.

Fig. 6. Summary of effects of mutating conserved MCK E-boxes on expression in transgenic mice and in cell cultures. Thick solid arrows represent effects that are large and statistically significant; thin solid arrows represent effects that are significant but of lower magnitude. Dashed arrows represent differences in median values in two independent comparisons of constructs (wild-type versus L mutant, L+P double mutant versus P mutant, Fig. 3) but which have not yet been shown to be different by statistical tests (Fig. 4). Downward and upward pointing arrows represent decreases and increases in expression, respectively. n.d., not determined. Note that the right E-box mutation was only tested by comparison of the double left-plus-promoter E-box mutant construct with the triple left-plus-right-plus-promoter mutant construct in the cases of EDL, soleus, and distal tongue. Note also that there are many zero values for the triple left-plus-right-plus-promoter E-box mutant expression in distal tongue; these cannot be plotted in Fig. 3, and, therefore, the distribution of values for the double left-plus-promoter E-box mutant looks deceptively similar to the values for the triple mutant. See Table I for the complete data.

The same MCK construct between different transgenic lines or founders (Table I and Fig. 3), we normalized early expression to the average adult values for the same anatomical muscle in each transgenic line, using quadriceps to normalize embryonic hindlimb values (Fig. 7). The three wild-type lines analyzed indicate the variability of expression, both within a line and between lines. If the hypothesis were correct that the three E-boxes contribute positively to early expression more than they contribute to adult steady-state levels, we would expect the triple E-box mutant construct to show lower early expression than that of the wild-type construct after the normalization. In fact, the values obtained for the triple mutant in developing hindlimb are not reduced (Fig. 7, upper panel), but instead fall mostly within the mid-range to highest levels.

As mentioned above, we had no a priori model for expression of MCK transgenes during development. We therefore tested several possible mathematical models by using a runs test (see...
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“Experimental Procedures”). Each of the four transgenic line hindlimb data sets fits well (lack of significant deviation from the model) to a nonlinear regression curve of exponential increase over time, calculated with error weighting of \( 1/\text{expression squared} \). When the rate constants of the curves were compared pairwise using a two-tailed Student’s \( t \) test, differences among the three wild-type lines were not significant \( (p > 0.05) \) in two cases and barely significant \( (p = 0.041) \) in the third comparison. In contrast, comparison of the triple E-box mutant line to each of the wild-type lines showed a significant difference in the rate constants \((p \text{ values } 0.0004, 0.0014, 0.0095)\). These statistical tests confirm that the transgene lacking the three conserved E-boxes has, in fact, higher early expression relative to adult levels than the three wild-type lines tested. Therefore, the hypothesis that the E-boxes collectively contribute to early expression more than to adult expression is not supported for hindlimb skeletal muscles.

The developmental effects of the triple E-box mutation are considerably different in ventricular muscle. Rather than an exponential rise over time, the earliest expression values measured cluster around the adult levels. In hindlimb, three out of four of the lines gave values statistically different from a linear regression as assessed by a runs test, but the data for ventricle are not significantly different from linear for any of the lines tested. Furthermore, as suggested by visual examination of the data (Fig. 7), the slopes for the mutant line and one of the wild-type lines are not significantly different from zero. Although two of the wild-type lines exhibit linear regression fits with positive slopes that are significantly different from zero, these slopes are shallow compared with the rapid increase in transgene expression occurring in hindlimb muscle. With all values normalized to mean adult levels, the ventricular expression of the triple E-box mutant is higher than that of the three wild-type constructs in the several days before birth, and lower in the 2 weeks following birth. However, because only one mutant line was available for developmental analysis, this may be a chance observation. Nonetheless, taken together, these data suggest that transgene expression in the ventricle reaches adult or near adult expression levels quite early and remains fairly constant over development and that the effect of the E-box mutations is similar in embryonic, young, and adult mice.

The developmental study of distal tongue reveals that expression of the triple E-box mutant transgene in this muscle is actually higher in fetal and young mice than in adult mice (Fig. 7). This contrasts with the fetal and young mice in three lines of wild-type mice, which exhibit values generally lower than or similar to adult levels.

In both of the skeletal muscles analyzed in this developmental study, hindlimb and tongue, the data suggest that the predominant effect of eliminating the three E-boxes may be to increase early expression.

DISCUSSION

The specificity of basic helix-loop-helix (bHLH) protein binding to E-boxes was first demonstrated when a myocyte-specific DNA binding activity, MEF1, which bound the right E-box of the MCK enhancer (20), was found to contain the bHLH muscle regulatory factor MyoD and pure MyoD was found to have high affinity for that site (7). Since that time, bHLH proteins and their E-box recognition sites have been recognized as crucial positive regulators of diverse biological processes including cell proliferation and death (42, 43) and the determination and/or differentiation of specialized cells such as skeletal, cardiac and smooth muscle, neurons, T-cells, oligodendrocytes, pancreas, retina, lung, and oocytes (44–55). In addition, bHLH proteins play roles in circadian rhythm and in the response to low oxygen levels (56–58). bHLH proteins and the related HLH Id proteins also play important repressor roles in many cellular processes (43, 59, 60).

As summarized in Fig. 6, the transcriptional consequences of mutating the conserved E-boxes of the mouse MCK promoter depend on the test system with very different results in transgenic mouse muscles versus muscle cell cultures. To determine whether these differences were caused by the integration state of transgenes, the skeletal muscle cell line MM14 was transfected and selected for pools of clones containing stably integrated wild-type or E-box mutated plasmids. The effects of mutations were similar to those observed in transiently transfected cell cultures (Fig. 2),4 revealing that the integration status of test genes is not responsible for the differences between transgenic and cell culture data. Differences relating to the transgenic mouse and cell culture test systems might also be attributed to the earlier stage in differentiation of cultures versus adult transgenic mouse muscles. However, our test of this hypothesis by measuring transgene expression in embryonic and young mice did not support such an explanation (Fig. 7). Because mutation of the left E-box appears to increase expression in adult skeletal muscle (Figs. 3 and 6), perhaps mutation of the left E-box also increases expression early in the development of both skeletal muscles examined, outweighing any potential negative effects of mutation of the right and promoter E-boxes. However, a pilot study of expression during mouse development yielded no significant differences in the onset of reporter expression between a construct with the right E-box mutated and a wild-type construct.5 It seems likely that differences between the expression of MCK gene enhancer and promoter constructs in cultured cells and transgenic animals are caused by signals from other cell types in the whole animal and/or to the effects of coordinated muscle contractions which are absent in cell cultures.

Our results show clear differences in the roles of the three conserved MCK E-boxes in muscle. In adult mice, transgenes containing the promoter E-box mutation are expressed at significantly reduced levels relative to the wild-type transgenes in all the muscles tested. This result was quite unexpected because no significant decrease was detected in cultured skeletal or cardiac myocytes. In contrast, the right E-box mutation caused a significant decrease in MCK transgene expression only in the distal tongue muscle of transgenic mice, whereas this mutation greatly reduced expression in cultured skeletal myocytes and myocardocytes. The left E-box mutation decreased expression in cultured skeletal muscle and in myocytes (Fig. 2 and Ref. 6) but increased expression in the soleus, the mouse muscle containing the highest proportion of slow fibers. Differential fiber-type transgene expression effects as a result of an E-box mutation have also been reported by Yan et al. (61), who found that mutation of an E-box in the 5′ untranslated region within mouse myoglobin exon-I increased the ratio of expression in the primarily fast fiber vastus lateralis muscle compared with the soleus.

The MCK enhancer left E-box is a weak binding site for bHLH proteins including MyoD and MyoD/E12/47 heterodimers (7, 62), but a DNA-binding factor with preference for the left E-box over the right E-box has been found in skeletal muscle nuclear extracts (63). This factor could be a repressor that is responsible for the negative role the left E-box exhibits in adult soleus muscle.

A number of regulatory elements have been identified as playing different roles in fast versus slow skeletal muscle in-

4 D. L. Gregory and S. D. Hauschka, unpublished results.
5 D. B. Donoviel and S. D. Hauschka, unpublished results.
including the rat “SURE” and quail “FIRE” clusters in slow and fast troponin I isoforms (64, 65). The E-box of SURE is required for high expression in cultured myotubes and in transgenic mouse muscle, but it is not responsible for differences between slow troponin I expression in slow and fast fiber types as evidenced by the continued preferential expression in soleus when the SURE E-box is replaced with the FIRE E-box (64). A fast fiber enhancer has also been reported in the upstream region of the rat MRF4 gene promoter but the elements responsible for fiber-type specificity were not analyzed (66).

There have been several reports that MRFs vary in their expression between skeletal muscle fiber types. For example, bovine muscles that are abundant in slow and intermediate myosin heavy chains have more MyoF than do muscles with faster myosin heavy chains (67). MyoD has been reported to be highest in the fastest muscle fibers in mouse (68, 69), whereas myogenin has been reported to be high in slow muscles (70). MRF4 has been suggested as important in maintaining the slow muscle phenotype (71, 72), which makes identification of an enhancer in its upstream region with specificity for fast fibers (as mentioned above; Ref. 66) intriguing. These differences in MRF abundance suggest that MRFs may play a direct role in modulating the expression of muscle genes in different fiber types. However, it has not yet been possible to correlate the sequence differences between the MCK enhancer-promoter E-boxes, the effects of mutating them in slow and fast muscle, or the MRF abundance in different fiber types with the preferential binding of these factors.

Analysis of many muscle genes reveals differences in the importance of specific control elements for their expression in heart or skeletal muscle. For example, in the MCK gene upstream enhancer, the CArG site is more important for expression in skeletal muscle (7, 20, 73), they are also expression in cultured myocardiocytes than in cultured skeletal myocytes (74), including the rat MRF4 gene promoter but the elements responsible for high expression in cultured myotubes and in transgenic mice (75). Because the skeletal muscle MRFs are not present in cardiac muscle, other factors must be responsible for the role of E-boxes in heart. Known cardiac E-box binding factors include the bHLH factors eHAND and dHAND (45, 46), and the PHD finger upstream stimulatory factor-1 (87).zipper factor upstream stimulatory factor-1 (87).
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Differences in the Function of Three Conserved E-boxes of the Muscle Creatine Kinase Gene in Cultured Myocytes and in Transgenic Mouse Skeletal and Cardiac Muscle
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