Molecular and Cellular Biology, Apr. 1996, p. 1649–1658 Vol. 16, No. 4
0270-7306/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Analysis of Muscle Creatine Kinase Gene Regulatory Elements in Skeletal and Cardiac Muscles of Transgenic Mice

DORIT B. DONOVIEL,[1]† MARGARET A. SHIELD,[1] JEAN N. BUSKIN,[1] HARALD S. HAUGEN,[2] CHRISTOPHER H. CLEGG,[2] and STEPHEN D. HAUSCHKA[1]*

Department of Biochemistry, University of Washington, Seattle, Washington 98195-7350,[1] and Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, Washington 98121[2]

Received 1 November 1995/Returned for modification 6 December 1995/Accepted 9 January 1996

Regulatory regions of the mouse muscle creatine kinase (MCK) gene, previously discovered by analysis in cultured muscle cells, were analyzed in transgenic mice. The 206-bp MCK enhancer at nt −1256 was required for high-level expression of MCK-chloramphenicol acetyltransferase fusion genes in skeletal and cardiac muscle; however, unlike its behavior in cell culture, inclusion of the 1-kb region of DNA between the enhancer and the basal promoter produced a 100-fold increase in skeletal muscle activity. Analysis of enhancer control elements also indicated major differences between their properties in transgenic muscles and in cultured muscle cells. Transgenes in which the enhancer right E box or CArG element were mutated exhibited expression levels that were indistinguishable from the wild-type transgene. Mutation of three conserved E boxes in the MCK 1,256-bp 5′ region also had no effect on transgene expression in thigh skeletal muscle expression. All of these mutations significantly reduced activity in cultured skeletal myocytes. However, the enhancer AT-rich element at nt −1195 was critical for expression in transgenic skeletal muscle. Mutation of this site reduced skeletal muscle expression to the same level as transgenes lacking the 206-bp enhancer, although mutation of the AT-rich site did not affect cardiac muscle expression. These results demonstrate clear differences between the activity of MCK regulatory regions in cultured muscle cells and in whole adult transgenic muscle. This suggests that there are alternative mechanisms of regulating the MCK gene in skeletal and cardiac muscle under different physiological states.

The muscle creatine kinase (MCK) gene is transcriptionally activated during striated muscle differentiation and is expressed at high levels in adult heart and skeletal muscles (11, 39). Previous cell culture analyses of MCK gene regulation have implicated both a 5′ muscle-specific enhancer (bp −1256 to −1050) and the adjacent 1-kb region of DNA (bp −1049 to +7) as playing important roles in expression of the MCK gene in skeletal and cardiac muscle (24, 29, 63). The MCK enhancer contains a number of conserved DNA motifs, which are also found in the regulatory regions of many other muscle-specific genes (reviewed in references 12, 22, and 49). These motifs bind *trans*-acting factors in vitro and are critical control elements in cultured muscle cells (2, 3, 8, 9, 24). The MCK enhancer sites include E boxes, which contain the core consensus binding sequence CANNTG for the myogenic basic helix-loop-helix (bHLH) proteins (MyoD, Myf-5, myogenin, and MRF-4) (for reviews, see references 17, 22, 49, and 72); a CArG element, containing the consensus serum response factor-binding sequence $CC(A/T)_6GG$ (68); and an AT-rich site, which has been shown in gel shift assays to bind ubiquitously expressed factors (1, 15a, 25), as well as MHox and MEF-2 (14). The 1-kb region of DNA between the enhancer and the basal promoter exhibits low-level activity in cultured skeletal myocytes and cardiomyocytes (2).

Mutation of each of the MCK enhancer sites results in altered expression of MCK constructs in cultured muscle cells but to different extents in skeletal myocytes and cardiomyocytes (2). E boxes are generally thought to be more important regulatory elements in skeletal muscle, where the myogenic bHLH factors are expressed, than in cardiac muscle, where these factors are not found. Mutation of the right E box (also called the MEF1 site) in the MCK enhancer has a more deleterious effect in cultured skeletal muscle cells than in primary rat cardiomyocytes. In contrast, CArG elements are critical for the regulation of a number of genes in cardiac muscle (12, 41, 52), as well as skeletal muscle (38, 43, 46, 71). Mutation of the MCK enhancer CArG site has a greater effect in cultured cardiomyocytes than in cultured skeletal myocytes. Another element, the AT-rich site in the MCK enhancer, seems to be important for transcription in both striated muscle types.

An initial study of MCK gene regulation in transgenic mice confirmed the importance of the 206-bp enhancer in striated muscle expression (30). Results from this earlier transgenic study also suggested that MCK sequences between the enhancer and the basal promoter were active in skeletal muscle but inactive in cardiac muscle. Combination of the enhancer and this 1-kb region appeared to confer no greater activity in either muscle type than did the enhancer alone. However, these conclusions were tentative because of the broad range of transgene activity observed in different lines of transgenic mice, presumably as a result of different sites of transgene integration (51), and the relatively small number of transgenic animals assayed.

The current study undertakes a further analysis of the regulatory role of the MCK enhancer and the 1-kb region of DNA between the enhancer and the basal promoter in transgenic muscle. The 1-kb region appears to play a more important role in regulation of the MCK gene than was suspected from cell culture analyses. We also examined the activity in adult skeletal and cardiac muscle of several MCK enhancer control elements which are binding sites for *trans*-acting factors. In contrast to their activity in cultured muscle cells, the CArG and

* Corresponding author. Mailing address: Department of Biochemistry, Box 357350, University of Washington, Seattle, WA 98195-7350. Phone: (206) 543-1797. Fax: (206) 685-1792.

† Present address: Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario M5G 1X5, Canada.

E-box elements were not critical for transgene activity. However, the MCK enhancer AT-rich element, which is a potential binding site for several transcription factors, is essential for enhancer function in skeletal muscle.

MATERIALS AND METHODS

MCK-CAT constructs. All constructs examined consist of mouse MCK 5′-flanking sequences in their native orientation fused to the 1.6-kb chloramphenicol acetyltransferase (CAT) structural gene-simian virus 40 small t intron-poly(A) sequence cassette (20) (Fig. 1A). 1256MCK-CAT (29), 1020MCK-CAT (29), and 117MCK-CAT (30) were constructed as previously described. enh117MCK-CAT and enh80MCK-CAT are constructs containing the 206-bp MCK enhancer linked to the MCK basal promoter, which is DNA sequences either from bp −117 to −1 (mice 43 and 44) (equivalent to E1-117MCK-CAT described by Johnson et al. [30]) or from bp −80 to +7 (mice 40 to 42) [equivalent to (+enh206)80MCK-CAT described by Amacher et al. (2)].

Constructs containing single mutations in enhancer elements in the context of 1256MCK-CAT are as previously described (2); sequences of wild-type and mutated sites are shown in Fig. 1B. The 1256[3E mut]MCK-CAT construct, which harbors mutations in three conserved E-box sites (at nucleotides [nt] −1178, −1153, and −249), was constructed by isolation of a *Hin*dIII-*Bam*HI fragment consisting of MCK sequences from bp −1256 to −1050 containing mutations in the left E box (Left mut 1) and right E box (MEF1 mut 1) from a mutated (+enh206)80MCK-CAT construct (2). Wild-type MCK sequences from bp −1050 to −492 were isolated from a partial *Bam*HI-*Nhe*I digestion of 1256MCK-CAT. These two fragments were sticky-end ligated into a *Hin*dIII-*Nhe*I-digested 776MCK-CAT vector (29) harboring a mutation in the E-box site at nt −249, regenerating the *Bam*HI site at nt −1050 and the *Nhe*I site at nt −492. The enh[right E mut]117MCK-CAT and enh[right E mut]80MCK-CAT constructs, containing the 206-bp enhancer harboring a mutation in the right E box linked to the MCK promoter, either bp −117 to −1 (mice 50 to 57) or bp −80 to +7 (mice 45 to 49), were formed from ligation of a blunt-ended *Sph*I-*Bam*HI fragment of the mutated pUC-E vector into *Sal*I-cut, blunt-ended 117MCK-CAT or ligation of a blunt-ended *Hin*dIII-*Bam*HI fragment of the right E box-mutated pUC-E vector (9) into *Sal*I cut, blunt-ended 80MCK-CAT, respectively. Correct initiation of transcription for the 1256MCK-CAT and enh117MCK-CAT constructs were confirmed by RNase protection analysis (31).

Production of transgenic mice. MCK-CAT sequences for microinjection were prepared such that only minimal polylinker sequences remained. In most cases, transgenes were separated from vector sequences by sucrose gradient centrifugation. The appropriate fractions were dialyzed against injection buffer (10 mM Tris-acetate [pH 7.8], 0.25 mM EDTA) and then precipitated with ethanol. Transgenes consisting of the enh117MCK-CAT, enh80MCK-CAT, enh[right E mut]117MCK-CAT, enh[right E mut]80MCK-CAT, and 1256[3E mut]MCK-CAT sequences were isolated by agarose gel electrophoresis followed by transfer to NA45 paper (Schleicher & Schuell) and further purification on Elutip columns (Schleicher & Schuell) as specified by the manufacturer. Most of the transgenic mice were derived from eggs of C57BL6/C3H F_1 matings by standard techniques (23). Founder mice 40, 42, 45 to 49 (see Table 1) were derived from C57BL6/DBA F_1 matings. Founder animals were identified by hybridization of tail DNA dots to a nick-translated ^{32}P-labeled CAT gene fragment. Some founder mice were outbred with DBA2 mice to generate lines (mice 18, 40, 45, and 46). No differences in transgene expression were apparent between different mouse strains. Founder animals were at least 4 weeks old when analyzed.

Copy number determination. High-molecular-weight genomic DNA was extracted from tissues digested with proteinase K and processed by standard techniques (23). Genomic DNA (20 μg), prepared for diagnostic Southern analysis as previously described (42), was cut with *Pvu*II and analyzed by Southern blots with Zeta-probe membranes (Bethesda Research Laboratories). A nick-translated ^{32}P-labeled *Bam*HI-*Hin*dIII fragment of pUC-E (9) containing the 206-bp MCK enhancer or a fragment containing MCK sequences from bp −238 to +1, was used as a probe to identify both transgene and endogenous MCK bands. For transgenes lacking the enhancer, a probe consisting of the *Eco*RI fragment of the CAT gene was used to detect the transgene band and a probe consisting of a 700-bp *Stu*I-*Bgl*II fragment from the mouse metallothionein I gene (75) was used for comparison with a single-copy endogenous gene. Identities of mutated 1256MCK-CAT transgenes were verified by diagnostic Southern analysis of at least one founder animal from each injection set, using the novel restriction site created by each mutation (16, 60).

Tissue extracts and CAT assays. Protein extracts were made from cardiac ventricles, thigh muscle (composed of the quadriceps and superficial gluteal muscles), liver, and kidney. Tissues were minced with a razor blade, homogenized in 250 mM Tris (pH 7.8)–5 mM EDTA, and then processed as previously described (30). Soluble protein concentrations were determined by Bradford assays (7). CAT assays were performed essentially as previously described (9). CAT standards were run in parallel with samples to ensure that activities were within the linear range of the assay. Counts per minute (cpm) measured from [*acetyl*-^{14}C]chloramphenicol in the CAT assay were converted to microunits of CAT activity, where 1 unit is the activity sufficient to acetylate 1 micromole of chloramphenicol per min at 37°C (59). Samples which exhibited less than twice the background cpm in the CAT assay are indicated as <0.2 μU/mg of protein in Table 1.

Statistical analysis. Statistical comparisons between transgenic data sets were performed by the Wilcoxon rank sum test (55). This procedure is the nonparametric analog of the *t* test for two independent samples, which is appropriate for analysis of data sets in which large variances prohibit a useful comparison of mean values. The test is based on the rank value of each observation within two data sets rather than the actual value. The sum of ranks for one of the data sets is compared with a table of critical values (55), which is based on the number of observations in each data set; this table specifies a *P* value for the null hypothesis that the data sets being compared are from the same distribution. A *P* value less than 0.05 was used as a criterion to reject the null hypothesis. In cases in which CAT activities below background levels (<0.2 μU/mg of protein) made up more than 25% of the data being compared, the χ^2 and Fisher exact tests (55) were used to compare the number of mice expressing the transgene with the number of nonexpressing mice. *P* values for statistical comparisons are indicated in the figures or text; except where noted, *P* values were based on the Wilcoxon rank sum test.

RESULTS

Muscle-specific expression of MCK-CAT transgenes. Transgenic mice carrying wild-type or mutated 5′-flanking regions of the mouse MCK gene were generated by using the nine MCK-CAT fusion genes illustrated in Fig. 1A. Individual founder mice, or multiple mice from transgenic lines, were analyzed for transgene activity (reported as microunits of CAT activity per milligram of protein) in thigh skeletal muscle, cardiac muscle, and nonmuscle tissues (liver data reported). Data for each of the 69 transgenic mice or lines are organized in order of decreasing CAT activity in skeletal muscle for each transgene (Table 1).

Nine founder animals carrying the wild-type 1256MCK-CAT transgene exhibited high-level CAT activity in skeletal and cardiac muscle but extremely low activity in liver or other nonmuscle tissues (Table 1). We observed a broad range of transgene activity (>100-fold) for different founder mice and different transgenic lines, which is thought to result from genomic effects at the sites of transgene integration (27, 51). No correlation between transgene copy number and CAT activity levels was observed. Because of the large range of transgene activity from mouse to mouse, we compared the activities of different transgenes by the Wilcoxon rank sum test. This statistical test utilizes data from all the animals rather than relying on the mean activity value for each data set. Additionally, relatively large numbers of different mouse lines or founder animals were analyzed for each transgene so that line-to-line variations in transgene activity would be less likely to affect data analysis. As a result of this analysis, several comparisons in this study clarify the activity of MCK regulatory regions which were examined in our earlier transgenic study (30).

MCK sequences in the 1-kb region between the enhancer and basal promoter act in concert with the 206-bp enhancer to produce high-level muscle-specific expression. In our previous transgenic-mouse study, comparison of activities of the 1256MCK-CAT transgene and a transgene in which the 206-bp MCK enhancer was linked to a basal promoter (enh117MCK-CAT or enh80MCK-CAT) (Fig. 1A) suggested that the two fusion genes had about the same level of activity in both skeletal and cardiac muscle (30). However, a significant difference between their activities became apparent when more independently generated mice carrying these two transgenes were analyzed (Fig. 2). The activity of the enhancer linked directly to the basal promoter was significantly lower and has a greater variance than that of 1256MCK-CAT in thigh skeletal muscle (P = 0.002) and in cardiac muscle (P = 0.02). These data suggest that sequences in the 1-kb MCK region between the enhancer and the basal promoter are important for high-level

A

MCK 5' region: -1256 [CArG | A/T-rich | Left | Right] -1050 ... -249 [E-box] ... -117 ... exon 1

206 bp 5' enhancer — 1 Kb region

transgene

1256MCK-CAT

1020MCK-CAT (-1020)

117MCK-CAT (-117)

1256[Right E mut]MCK-CAT

1256[3E mut]MCK-CAT

1256[CArG mut]MCK-CAT

1256[A/T mut]MCK-CAT

enh117MCK-CAT
enh80MCK-CAT (-117 or -80)

enh[Right E mut]117MCK-CAT
enh[Right E mut]80MCK-CAT (-117 or -80)

B

-1256 CArG
CCACTACGGGTCTAGGCTGC**CCATGTAAGG**AGGCAAGGCCTGGGGACACCCGAGATGCC
G G CC

A/T-rich Left E box Right E box
TGG**TTATAATTAA**CCCAGA**CATGTG**GCTGCCCCCCCCCCCCCAA**CACCTG**CTGCCTGAG
C GA CT TCT GA GGTAAC

CCTCACCCCCACCCCGGTGCCTGGGTCTTAGGCTCTGTACACCATGGAGGAGGAAGCTCG

-1050
CTCTAAAAATAACCCTGTCCCTGGTGGATCC. . . . .

-261 Proximal E box
. . . . .CCGGCGAAGGGC**CAGCTG**TCCCCCGCCA
GGTAA

FIG. 1. Diagram of MCK-CAT transgenes used to generate transgenic mice (A) and sequences of mutations introduced into MCK regulatory elements (B). See Materials and Methods for a description of the construction of MCK-CAT transgenes. All MCK 5′-flanking sequences are in their native 5′-to-3′ orientation. Solid boxes indicate elements which have been mutated. Mutated sequences are shown beneath the wild-type mouse sequence in panel B. Several mutations are the same as those described by Amacher et al. (2), with the following names: the right E-box mutation is MEF1 mut1, the CArG mutation is CArG mut 1, the AT-rich mutation is A/T mut 2, and the left E-box mutation used in 1256[3E mut]MCK-CAT is the same as Left mut 1.

TABLE 1. CAT activity in muscle and nonmuscle tissues of transgenic mice carrying wild-type and mutated enhancer and promoter regions of the mouse MCK gene[a]

Transgene[a]	Founder[b] or line	CAT activity (μU) per mg of protein[c] in:		
		Skeletal muscle	Cardiac muscle	Liver
Wild type				
1256MCK-CAT ($n = 9$)	1	6.0×10^{6}	8×10^{3}	20
	14266	1.6×10^{6}	8.0×10^{2}	27
	2	1.0×10^{6}	1.5×10^{3}	80
	14233	4.8×10^{5}	1.3×10^{4}	1
	3	2.0×10^{5}	20	10
	14227	1.3×10^{5}	9.6×10^{2}	0.8
	4	7.0×10^{4}	5	0.2
	5	6.0×10^{4}	10	0.2
	14324	5.9×10^{4}	10	0.3
1020MCK-CAT ($n = 7$)	6	2×10^{4}	<0.2[d]	0.6
	7	1.5×10^{3}	1.0×10^{3}	<0.2
	8	8.0×10^{2}	<0.2	<0.2
	9	2.0×10^{2}	<0.2	<0.2
	10	16	<0.2	<0.2
	11	16	<0.2	<0.2
	12	14	<0.2	<0.2
117MCK-CAT ($n = 5$)	13	0.4	<0.2	<0.2
	14	<0.2	<0.2	<0.2
	15	<0.2	<0.2	<0.2
	16	<0.2	<0.2	<0.2
	17	<0.2	<0.2	<0.2
enh117MCK-CAT[d] and enh80MCK-CAT ($n = 5$)	40	$(1 \pm 1) \times 10^{3}$[e,f]	11 ± 7	<0.2
	41	8.0×10^{2}	<0.2	<0.2
	43	25	3.1	<0.2
	42	20	0.6	0.3
	44	2.5	<0.2	<0.2
Mutated				
enh[Right E mut]117MCK-CAT and enh[Right E mut]80MCK-CAT ($n = 13$)	50	3.0×10^{4}	20	0.2
	51	1.0×10^{4}	2	<0.2
	52	1.0×10^{4}	0.7	<0.2
	53	3.0×10^{3}	5	3.0
	54	7.0×10^{2}	1.4	<0.2
	45	$4.0 \times 10^{2} \pm 20$	2.0 ± 2.0	<0.2
	55	3.0×10^{2}	2.5	<0.2
	46	$3.0 \times 10^{2} \pm 30$	0.5	<0.2
	47	2.0×10^{2}	<0.2	<0.2
	56	1.0×10^{2}	0.7	<0.2
	57	65	0.2	<0.2
	48	17	1.6	<0.2
	49	0.5	<0.2	<0.2
1256[CArG mut]MCK-CAT ($n = 10$)	23	2.0×10^{6}	1.0×10^{3}	30
	24	1.0×10^{6}	4.0×10^{2}	80
	25	1.0×10^{6}	1.6×10^{2}	10
	26	7.5×10^{5}	1.7×10^{2}	1.0×10^{2}
	27	7.5×10^{5}	10	4
	28	4.0×10^{5}	70	5
	29	2.0×10^{5}	40	2
	30	7.0×10^{4}	4	10
	31	5.0×10^{4}	5	0.2
	32	1.7×10^{4}	0.2	0.2
1256[Right E mut]MCK-CAT ($n = 5$)	18	$(3.0 \pm 3.0) \times 10^{7}$[f]	$(1.0 \pm 1.0) \times 10^{3}$[f]	1.0×10^{2}
	19	7.0×10^{6}	8.0×10^{3}	1.0×10^{2}
	20	4.0×10^{6}	4.0×10^{3}	2.0×10^{2}
	21	7.0×10^{5}	6.0×10^{2}	40
	22	3.0×10^{3}	0.2	0.2

Continued on following page

TABLE 1—*Continued*

Transgene[a]	Founder[b] or line	CAT activity (μU) per mg of protein[c] in:		
		Skeletal muscle	Cardiac muscle	Liver
1256[3E mut]MCK-CAT ($n = 8$)	11995	1.6×10^6	NS[g]	10
	12073	1.3×10^6	NS	24
	12075	6.3×10^5	NS	5
	12070	4.7×10^5	NS	1
	30073	1.4×10^5	NS	0.5
	12023	6.8×10^4	NS	<0.2
	12005	3.3×10^3	NS	<0.2
	12056	3.1×10^2	NS	<0.2
1256[A/T mut]MCK-CAT ($n = 7$)	33	3.0×10^3	10	<0.2
	34	1.0×10^3	3.0×10^2	2.0×10^2
	35	3.0×10^2	0.2	<0.2
	36	1.0×10^2	20	<0.2
	37	50	2.0×10^2	<0.2
	38	10	10	<0.2
	39	<0.2	<0.2	<0.2

[a] See Fig. 1 for transgene descriptions.
[b] Copy numbers ranged from 1 to 40, with the exception of one mouse (mouse 13), which had a copy number of approximately 1,000. No correlation between copy number and CAT activity level was observed.
[c] One unit of CAT activity is the amount sufficient to acetylate 1 μmol of chloramphenicol per min at 37°C. Data for three transgenic lines carrying the 1256MCK-CAT transgene and eight lines carrying the enh117MCK-CAT transgene which were previously published (30) are not shown here but are presented in Fig. 2 to 4, with distinguishing markers as indicated in the figure legends.
[d] Activities lower than 1 μU/mg are consider below background.
[e] For activities in which standard deviations are reported, at least three animals were assayed for each line of mice. All other values are from founder mice.
[f] Mean CAT activities for these mice were equal to the standard deviations.
[g] NS, data which are reported elsewhere (60).

expression of the MCK gene in skeletal and cardiac muscle in vivo.

MCK sequences between bp −1020 and −118 confer skeletal muscle-specific but not cardiac muscle-specific expression. The results described in the previous section concur with those from transient transfections of the same MCK gene regions in cardiomyocytes (2); however, the results obtained with transgenic mice differ from those in studies of cultured skeletal muscle cells, in which the 1 kb of DNA between the enhancer and basal promoter was found to be nonessential for enhancer activity (2, 29). To directly test this regulatory region, we generated mice with a 1020MCK-CAT transgene (Fig. 1A).

Mice carrying the 1020MCK-CAT transgene exhibited substantial CAT activity in skeletal muscle but at a significantly lower level than that of mice carrying the 1256MCK-CAT transgene ($P < 0.01$) (Fig. 2A; Table 1). The activity of 1020MCK-CAT in skeletal muscle appeared to be similar to that of transgenes in which the enhancer is linked directly to the basal promoter ($P > 0.10$) (Fig. 2A). In cardiac muscle, however, 1020MCK-CAT was inactive (Fig. 2B). The five animals carrying a transgene consisting of only the TATA box-containing promoter region (117MCK-CAT) exhibited no CAT activity in any tissue type examined (Table 1; Fig. 2). These results suggest that MCK sequences between bp −1020 and −117 contain skeletal muscle-specific elements. These data are in agreement with the earlier transgenic analysis (30) in which a transgene containing MCK sequences from bp −723 to +7 exhibited activity similar to that of the 1020MCK-CAT transgene reported here. The combined data from the 1256MCK-CAT, 1020MCK-CAT, and enh117MCK-CAT/enh80MCK-CAT transgenes indicate that interaction(s) between the enhancer and 1-kb region results in at least 10-fold-higher transcriptional activity than the sum of their individual activities in either striated muscle type.

The MCK enhancer CArG and right E box sites are not critical for transgene expression in skeletal and cardiac muscle. A number of individual elements within the MCK enhancer (such as E-box and CArG sites) are found in the control regions of many muscle-specific genes and appear to be important for transcriptional activity of the MCK gene in transiently transfected skeletal myocytes and primary cardiomyocytes (2, 3, 9). To examine the role of these elements in regulating the MCK gene in vivo, we analyzed the activities of 1256MCK-CAT transgenes harboring individual mutations in the enhancer CArG or right E box (Fig. 1). In contrast to their activities in cell culture analysis, mutation of either site did not dramatically reduce expression of the transgenes. Both the mutated 1256MCK-CAT transgenes exhibited activity levels in thigh skeletal muscle and in heart muscle which were statistically indistinguishable from that of the wild-type transgene ($P > 0.10$ for all comparisons) (Fig. 3; Table 1). The range of transgene activities exhibited by different founder animals carrying wild-type or mutated transgenes may have obscured small differences in transcriptional activity; however, these specific mutations did not dramatically alter transgene expression.

To determine whether the right E-box mutation might be more deleterious in the absence of cooperative transcriptional activity provided by the 1-kb upstream regulatory region, we examined the same mutation in the context of the enh117MCK-CAT and enh80MCK-CAT transgenes (Fig. 1A). This seemed a reasonable possibility because in cultured skeletal muscle cells, the effect of mutating the right E-box site is even larger in the context of the enhancer linked to the basal promoter (100-fold) than in the context of the 1256MCK-CAT construct (33-fold) (2). However, in transgenic mice, the activity of the wild-type and right E-box-mutated transgenes in which the enhancer was linked directly to the basal promoter was statistically identical in both thigh skeletal muscle and heart muscle ($P > 0.20$ for both comparisons) (Fig. 3; Table 1). Mutation of the right E-box site, which is a critical control element for high-level expression in skeletal muscle cultures, thus has no effect on steady-state expression of the transgene in thigh skeletal muscle.

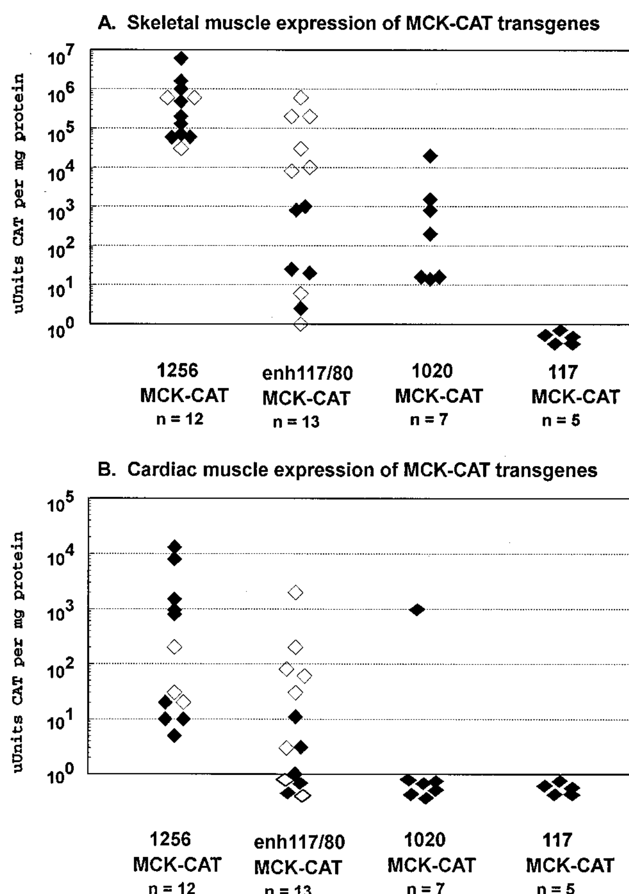


FIG. 2. Expression of MCK-CAT transgenes in skeletal (A) and cardiac (B) muscle. Each diamond represents CAT activity measured from an individual founder mouse (or line) in the tissue indicated. Open diamonds represent data previously reported by Johnson et al. (30). These values were adjusted down 10-fold to correct for a mathematical error that was included in all previously published values (30). Activities are reported in microunits of CAT per milligram of protein (note that the abscissa is in log units). enh117/80MCK-CAT denotes the enh117MCK-CAT and enh80MCK-CAT transgenes. Statistical comparison of activity of each transgene with that of 1256MCK-CAT gave the following P values: $P = 0.002$ for enh117/80MCK-CAT in skeletal muscle, $P = 0.02$ for enh117/80MCK-CAT in cardiac muscle, and $P < 0.01$ for 1020MCK-CAT in both muscle types. Comparison of the activity of 1020MCK-CAT and 117MCK-CAT in skeletal muscle yields $P < 0.01$.

Simultaneous mutation of three conserved MCK E-box sites has no apparent effect on skeletal muscle expression of a 1256MCK-CAT transgene. The MCK enhancer contains two adjacent control elements containing the CANNTG consensus sequence, called the left and right E boxes. It was therefore conceivable that any effect of mutating the right E box was compensated by the presence of the left E box. In addition to the enhancer E boxes, a third E box at nt −249 is conserved in MCK 5′ sequences from mice (28), rats (24), rabbits (78), and humans (67); therefore, it was possible that the E box at nt −249 also compensated for the right E-box mutation. This hypothesis was examined by construction of a MCK-CAT fusion gene containing simultaneous mutations in all three conserved E boxes (1256[3E mut]MCK-CAT [Fig. 1A]). This construct had virtually no activity when tested in MM14 skeletal muscle cultures (data not shown). Surprisingly, mutation of all three conserved E-box sites did not affect expression of the transgene in thigh skeletal muscle of transgenic mice ($P > 0.10$) (Fig. 3A; Table 1). Of 8 founder animals, 6 exhibited CAT activity at the same level as the 12 wild-type 1256MCK-CAT mice, and all the 1256[3E mut]MCK-CAT mice exhibited significant levels of CAT expression in thigh skeletal muscle. Thus, in contrast to its behavior in cultured skeletal muscle cells, steady-state activity of the MCK enhancer in adult skeletal muscle is not dependent on these bHLH factor-binding sites. Interestingly the transgene containing three E-box mutations was largely inactive in heart muscle (reference 61 and data not shown).

Mutation of the MCK enhancer AT-rich site reduces transgene activity in skeletal muscle but not in cardiac muscle. As the right E box and CArG element were not critical for MCK enhancer activity in adult skeletal muscle, we examined whether the AT-rich site was required. In transiently transfected muscle cultures, mutation of the MCK enhancer AT-rich element produces a significant loss of transcriptional activity in both skeletal myocytes and cardiomyocytes (2). Transgenic analysis of the same AT-rich site mutation in the 1256MCK-CAT context (Fig. 1A) showed a significant loss of transcriptional activity in thigh skeletal muscle ($P < 0.01$) in comparison with the wild-type 1256MCK-CAT transgene (Fig. 4A). How-

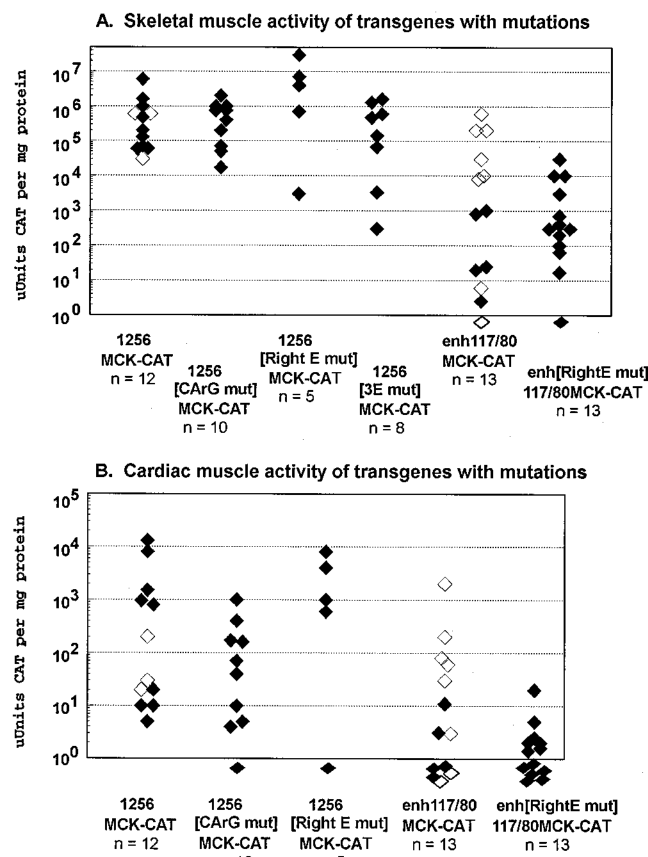
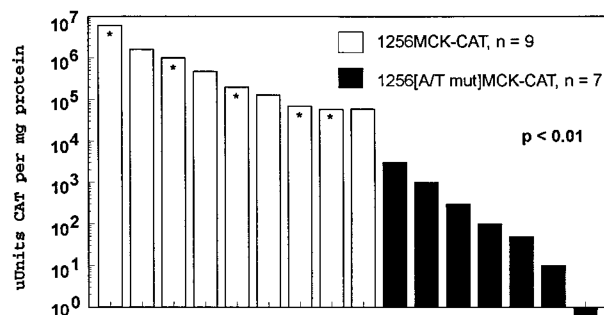


FIG. 3. Activity of MCK-CAT transgenes with mutations of putative regulatory elements in skeletal muscle (A) and cardiac muscle (B). Each diamond represents CAT activity measured from an individual founder mouse (or line) in the tissue indicated. Open diamonds represent data reported by Johnson et al. (30). enh117/80MCK-CAT and enh[Right E mut]117/80MCK-CAT denote the wild-type and right E-box mutated enh117MCK-CAT and enh80MCK-CAT transgenes, respectively. Statistical comparison of activity of the CArG or E-box mutated 1256MCK-CAT transgenes with that of the wild-type 1256MCK-CAT yielded $P > 0.10$ in both muscle types for all transgenes. Comparison of activities of the right E-box mutated and wild-type enh117/80MCK-CAT transgenes also yields $P > 0.20$ in both muscle types.

A. Skeletal muscle activity of transgene with A/T-rich site mutation



B. Cardiac muscle activity of transgene with A/T-rich site mutation

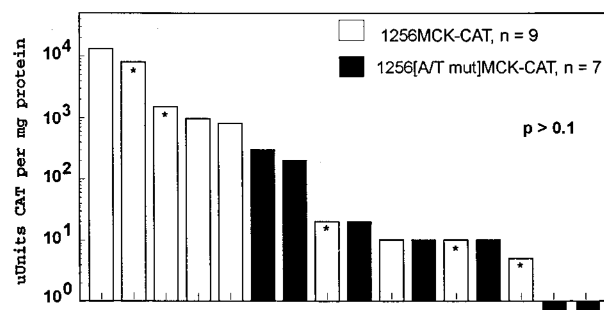


FIG. 4. Effect of mutation of the MCK enhancer AT-rich site on transgene expression in skeletal (A) and cardiac (B) muscle. Each bar represents CAT activity measured for each founder animal or transgenic line in the tissue indicated. Activities for mice carrying the wild-type 1256MCK-CAT transgene are indicated by open bars, and activities for mice carrying the AT-rich-site mutant transgene are indicated by solid bars. 1256MCK-CAT bars with asterisks are values from transgenic mice analyzed by Johnson et al. (30). $P < 0.01$ for comparison of activities of these two transgenes in skeletal muscle, whereas in cardiac muscle $P > 0.10$.

ever, mutation of the MCK enhancer AT-rich site did not result in a dramatic effect on transgene expression in cardiac muscle (Fig. 4B). Although several of the mice carrying the wild-type transgene exhibited higher CAT activity than did any of the mice carrying the AT-rich site-mutated transgene, statistical analysis of the data indicated that cardiac expression of the two transgenes was indistinguishable ($P > 0.10$). The AT-rich mutated transgene retains skeletal muscle-specific expression (Table 1); however, this tissue specificity could be due to activity of sequences outside the enhancer because the activity of the AT-rich mutated transgene was at the same level as that of the 1020MCK-CAT transgene ($P > 0.10$), which lacks the entire enhancer region.

DISCUSSION

Activity of MCK enhancer and 1-kb 5′-flanking region in skeletal and cardiac muscle. This study analyzed the regulation of the mouse MCK gene in transgenic mice. The role of MCK sequences from bp −1020 to −118 was clarified by examination of transgenes which contained either 1,256 bp of 5′-flanking sequences (encompassing the MCK upstream enhancer in its native orientation and position), 1,020 bp of 5′-flanking sequences lacking the enhancer, or the 206-bp MCK 5′ enhancer linked to the basal promoter (117 or 80 bp of MCK 5′-flanking sequence) (Fig. 1A). Our results indicate that the 1-kb MCK region alone exhibits skeletal muscle-specific activity at a level equivalent to that of the 206-bp enhancer linked to the basal promoter. However, when the 206-bp enhancer is combined with the 1-kb region, the resulting transcriptional activity in both skeletal and cardiac muscle is greater than that of either region alone (Table 1; Fig. 2). Interestingly, transgenes containing the 1-kb region alone or the enhancer linked to the basal promoter exhibit roughly 10^3- and 10^6-fold variations in expression levels whereas variation of CAT activities in the 1256MCK-CAT data set is only about 200-fold. This suggests that combination of the enhancer and the 1-kb region reduces the effect(s) of site of integration on transgene expression.

These results suggest that MCK sequences in the 1-kb region (bp −1020 to −118) contain skeletal muscle-specific elements that act independently as well as in concert with enhancer elements. The MCK 1-kb region does not exhibit transcriptional activity in cardiac muscle independently but appears to contain some cardiac muscle-specific elements that contribute to transcriptional activity when the enhancer is present. The transgenic-animal results differ from cell culture analysis in skeletal myocytes in which the 1-kb region is not required for full activity of the MCK enhancer and has only about 2% of the activity of 1256MCK-CAT, which includes the enhancer (2). However, they are in agreement with results from cultured primary cardiomyocytes, in which both regions are required for maximal transcriptional activity (2).

Activity of MCK enhancer sites in transgenic mice. The activities of several sites in the MCK enhancer—the right E box, CArG site, and AT-rich site—were examined in transgenic mice. These DNA motifs were targeted because they reside in the control regions of many muscle-specific genes and have been shown to be binding sites for *trans*-acting factors in gel shift assays. Furthermore, mutations in these sites resulted in the largest relative decreases in expression of MCK constructs in either cultured cardiac or skeletal myocytes (2).

Mutation of the MCK enhancer CArG element, a potential binding site for serum response factor (36, 56, 68), had no dramatic effect on transgene expression in either adult cardiac or skeletal muscle. Although it is possible that a conserved CArG consensus sequence at nt −177 compensated for the mutation of the enhancer CArG element, this compensation clearly fails to occur in cultured muscle cells, in which the same mutation in the enhancer CArG site produced a loss of transcriptional activity in both cardiomyocytes and skeletal myocytes (2). The activity of 5′-flanking sequences of the rabbit MCK gene, as measured by direct DNA injection into adult rat hearts, suggested that the promoter region which includes this CArG site exhibits activity in heart muscle (69). However, under those experimental conditions, the enhancer appeared to exhibit no cardiac muscle-specific activity at all. This finding is in contrast to the results of this study and our earlier transgenic analysis (30) in which the mouse MCK enhancer is required for expression in adult mouse heart muscle. Results from this transgenic-mouse study suggest that even though CArG sites have been implicated in the transcription of other muscle-specific genes (36, 41, 43, 52), the CArG site in the MCK enhancer is not a critical regulatory element by itself in either adult cardiac muscle or thigh skeletal muscle.

The right E box in the MCK enhancer is critical for expression of MCK constructs in cultured skeletal myocytes (2, 3, 8, 9, 24) and has been shown to be a binding site for heterodimers of the myogenic bHLH factors (34, 44, 45, 73), including MRF4, which is the predominant myogenic bHLH factor in adult skeletal muscle (54). The adjacent left E box also exhibits transcriptional activity in skeletal and cardiac muscle cultures (2); however, it has functional and binding properties that are distinct from those of the right E box (3) and cannot compen-

sate for mutation of the right E box in cultured cells. To test the activity of these E boxes in vivo, we examined the activity of transgenes with a mutation in the right E box alone or concomitant mutations in three E box sites: the right and left E boxes and a conserved E box at nt −249. All of the transgenes carrying E-box mutations exhibited wild-type levels of activity in adult skeletal muscle. Activities of the E-box-mutated 1256MCK-CAT transgenes were greater than those of transgenes consisting of the 1-kb region alone or the enhancer linked to the basal promoter, suggesting that the right E box alone or in combination with the other two E boxes cannot account for the synergistic activity of these two regions. Mutation of the right E-box site alone also had no significant effect on transgene expression in cardiac muscle.

The myogenic bHLH factors (MyoD, Myf-5, myogenin, and MRF-4) are key regulatory factors in muscle differentiation (for reviews, see references 17, 50, and 72). Analyses of other muscle-specific genes in cultured muscle cells have shown that E-box sites, which bind the myogenic bHLH factors, often appear to be required for muscle-specific transcription (6, 10, 18, 37, 38, 53, 57, 62, 71), although in many other cases they are dispensable (40, 47, 66). However, few of these E-box sites have been mutagenized and analyzed in transgenic mice. Transgenic-mouse analysis showed that both an E-box site and an AT-rich sequence are required for proper expression of the myogenin gene in developing mouse muscles (77). Analysis of the MyoD promoter, however, indicated that mutation of three conserved E-box sites in the distal enhancer region has no apparent effect on embryonic expression in transgenic mice (19). A transgenic-mouse analysis of the cardiac myosin light-chain 2 gene concludes that mutation of an E box exerts only a minor negative effect on ventricular expression of that gene (35); however, the role of an additional E-box site, which is important for transcriptional activation in cultured rat ventricular muscle cells (48), has not yet been examined in transgenic mice. Thus, in a number of cases, the functional importance of E-box sequences appears to depend upon their gene context. Further regulatory studies of these genes in transgenic animals may disclose yet other determinants of E box function.

What could account for the difference between the critical role of the MCK E boxes in cultured muscle cells and their apparent lack of function in vivo? First, our transgenic-animal data represent steady-state expression levels in an adult muscle environment and do not exclude the possibility that these E boxes play important roles under other physiological conditions. Second, data from virtually all skeletal muscle culture studies may more closely reflect the developmental program of MCK gene activation, since in transient- as well as stable-transfection studies, cells are harvested for CAT assays within several days of being induced to differentiate. Similarly, cardiomyocytes cultured from 2-day-old rats used in the cell culture assays represent a different developmental time point from adult heart muscle. This difference between the experimental systems may be especially important for the evaluation of E-box site activity because the myogenic bHLH factors are expressed in different relative amounts in developing muscle and in different adult skeletal muscles (26, 58, 70). To address this issue, MCK transgenes could be assayed in developing muscle of embryos when myoblasts are known to undergo early stages of differentiation. Preliminary analysis of fetal and postnatal mice, however, showed no evidence of altered transgene expression in developing hindlimb muscle as a result of mutation of the right E box (16). Third, quantitative differences in transcription factors may occur when cells are cultured, as has been observed for some other binding factors (76). Consistent with this idea, a recent study of the myosin light-chain 1A gene reported that mutation of an E box had a less dramatic effect in primary mouse skeletal muscle cultures than in the C2 or Sol8 muscle cell lines (10). In addition, most skeletal muscle cell lines, including the MM14 line used in our cell culture analyses, are derived from satellite cells, which may be phenotypically distinct from other skeletal myoblasts. The apparent lack of function of the conserved MCK E boxes in thigh skeletal muscle suggests that in this adult mouse muscle type, other tissue-specific or ubiquitous factors are more important for steady-state expression of the MCK gene. However, while the three conserved MCK E-box sites are not required for expression in thigh skeletal muscle, they do play roles in cardiac muscle as well as in certain other adult skeletal muscles (61).

MCK enhancer AT-rich element. The AT-rich site (TTATA ATTAA) has been shown to contribute substantially to activity of the MCK enhancer in both skeletal and cardiac muscle cell cultures (2). Mutation of the AT-rich site caused a dramatic loss of activity in transgenic thigh skeletal muscle (Table 1; Fig. 4A), resulting in an activity level similar to that of the 1020MCK-CAT transgene, which lacks the MCK 5′ enhancer. However, and in contrast to results from cultured cardiomyocytes (2), the AT-rich element does not appear to be as critical for expression of the 1256MCK-CAT transgene in adult transgenic cardiac muscle (Fig. 4B).

This suggests that an intact AT-rich site is a major requirement for steady-state activity of the MCK enhancer in thigh skeletal muscle. The MCK AT-rich site has been shown to bind at least three factors in in vitro assays: MHox, MEF-2, and Oct-1 (1, 13, 14), and experiments involving the mouse C2 skeletal muscle cell line suggest that MEF-2 is the factor which mediates its transcriptional activation (14). Because the AT-rich site in the MCK enhancer exhibits such complex binding properties, it seemed critical to examine its activity in whole muscle tissues, in which subtle differences in the relative amounts of these different factors might be more crucial. Such subtle differences may explain why mutation of the MCK AT-rich site exhibited a significant effect in cultured newborn rat cardiomyocytes (2) but not in whole adult transgenic mouse heart muscle (Fig. 4B). The MCK enhancer also contains a MEF-2 site (TAAAAATAA), located 3′ of the other enhancer elements, which is bound in gel shift assays by some of the same factors which recognize the AT-rich site (14, 25); however, the MEF-2 factor exhibits preferential binding to the MEF-2 site over the AT-rich site (14). In addition, our results with transgenic animals suggest that the presence of the enhancer MEF-2 site does not compensate for a mutation of the AT-rich site. The importance of the AT-rich site does not exclude the possibility that other enhancer elements function to a lesser extent or interact with the AT-rich site to produce the full transcriptional activity of the MCK enhancer.

Muscle specificity of MCK transgenes. All of the MCK-CAT transgenes examined in this study, with the exception of the basal promoter 117MCK-CAT construct, were expressed in a muscle-specific manner. Transgene activity in liver tissue was typically several orders of magnitude lower than activity in skeletal or cardiac muscle. This observation is evidence that the transgenes examined are not exhibiting activity as a result of fortuitous integration into active chromatin sites. Furthermore, since none of the mutations or truncations of MCK 5′-flanking sequences produced greater activity in liver tissue, this study offers no evidence of transcriptional repressor sites present in the MCK 1,256-bp 5′ region. Mutation of the AT-rich site did, however, result in a loss of activity in liver (compare liver activities of 1256MCK-CAT and 1256[A/T mut] MCK-CAT in Table 1). This difference in expression was confirmed by statistical analysis ($P = 0.006$ by the χ^2 and Fisher

exact tests). Binding of ubiquitous factors to the MCK AT-rich site, as has been observed in gel shift binding studies (1, 15a, 25), may contribute to the extremely low-level expression from the bp −1256 to +7 MCK gene fragment in nonmuscle tissues.

A number of tissue-specific gene-regulatory regions exhibit properties in transgenic tissues different from those in cultured cells from the same tissue types (4, 5, 15, 32, 33, 64, 65). This is not surprising, because in transgenic mice the introduced genes are integrated in chromatin during embryogenesis and exposed to a normal array of physiological signals. Transgenic analysis of the mouse MCK gene presents a significantly different picture of the activity of 5′-flanking sequences, especially enhancer elements, from that suggested by the results of transient-transfection assays in cultured muscle cells. These differences cannot be easily explained by some of the technical differences between the two experimental systems. For example, integration of the transgenes into chromatin does not seem to produce these effects, because MCK constructs exhibit the same activities in stably transfected muscle cultures as in transiently transfected cultures (21). Rather, the differences we observed between transgenic and cell culture analyses probably reflect multiple mechanisms for transcriptional regulation of the MCK gene depending on the repertoire and concentration of available transcription factors. This, in turn, is dependent on the developmental and physiological state of muscle. Transcriptional regulation of muscle-specific genes involves a complex array of muscle-specific and ubiquitous factors which may be affected by a range of physiological signals. In vivo analysis of gene-regulatory regions is thus essential for understanding mechanisms of gene regulation in different muscle contexts.

ACKNOWLEDGMENTS

We thank Sharon Amacher for supplying plasmid preparations of various constructs and Joan Carpenter for generation of some of the wild-type and enh[Right E mut]117MCK-CAT animals. We also thank Flora Chen, Mike Donoviel, and Jeff Ruffles for technical assistance and members of the Biostatistics Department at the University of Washington for assistance with statistical analyses. We also appreciate the helpful comments on this manuscript made by Michael Donoviel and Brian Zambrowicz. Gary Lyons is also acknowledged for sharing useful information prior to publication.

This work was supported by National Institutes of Health grants HL 39070 and AR 18860 and PO1-HL03174 and a grant from the Muscular Dystrophy Association to S.D.H. D.B.D. was supported by Public Health Service grant NRSA T32 GM07270 from NIGMS. M.A.S. was supported by National Research Service Award 5T32GMO7270-15.

REFERENCES

1. **Amacher, S. L.** 1993. Ph.D. thesis. University of Washington, Seattle.
2. **Amacher, S. L., J. N. Buskin, and S. D. Hauschka.** 1993. Multiple regulatory elements contribute differentially to muscle creatine kinase enhancer activity in skeletal and cardiac muscle. Mol. Cell. Biol. **13:**2753–2764.
3. **Apone, S., and S. D. Hauschka.** 1995. Muscle gene E-box control elements: evidence for quantitatively different transcriptional activities and the binding of distinct regulatory factors. J. Biol. Chem. **270:**21420–21427.
4. **Aronow, B. J., R. N. Silbiger, M. R. Dusing, J. L. Stock, K. L. Yager, S. S. Potter, J. J. Hutton, and D. A. Wiginton.** 1992. Functional analysis of the human adenosine deaminase gene thymic regulatory region and its ability to generate position-independent transgene expression. Mol. Cell. Biol. **12:** 4170–4185.
5. **Bedalov, A., D. T. Breault, B. P. Sokolov, A. C. Lichtler, I. Bedalov, S. H. Clark, K. Mack, J. S. Khillian, C. O. Woody, B. E. Kream, and D. W. Rowe.** 1994. Regulation of the α1(I) collagen promoter in vascular smooth muscle cells. Comparison with other α1(I) collagen-producing cells in transgenic animals and cultured cells. J. Biol. Chem. **269:**4903–4909.
6. **Bouvagnet, P. F., E. E. Strehler, G. E. White, M. A. Strehler-Page, B. Nadal-Ginard, and V. Mahdavi.** 1987. Multiple positive and negative 5′ regulatory elements control the cell-type-specific expression of the embryonic skeletal myosin heavy-chain gene. Mol. Cell. Biol. **7:**4377–4389.
7. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
8. **Brennan, T. J., and E. Olson.** 1990. Myogenin resides in the nucleus and acquires high affinity for a conserved enhancer element upon dimerization. Genes Dev. **4:**582–595.
9. **Buskin, J. N., and S. D. Hauschka.** 1989. Identification of a myocyte-specific nuclear factor that binds to the muscle-specific enhancer of the mouse muscle creatine kinase gene. Mol. Cell. Biol. **9:**2627–2640.
10. **Catala, F., R. Wanner, P. Baron, A. Cohen, W. Wright, and M. Buckingham.** 1995. A skeletal muscle-specific enhancer regulated by factors binding to E and CArG boxes is present in the promoter of the mouse myosin light-chain 1A gene. Mol. Cell. Biol. **15:**4585–4596.
11. **Chamberlain, J. S., J. B. Jaynes, and S. D. Hauschka.** 1985. Regulation of creatine kinase induction in differentiating mouse myoblasts. Mol. Cell. Biol. **5:**484–492.
12. **Chien, K. R., H. Zhu, K. U. Knowlton, W. Miller-Hance, M. van-Bilsen, T. X. O'Brien, and S. M. Evans.** 1993. Transcriptional regulation during cardiac growth and development. Annu. Rev. Physiol. **55:**77–95.
13. **Cserjesi, P., B. Lilly, L. Bryson, Y. Wang, D. A. Sasson, and E. N. Olson.** 1992. MHox: a mesodermally restricted homeodomain protein that binds an essential site in the muscle creatine kinase enhancer. Development **115:** 1087–1101.
14. **Cserjesi, P., B. Lilly, C. Hinkley, M. Perry, and E. N. Olson.** 1994. Homeodomain protein MHox and MADS protein myocyte enhancer-binding factor-2 converge on a common element in the muscle creatine kinase enhancer. J. Biol. Chem. **269:**16740–16745.
15. **Dandoy-Dron, F., E. Monthioux, J. Jami, and D. Bucchini.** 1991. Regulatory regions of rat insulin I gene necessary for expression in transgenic mice. Nucleic Acids Res. **19:**4925–4930.

15a. **Donoviel, D.** Unpublished data.

16. **Donoviel, D. B.** 1993. Ph.D. thesis. University of Washington, Seattle.
17. **Edmondson, D. G., and E. N. Olson.** 1993. Helix-loop-helix proteins as regulators of muscle-specific transcription. J. Biol. Chem. **268:**755–758.
18. **French, B. A., K. L. Chow, E. N. Olson, and R. J. Schwartz.** 1991. Heterodimers of myogenic helix-loop-helix regulatory factors and E12 bind a complex element governing myogenic induction of the avian cardiac α-actin promoter. Mol. Cell. Biol. **11:**2439–2450.
19. **Goldhamer, D. J., B. P. Brunk, A. Faerman, A. King, M. Shani, and C. P. Emerson.** 1995. Embryonic activation of the MyoD gene is regulated by a highly conserved distal control element. Development **121:**637–649.
20. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
21. **Gregory, D., J. N. Buskin, D. Donoviel, and S. D. Hauschka.** 1994. Comparison of muscle creatine kinase gene regulation in stable transfections, transient transfections and transgenic mice, abstr. W221. J. Cell. Biochem. **18D:** 497.
22. **Hauschka, S. D.** 1994. The embryonic origin of muscle, p. 3–73. *In* A. G. Engel and C. Franzini-Armstrong (ed.), Myology, 2nd ed. McGraw-Hill, New York.
23. **Hogan, B., F. Constantini, and E. Lacy.** 1986. Manipulating the mouse embryo: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
24. **Horlick, R. A., and P. A. Benfield.** 1989. The upstream muscle-specific enhancer of the rat muscle creatine kinase gene is composed of multiple elements. Mol. Cell. Biol. **9:**2396–2413.
25. **Horlick, R. A., G. M. Hobson, J. H. Patterson, M. T. Mitchell, and P. A. Benfield.** 1990. Brain and muscle creatine kinase genes contain common TA-rich recognition protein-binding regulatory elements. Mol. Cell. Biol. **10:** 4826–4836.
26. **Hughes, S. M., J. M. Taylor, S. J. Tapscott, C. M. Gurley, W. J. Carter, and C. A. Peterson.** 1993. Selective accumulation of MyoD and myogenin mRNA in fast and slow adult skeletal muscle is controlled by innervation and hormones. Development **118:**1137–1147.
27. **Jaenisch, R.** 1988. Transgenic animals. Science **240:**1468–1474.
28. **Jaynes, J. B., J. S. Chamberlain, J. N. Buskin, J. E. Johnson, and S. D. Hauschka.** 1986. Transcriptional regulation of the muscle creatine kinase gene and regulated expression in transfected mouse myoblasts. Mol. Cell. Biol. **6:**2855–2864.
29. **Jaynes, J. B., J. E. Johnson, J. N. Buskin, C. L. Gartside, and S. D. Hauschka.** 1988. The muscle creatine kinase gene is regulated by multiple upstream elements, including a muscle-specific enhancer. Mol. Cell. Biol. **8:** 62–70.
30. **Johnson, J., B. J. Wold, and S. D. Hauschka.** 1989. Muscle creatine kinase sequence elements regulating skeletal and cardiac muscle expression in transgenic mice. Mol. Cell. Biol. **9:**3393–3399.
31. **Johnson, J. E.** 1988. Ph.D. thesis. University of Washington, Seattle.
32. **Kitsis, R. N., and L. A. Leinwand.** 1992. Discordance between gene regulation *in vitro* and *in vivo*. Gene Expression **2:**313–318.
33. **Knotts, S., H. Rindt, J. Neumann, and J. Robbins.** 1994. *In vivo* regulation of the mouse β myosin heavy chain gene. J. Biol. Chem. **269:**31275–31282.
34. **Lassar, A. B., J. N. Buskin, D. Lockshon, R. L. Davis, S. Apone, S. D.**

Hauschka, and H. Weintraub. 1989. MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. Cell **58:**823–831.
35. **Lee, K. J., R. Hickey, H. Zhu, and K. R. Chien.** 1994. Positive regulatory elements (HF-1a and HF-1b) and a novel negative regulatory element (HF-3) mediate ventricular muscle-specific expression of myosin light-chain 2-luciferase fusion genes in transgenic mice. Mol. Cell. Biol. **14:**1220–1229.
36. **Lee, T. C., K. L. Chow, P. Fang, and R. J. Schwartz.** 1991. Activation of skeletal α-actin gene transcription: the cooperative formation of serum response factor-binding complexes over positive *cis*-acting promoter serum response elements displaces a negative-acting nuclear factor enriched in replicating myoblasts and nonmyogenic cells. Mol. Cell. Biol. **11:**5090–5100.
37. **Li, H., and Y. Capetanaki.** 1994. An E box in the desmin promoter cooperates with the E box and MEF-2 sites of a distal enhancer to direct muscle-specific transcription. EMBO J. **13:**3580–3589.
38. **Lin, H., K. E. Yutzey, and S. F. Konieczny.** 1991. Muscle-specific expression of the troponin I gene requires interaction between helix-loop-helix muscle regulatory factors and ubiquitous transcription factors. Mol. Cell. Biol. **11:** 267–280.
39. **Lyons, G. E., S. Muhlebach, A. Moser, R. Masood, B. M. Paterson, M. E. Buckingham, and J.-C. Perriard.** 1991. Developmental regulation of creatine kinase gene expression by myogenic factors in embryonic mouse and chick skeletal muscle. Development **113:**1017–1029.
40. **Mar, J. H., and C. P. Ordahl.** 1988. A conserved CATTCCT motif is required for skeletal muscle-specific activity of the cardiac troponin T gene promoter. Proc. Natl. Acad. Sci. USA **85:**6404–6408.
41. **Miwa, T., and L. Kedes.** 1987. Duplicate CArG box domains have positive and mutually dependent regulatory roles in expression of the human α-cardiac actin gene. Mol. Cell. Biol. **7:**2803–2813.
42. **Monnat, R. J.** 1989. Molecular analysis of spontaneous hypoxanthine phosphoribosyl transferase mutations in thioguanine resistant HL-60 human leukemia cells. Cancer Res. **49:**81–87.
43. **Moss, J. B., T. C. McQuinn, and R. J. Schwartz.** 1994. The avian cardiac α-actin promoter is regulated through a pair of complex elements composed of E boxes and serum response elements that bind both positive and negative acting factors. J. Biol. Chem. **269:**12731–12740.
44. **Murre, C., P. S. McCaw, H. Vaessin, M. Caudy, L. Y. Jan, C. V. Cabrera, J. N. Buskin, S. D. Hauschka, A. B. Lassar, H. Weintraub, and D. Baltimore.** 1989. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell **58:** 537–544.
45. **Murre, C., A. Voronova, and D. Baltimore.** 1991. B cell- and myocyte-specific E2-box binding factors contain E12/E47-like subunits. Mol. Cell. Biol. **11:** 1156–1160.
46. **Muscat, G. E., T. A. Gustafson, and L. Kedes.** 1988. A common factor regulates skeletal and cardiac α-actin gene transcription in muscle. Mol. Cell. Biol. **8:**4120–4133.
47. **Nakatsuji, Y., K. Hidaka, S. Tsujino, Y. Yamamoto, T. Mukai, T. Yanagihara, T. Kishimoto, and S. Sakoda.** 1992. A single MEF2 site is a major positive regulatory element required for transcription of the muscle-specific subunit of the human phosphoglycerate mutase gene in skeletal and cardiac muscle gene expression. Mol. Cell. Biol. **12:**4384–4390.
48. **Navankasattusas, S., M. Sawadogo, M. van Bilsen, C. V. Dang, and K. R. Chien.** 1994. The basic helix-loop-helix protein upstream stimulating factor regulates the cardiac ventricular myosin light-chain 2 gene via independent *cis* regulatory elements. Mol. Cell. Biol. **14:**7331–7339.
49. **Olson, E. N.** 1993. Regulation of muscle transcription by the MyoD family. The heart of the matter. Circ. Res. **72:**1–6.
50. **Olson, E. N., and W. H. Klein.** 1994. bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. Genes Dev. **8:**1–8.
51. **Palmiter, R. D., and R. L. Brinster.** 1986. Germ-line transformation of mice. Annu. Rev. Genet. **20:**465–499.
52. **Pari, G., K. Jardine, and M. W. McBurney.** 1991. Multiple CArG boxes in the human cardiac actin gene promoter required for expression in embryonic cardiac muscle cells developing in vitro from embryonal carcinoma cells. Mol. Cell. Biol. **11:**4796–4803.
53. **Piette, J., J. L. Bessereau, M. Huchet, and J.-P. Changeux.** 1990. Two adjacent MyoD1-binding sites regulate expression of the acetylcholine receptor α-subunit gene. Nature (London) **345:**353–355.
54. **Rhodes, S. J., and S. F. Konieczny.** 1989. Identification of MRF4: a new member of the muscle regulatory factor gene family. Genes Dev. **3:**2050–2061.
55. **Rosner, B.** 1990. Fundamentals of biostatistics, 3rd ed., p. 304–308 and Appendix 1. PWS-KENT Publishing, Boston.
56. **Santoro, I. M., and K. Walsh.** 1991. Natural and synthetic DNA elements with the CArG motif differ in expression and protein-binding properties. Mol. Cell. Biol. **11:**6296–6305.
57. **Sartorelli, V., K. A. Webster, and L. Kedes.** 1990. Muscle-specific expression of the cardiac α-actin gene requires MyoD1, CArG-box binding factor, and Sp1. Genes Dev. **4:**1811–1822.
58. **Sassoon, D., G. Lyons, W. E. Wright, V. Lin, A. Lassar, H. Weintraub, and M. Buckingham.** 1989. Expression of two myogenic regulatory factors myogenin and MyoD1 during mouse embryogenesis. Nature (London) **341:**303–307.
59. **Shaw, W. V., and R. F. Brodsky.** 1968. Characterization of chloramphenicol acetyltransferase from chloramphenicol-resistant *Staphylococcus aureus*. J. Bacteriol. **95:**28–36.
60. **Shield, M. A.** 1995. Ph.D. thesis. University of Washington, Seattle.
61. **Shield, M. A., H. S. Haugen, C. H. Clegg, and S. D. Hauschka.** Submitted for publication.
62. **Skerjanc, I. S., and M. W. McBurney.** 1994. The E box is essential for activity of the cardiac actin promoter in skeletal but not in cardiac muscle. Dev. Biol. **163:**125–132.
63. **Sternberg, E. A., G. Spizz, W. M. Perry, D. Vizard, T. Weil, and E. N. Olson.** 1988. Identification of upstream and intragenic regulatory elements that confer cell-type-restricted and differentiation-specific expression on the muscle creatine kinase gene. Mol. Cell. Biol. **8:**2896–2909.
64. **Subramaniam, A., J. Gulick, J. Neumann, S. Knotts, and J. Robbins.** 1993. Transgenic analysis of the thyroid-responsive elements in the α-cardiac myosin heavy chain gene promoter. J. Biol. Chem. **268:**4331–4336.
65. **Swift, G. H., F. Kruse, R. J. MacDonald, and R. E. Hammer.** 1989. Differential requirements for cell-specific elastase I enhancer domains in transfected cells and transgenic mice. Genes Dev. **3:**687–696.
66. **Thompson, W. R., B. Nadal-Ginard, and V. Mahdavi.** 1991. A MyoD1-independent muscle-specific enhancer controls the expression of the β-myosin heavy chain gene in skeletal and cardiac muscle cells. J. Biol. Chem. **266:** 22678–22688.
67. **Trask, R. V., A. W. Strauss, and J. J. Billadello.** 1988. Developmental regulation and tissue-specific expression of the human muscle creatine kinase gene. J. Biol. Chem. **263:**17142–17149.
68. **Treisman, R.** 1992. The serum response element. Trends Biochem. Sci. **17:** 423–426.
69. **Vincent, C. K., A. Gualberto, C. V. Patel, and K. Walsh.** 1993. Different regulatory sequences control creatine kinase-M gene expression in directly injected skeletal and cardiac muscle. Mol. Cell. Biol. **13:**1264–1272.
70. **Voytik, S. L., M. Przyborski, S. F. Badylak, and S. F. Konieczny.** 1993. Differential expression of muscle regulatory factor genes in normal and denervated adult rat hindlimb muscles. Dev. Dyn. **198:**214–224.
71. **Walsh, K., and P. Schimmel.** 1987. Two nuclear factors compete for the skeletal muscle actin promoter. J. Biol. Chem. **262:**9429–9432.
72. **Weintraub, H.** 1993. The MyoD family and myogenesis: redundancy, networks, and thresholds. Cell **75:**1241–1244.
73. **Weintraub, H., R. Davis, D. Lockshon, and A. Lassar.** 1990. MyoD binds cooperatively to two sites in target enhancer sequence: occupancy of two sites is required for activation. Proc. Natl. Acad. Sci. USA **87:**5623–5627.
74. **Wentworth, B. M., M. Donoghue, J. C. Engert, and E. B. Berglund.** 1991. Paired MyoD-binding sites regulate myosin light chain gene expression. Proc. Natl. Acad. Sci. USA **88:**1242–1246.
75. **Wilkie, T. M., and R. D. Palmiter.** 1987. Analysis of the integrant in MyK-103 transgenic mice in which males fail to transmit the integrant. Mol. Cell. Biol. **7:**1646–1655.
76. **Xanthopoulos, K. G., J. Mirkovitch, T. Decker, C. F. Kuo, and J. E. Darnell, Jr.** 1989. Cell-specific transcriptional control of the mouse DNA-binding protein mC/EBP. Proc. Natl. Acad. Sci. USA **86:**4117–4121.
77. **Yee, S. P., and P. W. Rigby.** 1993. The regulation of myogenin gene expression during the embryonic development of the mouse. Genes Dev. **7:**1277–1289.
78. **Yi, T.-M., K. Walsh, and P. Schimmel.** 1991. Rabbit muscle creatine kinase: genomic cloning, sequencing, and analysis of upstream sequences important for expression in myocytes. Nucleic Acids Res. **19:**3027–3033.