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The Upstream Muscle-Specific Enhancer of the Rat Muscle Creatine Kinase Gene Is Composed of Multiple Elements

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A series of constructs that links the rat muscle creatine kinase promoter to the bacterial chloramphenicol acetyltransferase gene was generated. These constructs were introduced into differentiating mouse C2C12 myogenic cells to localize sequences that are important for up-regulation of the creatine kinase gene during myogenic differentiation. A muscle-specific enhancer element responsible for induction of chloramphenicol acetyltransferase expression during myogenesis was localized to a 159-base-pair region from 1,031 to 1,190 base pairs upstream of the transcription start site. Analysis of transient expression experiments using promoters mutated by deletion indicated the presence of multiple functional domains within this muscle-specific regulatory element. A DNA fragment spanning this region was used in DNase I protection experiments. Nuclear extracts derived from C2 myotubes protected three regions (designated E1, E2, and E3) on this fragment from digestion, which indicated there may be three or more *trans*-acting factors that interact with the creatine kinase muscle enhancer. Gel retardation assays revealed that factors able to bind specifically to E1, E2, and E3 are present in a wide variety of tissues and cell types. Transient expression assays demonstrated that elements in regions E1 and E3, but not necessarily E2, are required for full enhancer activity.

Creatine kinase (CK) is a small soluble enzyme with a monomer molecular weight of 41,000. Its activity is found widely distributed in many different tissues (71) and has been postulated to play an important role in regenerating ATP from stores of creatine phosphate within contractile and transport systems (50, 73, 76). Three isoforms of the protein are currently recognized: brain CK (CKB), muscle CK (CKM), and mitochondrial CK. There is evidence in chicken that mitochondrial CK also exists as brain and muscle variants (31). The active form of the enzyme is a dimer.

The *ck* genes belong to a small gene family that is differentially regulated during tissue differentiation. The two cytoplasmic isoforms of CK, CKM and CKB, appear to be the products of single-copy unlinked genes (47). We have recently isolated and characterized four *ck* genomic sequences from rats that correspond to the muscle and brain *ck* genes, a *ckb*-processed pseudogene and a potential mitochondrial gene (4). CKM expression is usually restricted to differentiated skeletal and cardiac muscle (71), although it has also been reported to be expressed in the lens of the eye (M. B. Perryman, personal communication) and in certain carcinoma cells (35). CKB is expressed in a much broader range of tissues but at highest levels in brain. Immature muscle cells or myoblasts express CKB. The *ckm* gene is up-regulated as these myoblasts withdraw from the cell cycle, terminally differentiate, and fuse to form myotubes (8, 49). This up-regulation is controlled in part at the level of transcription (33). The isoform switch is coordinated with the induction of an extensive set of muscle-specific functions that includes contractile proteins, metabolic enzymes, and cell surface molecules and receptors (16, 26, 28, 48). To begin to understand the molecular nature of this coincident induction of many differentiation-specific genes, Gunning et al. (26) have recently studied in detail the kinetics of the appearance and disappearance of steady-state transcripts from more than 20 genes that are regulated during myogenesis. They found each transcript to have its own unique pattern of expression. The *ck* genes therefore provide an interesting model system with which to study differential gene expression during development and coordinate regulation of genes in a particular developmental progression.

The regulatory apparatus has been studied for several muscle-specific genes that are coregulated with *ckm*, e.g., α-actin (5, 44, 45, 63), myosin light chains 1 and 3 (17a), myosin heavy chain (7, 39), and troponin I (37). To date, no obvious commonly shared regulatory elements have been identified, although a few sequences that appear to be important for up-regulation during myogenesis for several genes, e.g., the CArG box (42), have been described.

Two groups have previously described a muscle-regulatory element upstream of the mouse muscle *ck* gene (34, 69). This sequence has the properties of a muscle-specific enhancer element. Muscle-specific enhancerlike elements have previously been described for the rat skeletal muscle α-actin gene (47a), and the rat myosin light-chain 1/3 gene, but all are incompletely characterized. In this paper, we characterize a muscle-specific enhancer element upstream of the rat *ckm* gene. This enhancer element shares homology with that previously described for the mouse *ckm* gene and is similarly located with respect to the transcript start point. We show that, like many enhancers, this enhancer is composed of multiple domains (25, 29, 51, 52, 60, 61, 79). Each of these domains appears to be able to interact with a sequence-specific binding protein(s) that is a candidate for a component of a tissue-specific regulatory apparatus.

More complete characterization of this enhancer should lead to a better understanding of enhancer function. It should also contribute to our understanding of gene regulation during myogenesis and of the mechanisms of coordinate regulation of gene sets.

MATERIALS AND METHODS

Sequencing. Sequencing of the rat *ckm* promoter was performed by the procedure of Sanger et al. (59). Appropri-

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ate subclones of the *ckm* promoter in M13mp10 and M13mp11 were used as substrates for chain termination reactions.

Construction of test plasmids. Test chloramphenicol acetyltransferase (CAT) plasmids were constructed by standard methods (38). A parent *ck* CAT construct was generated by insertion of a 6.0-kilobase-pair (kbp) promoter fragment from the unique *Xma*I site at the start point of transcription to a unique *Sal*I site 6.0 kbp upstream, into $pUC_{PL}CAT$. $pUC_{PL}CAT$, obtained from Beverly Bond (Department of Biology, University of California at Los Angeles), contains the bacterial CAT gene and the simian virus (SV40) splice signals and poly(A) addition site inserted into plasmid vector pUC18. This vector contains, in addition, an artificial polylinker-generated set of cloning sites upstream of the CAT gene. The *ckm* promoter fragment was inserted between the *Sal*I-compatible 5′ *Xho*I site and the 3′ *Bgl*II site in this polycloning region. At the 3′ end of the *ckm* insert, an 18-bp synthetic oligodeoxynucleotide was used to link the *Xma*I site in the *ckm* promoter to the *Bgl*II site 5′ to the CAT gene. This synthetic oligodeoxynucleotide, which has the sequence 5′-CCGGGTCACCACCACCTA-3′, was designed to regenerate the correct start point of transcription and bases +1 to +14 of the wild-type *ckm* gene. In this way, a conserved box c sequence motif previously reported to overlap the start point of transcription (33) for the *ckm* gene was retained in the CAT constructs. The *Bgl*II site at +14 is therefore synthetic and is not present in the wild-type *ckm* gene. Thereafter, deleted promoter constructs were generated by cleavage of this parent construct with appropriate restriction endonucleases, filling in with the Klenow fragment of DNA polymerase I, and ligation with T4 DNA ligase. Plasmids are named after the restriction sites used in their construction (see Fig. 2).

Cell culture and transfections. Mouse C2C12 cells were grown as described previously (6, 78). Before transfection, cells were plated at a density of 3×10^5/85-mm-diameter plate. At 20 h after plating, cells were transfected by the calcium phosphate method as described previously (24). Each construct was transfected onto duplicate plates. DNA was removed from cells after 17 h by washing plates twice with 5 ml of Dulbecco phosphate-buffered saline containing 1 mM EDTA, and cells were trypsinized in the presence of 1 mM EDTA. For each construct, cells from the duplicate plates were pooled, distributed onto two new plates at a ratio of 9:1, and allowed to fuse into myotubes or to continue to proliferate as myoblasts. Thus, results from myoblasts and myotubes within a construct are derived from the same transfection, providing an internal control for transfection efficiency. The more densely populated (myotube) plates were refed 4 h after plating with Dulbecco modified essential medium containing 2% horse serum. All plates were harvested at 40 to 48 h after replating. Cells were collected and CAT assays were performed as described previously (22). Assays were performed on the same amount of protein per extract. Each assay was repeated at least three times. Results were quantitated by removal of radioactive spots from thin-layer chromatography plates and liquid scintillation counting.

DNase I footprinting. DNase I footprinting experiments were modified from the method of Babiss et al. (2). The *Xba* 1.5-kbp construct (see Fig. 2) was digested with *Eco*RI plus *Bal*I, blunt ended with Klenow polymerase, and religated. The resulting plasmid was cut with *Eco*RI, treated with calf intestinal phosphatase, and recut with *Pvu*II. The 310-bp fragment was used for subsequent end labeling by kinase or by filling reactions, using reverse transcriptase (38). Alternatively, a smaller fragment from the *Stu*I site to the *Bam*HI site was used as a substrate (see Fig. 4). This fragment was 5′ end labeled at the *Stu*I site with polynucleotide kinase.

For protected samples, between 1 and 10 μg of nuclear extract in 6 μl of dialysis buffer, 4 μg of poly(dI-dC), and 4 μl of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4)–50 mM KCl–5 mM $MgCl_2$–1 mM dithiothreitol were incubated on ice for 15 min to allow nonspecific DNA-binding proteins to be adsorbed. For unprotected samples, the same reaction was carried out exactly as described above, but with 6 μl of dialysis buffer without nuclear extract. When a kinase-labeled fragment was used, 2 μg of tRNA was added to titrate out endogenous phosphatases. Approximately 1 ng of end-labeled fragment was added to this mixture, for a total reaction volume of 20 μl, and incubation was continued for 15 min at room temperature. DNase I (4 μl) diluted to the appropriate concentration in 20 mM Tris (pH 7.6)–50 mM NaCl–1 mM dithiothreitol–100 μg of bovine serum albumin per ml–50% glycerol was added to each sample. Digestion reactions were terminated after 90 s by addition of 40 μl of 50 mM EDTA, 0.2% sodium dodecyl sulfate, 100 μg of tRNA per ml, and 100 μg of proteinase K per ml, followed by incubation at 42°C for 45 min. Samples were extracted with phenol-chloroform, precipitated in 2.0 M ammonium acetate, and run on 7% DNA-sequencing gels.

For marker lanes, a chemical degradation procedure for sequencing was carried out on DNA immobilized on DEAE paper, using a modification of the reaction conditions of Maxam and Gilbert (40) as described by Chuvpilo and Kravchenko (11). Identical amounts of end-labeled DNA were spotted on DEAE strips (2 by 5 mm; Whatman, Inc., Clifton, N.J.), each of which was then placed in a microdilution dish well. The G reaction was incubated with 0.25% dimethyl sulfate in cacodylate buffer (pH 8.0), the G+A reaction was incubated with 63% aqueous formic acid, the T+C reaction was incubated with 60% aqueous hydrazine, and the C reaction was incubated with 60% aqueous hydrazine plus 1.5 M NaCl. Each strip was incubated with just enough reagent to cover it. Reactions were terminated at 5 min by rinsing rapidly five times each with 100% ethanol (EtOH), 70% EtOH, and H_2O and once with 100% EtOH. Strips were then air dried and pooled into one screw-cap Sarstedt tube. Strips were incubated in 200 μl of 1 M piperidine for 30 min at 90°C and then washed five times each with 100% EtOH, 70% EtOH, and H_2O and once in 100% EtOH. Each strip was placed into its own yellow Eppendorf pipette tip, and DNA was eluted for 10 min at 65°C in 50 μl of 1 M NaCl–10 mM EDTA–0.1 μg of tRNA per ml. This last step was repeated, and both eluted fractions were pooled, ethanol precipitated, and washed once with 80% EtOH. Pellets were suspended in 5 μl of water and lyophilized to remove any potential residual piperidine. Samples were suspended in 99% formamide–10 mM EDTA–0.2% bromophenol blue–xylene cyanol and run on sequencing gels next to the DNase I protection samples.

Preparation of nuclear extracts. Nuclear extracts were prepared either from undifferentiated C2C12 myoblasts or from well-differentiated myotubes either by the method of Dignam et al. (17) or by the method of Shapiro et al. (65). In each case, cells were harvested by trypsinization and washed three times with phosphate-buffered saline containing 100 μg of soybean trypsin inhibitor per ml before continuation of the preparation. When extracts were prepared by the method of Dignam et al. (17), we found that

nuclei did not lyse in the presence of the 0.42 M KCl salt wash. We therefore prepared a 0.42 KCl salt wash, followed by 0.5 and 0.6 M KCl salt washes until nuclear lysis occurred. For C2 cells, this usually corresponded to a 0.50 M salt wash. Results with the 0.42 and 0.50 M salt washes were similar (data not shown). Extracts from HeLa cells were prepared as described by Shapiro et al. (65), and extracts from rat brain and rat liver were prepared by the procedure of Gorski et al. (23).

Gel retardation assays. Gel retardation assays were performed as described by Singh et al. (66). A 0.1-ng sample of ^{32}P-5′-end-labeled probe DNA was incubated with 3 to 10 μg of nuclear extract in the presence of 4 μg of poly(dI-dC) (Pharmacia, Inc., Piscataway, N.J.) as competitor. The precise origin of probes used is indicated where appropriate. The binding buffer was 50 mM NaCl–100 mM Tris hydrochloride (pH 7.5)–2 mM dithiothreitol–20% (vol/vol) glycerol. Binding reactions were performed for 30 min at room temperature in a total volume of 30 μl. For oligodeoxynucleotide competition, increasing amounts of an appropriate synthetic oligodeoxynucleotide were included in the reaction. Nuclear extract was always added last. At the end of the incubation period, 3 μl of load dye (25% Ficoll [Pharmacia], 0.1% each bromophenol blue and xylene cyanol FF) was added, and samples were electrophoresed on 6% (wt/vol) polyacrylamide gels in Tris acetate buffer (6.7 mM Tris [pH 7.9], 3.3 mM sodium acetate, 1 mM EDTA), with buffer recirculation. Gels were prerun for 30 min before loading. Gels were electrophoresed for 2 to 3 h at 30 mA. After electrophoresis, gels were dried and subjected to autoradiography, using XAR5 or XRP film (Eastman Kodak Co., Rochester, N.Y.).

RESULTS

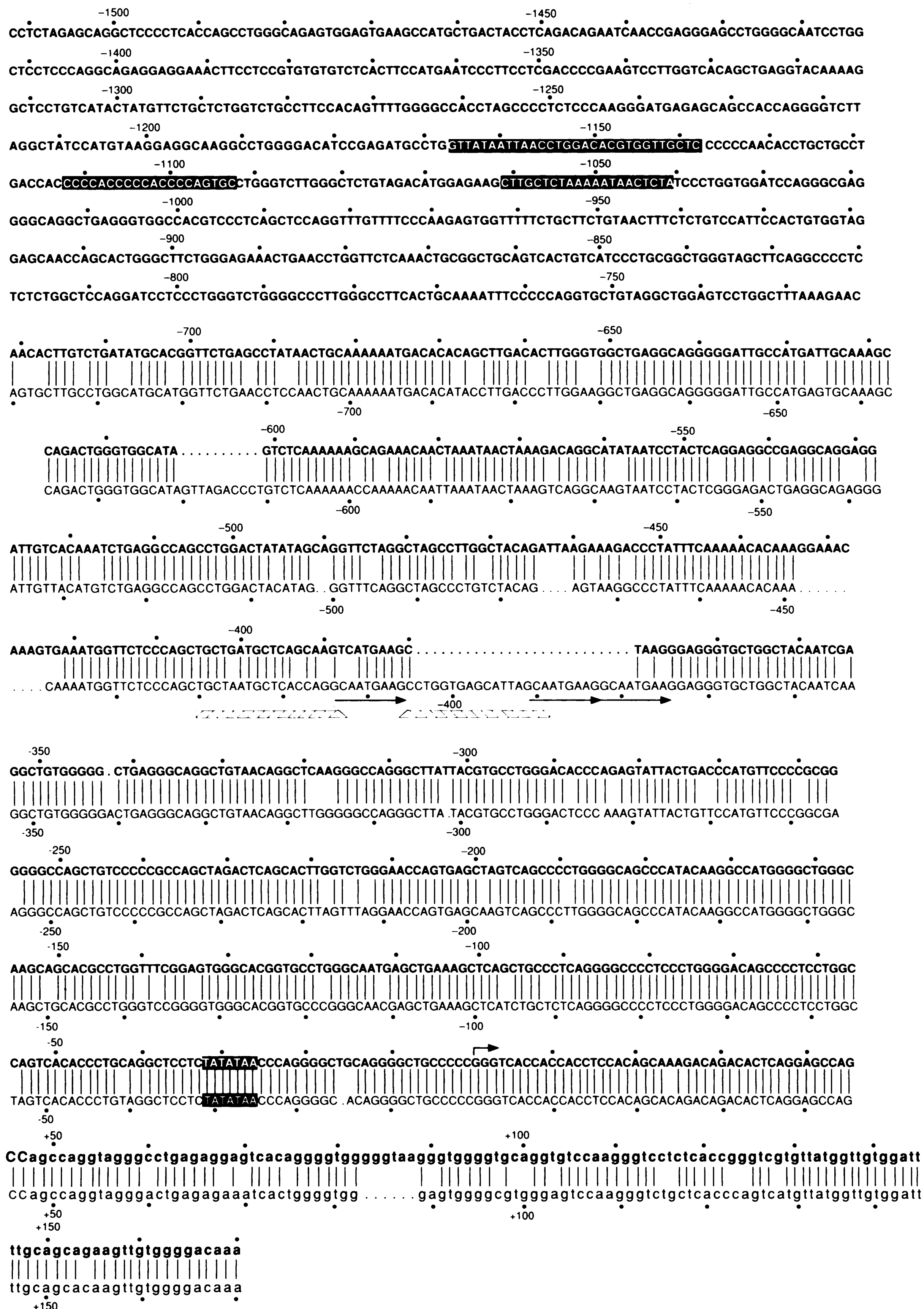
Sequence conservation in the *ckm* promoter region. The sequence in the 5′ region of rat *ckm* was determined and compared with that previously presented for the equivalent mouse gene (Fig. 1) (33, 69). The two sequences are highly homologous, not only in the promoter region but also in the sequence that extends into the first intron. For the first 379 bases upstream of the transcript start point, the sequences are 92% conserved. Further upstream of position −379, gaps need to be introduced into the mouse or rat sequence to obtain maximum alignment, but the ungapped sequence regions are still 87% conserved. Sequence variation appears to be random, and mutations are not clustered in any one region. Thus, no candidate regions for regulatory sequences are eliminated on the basis of particularly low degrees of sequence conservation. Several sequence features previously noted in the mouse *ckm* promoter-enhancer region are highly conserved in the rat *ckm* gene (33, 69). These include CArG-like motifs, SV40, immunoglobulin K (IgK) and IgH-like enhancer domains, including a potential AP-2 recognition sequence (43), an *Alu*-like sequence, a sequence showing homology with the acetylcholine receptor gene (36), and a repeated 17-bp motif in the immediate promoter region (33). However, a set of sequence repeats and an inverted repeat previously noted for mouse *ckm* (33) are not found in the rat sequence. Another notable sequence difference is the replacement of a C by a T at position −111 in the rat sequence that creates a CCAAT-like motif that is absent in the mouse sequence. Oligodeoxynucleotides that include this region in rat *ckm* have been found to compete for binding of proteins that recognize a CCAAT motif in the rat *ckb* promoter (30). Even in the 5′ region of the first intron, 87% sequence conservation is observed. Thus, the rat and mouse *ckm* genes are highly conserved in their 5′ regions.

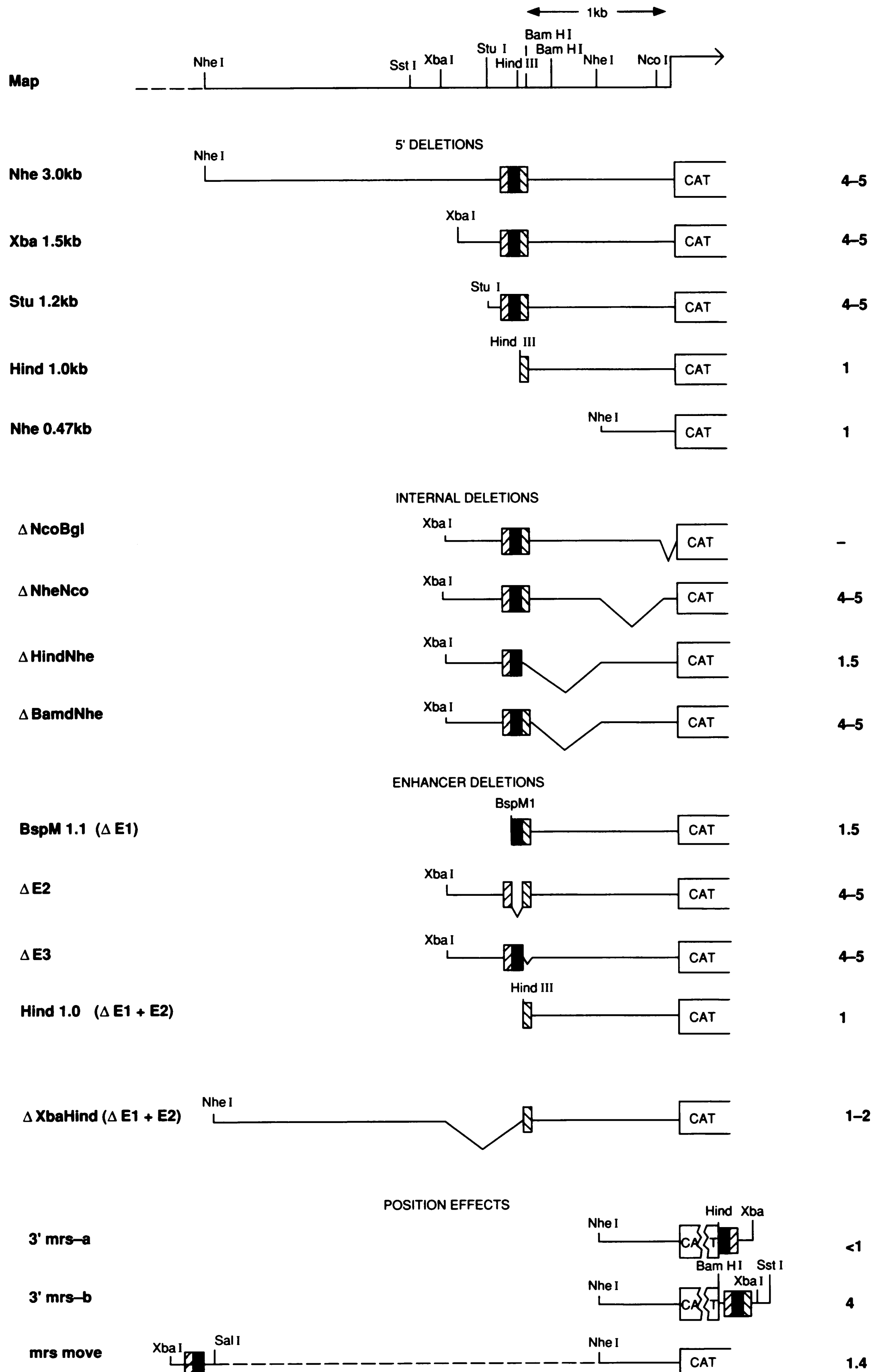
Identification and localization of a muscle-regulatory sequence (MRS) element. To identify sequences important for up-regulation of the rat *ckm* gene during myogenesis, a series of constructs that linked portions of the *ckm* promoter to the bacterial CAT gene was generated (Fig. 2). These constructs were then introduced into differentiating C2 mouse myogenic cells, and CAT activity was measured as a function of differentiation. Introduction of a construct that contains 3.0 kbp of promoter (*Nhe* 3.0 kbp) linked to CAT into C2 cells allowed for induction of CAT expression upon differentiation by about four- to fivefold under the assay conditions used here. Thus, sequences able to confer myogenic regulation lie within this 3.0-kbp segment. Progressive 5′ deletions up to and including the *Stu*I site at −1.2 kbp had no effect on this regulatory behavior. However, further 5′ deletions to the *Bsp*MI site at −1.1 kbp resulted in loss of the ability of *ckm* promoter DNA to regulate the introduced CAT gene. Thus, the *Stu*I site at −1190 marks the 5′ boundary of an MRS element in this *ckm* promoter.

A series of internal deletions was then similarly tested to locate the 3′ boundary of the MRS. As expected, deletion of the immediate promoter region from −168 to +14, which included the putative TATA box (Δ*Nco-Bgl*), led to complete loss of CAT activity in both differentiated and undifferentiated cells. However, deletion of sequences from −480 to −168 (Δ*Nhe-Nco*) or from −1031 to −480 (Δ*Bam*d-*Nhe*) had no effect on the ability of the *ckm* promoter to support regulated CAT expression during myogenesis. Conversely, deletion from −1063 to −480 (Δ*Hin*d-*Nhe*) impaired myogenic regulation. Taken together, these results suggest that sequences important for myogenic regulation lie between the *Stu*I site at −1190 and the distal *Bam*HI site at −1031. In support of this conclusion, deletions that extended into this region resulted in loss of regulatory activity. A 5′ deletion to the *Bsp*MI site at −1.1 kbp (*Bsp*MI 1.1 kbp) or a 3′ deletion from the *Nhe*I site at −0.48 kbp to the *Hin*dIII site at −1063 (Δ*Hin*d-*Nhe*) profoundly affected myogenic regulation.

The MRS is a muscle-specific enhancer element. To test whether the MRS (the sequence between the *Stu*I site at −1190 and the *Bam*HI site at −1031) was an enhancerlike element, constructs were made in which the MRS was placed either 3′ to the CAT gene or further 5′ to the CAT gene. Because of the circular nature of the introduced plasmid DNA, the 3′ MRS constructs can also be viewed as constructs that move the MRS further 5′ to the CAT gene. The 3′ MRS constructs were therefore designed so that the MRS orientation was inverted in the 5′ context. In each of

FIG. 1. Comparative sequence analysis in the 5′ region of the rat and mouse *ckm* genes. The potential TATA box and three footprinted regions (E1, E2, and E3) within the rat muscle *ckm* enhancer are highlighted. Sequence for the rat *ckm* gene runs from −1512 to +170, whereas that for the mouse *ckm* gene runs from −740 to +170. Where both sequences are presented, the rat sequence is on the top line in bold type and the mouse sequence is on the bottom line. Sequences are numbered, and a dot is placed every 10 bp. The bent arrow at +1 indicates the start point of transcription. The junction between the first exon and the first intron is at position +47, and intron sequences thereafter are shown in lowercase.




1kb
Map
Nhe I
Sst I
Xba I
Stu I
Bam H I
Hind III
Bam H I
Nhe I
Nco I
5' DELETIONS
Nhe 3.0kb
CAT
4–5
Xba 1.5kb
4–5
Stu 1.2kb
4–5
Hind 1.0kb
1
Nhe 0.47kb
1
INTERNAL DELETIONS
Δ NcoBgl
-
Δ NheNco
4–5
Δ HindNhe
1.5
Δ BamdNhe
4–5
ENHANCER DELETIONS
BspM1
BspM 1.1 (Δ E1)
1.5
Δ E2
4–5
Δ E3
4–5
Hind 1.0 (Δ E1 + E2)
1
Δ XbaHind (Δ E1 + E2)
1–2
POSITION EFFECTS
3' mrs–a
Hind Xba
<1
3' mrs–b
Bam H I Sst I
4
mrs move
Sal I
1.4

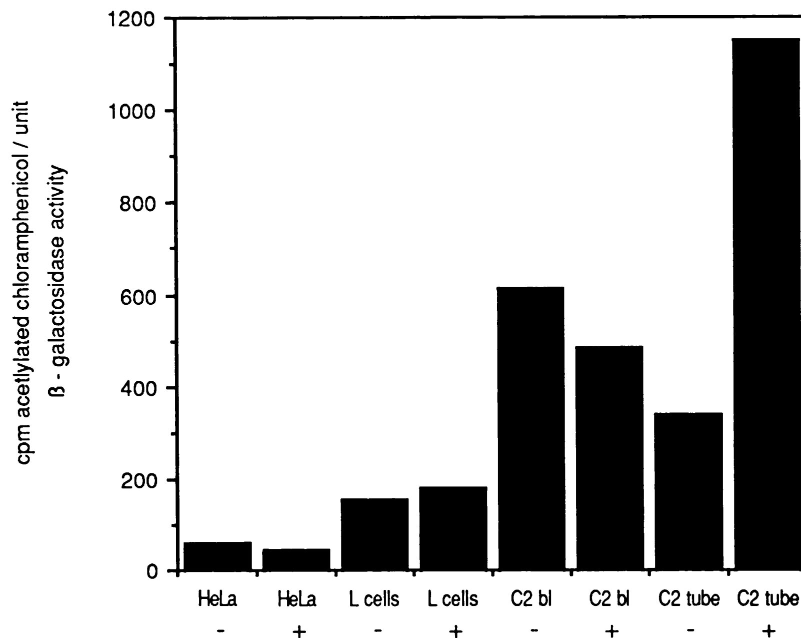


FIG. 3. Transient expression of CAT activity under control of the *ckm* promoter in various cell lines. Constructs containing (+; *Xba* 1.5 kbp) or deleted in (−; *Nhe* 0.5 kbp) the enhancer were transfected into cells in the presence of plasmid pTB-1 as an internal control. Plasmid pTB1 contains the Rous sarcoma virus enhancer-promoter linked to the β-galactosidase gene and was obtained from Terete Borras, National Institutes of Health, Bethesda, Md. For each transfection, 20 μg of test plasmid and 1 μg of control plasmid pTB1 were used per 3×10^5 cells. C2 cells were split 24 h posttransfection and maintained as myoblasts (bl) or allowed to fuse to myotubes (tube) (see Materials and Methods). Cells were harvested 68 h posttransfection and assayed for β-galactosidase and CAT activities. Acetylated chloramphenicol was quantitated by removal of spots from thin-layer plates and liquid scintillation counting. CAT activities are represented as average counts per minute of acetylated chloramphenicol per unit of β-galactosidase activity for three experiments. The relative levels of expression between myotubes and myoblasts are probably artificially lowered, since the control Rous sarcoma virus promoter-enhancer is up-regulated during myogenesis.

these constructs, the promoter region was provided by DNA that extended from the transcript start point to the *Nhe*I site at −0.48 kbp (Fig. 2). In a position 3′ to the CAT gene (3′ MRS-b), the MRS was able to confer myogenic regulation upon the CAT gene. Therefore, by virtue of the fact that it was position and orientation independent, the MRS appeared to have the properties of an enhancer element. This conclusion is in agreement with previous results presented for the mouse *ckm* gene (34, 69). A shorter version of the MRS that lacks sequence 3′ to the *Hin*dIII site at −1063 was, however, lacking in regulatory activity when placed 3′ to the CAT gene (MRS-a) or further 5′ to the CAT gene (MRS-move). This result suggests that sequences 3′ to the *Hin*dIII site at −1063 and 5′ to the distal *Bam*HI site at −1031 are important for enhancer function in the context of the *Nhe*I (0.48-kbp) promoter and supports the conclusions derived by comparing the regulatory activities of the Δ*Hind*-*Nhe* and Δ*Bam*d-*Nhe* constructs. However, deletion of this sequence alone (ΔE3) in the context of a longer promoter was without effect.

Although the MRS affected differential expression of an attached CAT gene in myogenic cells, it had no effect on CAT expression in nonmyogenic cells (L cells and HeLa cells) (Fig. 3). Therefore, the MRS is a muscle-specific enhancer element. It is not promoter specific, since replacement of the *ckm* promoter region downstream of the *Nco*I site with the herpes simplex virus *tk* promoter still resulted in increased regulated CAT expression (by a factor of 5) in differentiating C2 cells (data not shown).

Localization of protein-binding sites in the *ckm* enhancer region by DNase I footprinting. DNase I footprinting experiments were performed on the *ckm* enhancer to localize binding sites for potential regulatory proteins, using nuclear extracts prepared by two different protocols. Both extracts were prepared from well-differentiated myotubes. Extract CT1 was prepared by the method of Dignam et al. (17), using a 0.5 M KCl salt wash, whereas CT6 was prepared by the method of Shapiro et al. (65). The probe was either a 159-bp *Stu*I-*Bam*HI fragment that extended from −1190 to −1031 or a longer *Pvu*II-*Bal*I fragment that extended from −1326 to −1001. Each contained the entire *ckm* enhancer region. The two extracts generated different degrees of protection. First, an extract (CT6) prepared by the method of Shapiro et al. (65) was tested. On the coding strand, modifications to the

FIG. 2. CAT constructs used in transfection assays. A map of the rat *ckm* promoter region is shown at the top. The bent arrow denotes the start point of transcription. Constructs are named as indicated at the left of the figure according to the restriction sites by which they were generated. Maps of the various deletions linked to the CAT gene are shown in the center. The enhancer region is indicated by a box; for clarity, the E1-, E2-, and E3-containing regions are shown as differently hatched elements. The degree of CAT induction as assayed by transfection into C2 cells, i.e., CAT activity in myotubes/CAT activity in myoblasts, is shown on the right. In the MRS-move construct, the enhancer region is placed 5′ to a 6-kbp portion of *ckm* promoter DNA that has been internally deleted for the 3-kbp *Nhe*I fragment. It is therefore now 3 kbp upstream of the transcript start point, using the *Nhe* 0.5-kbp region as the promoter. Each construct was assayed at least three times. Results are quantitated by excision of radioactive spots from thin-layer plates and liquid scintillation counting.

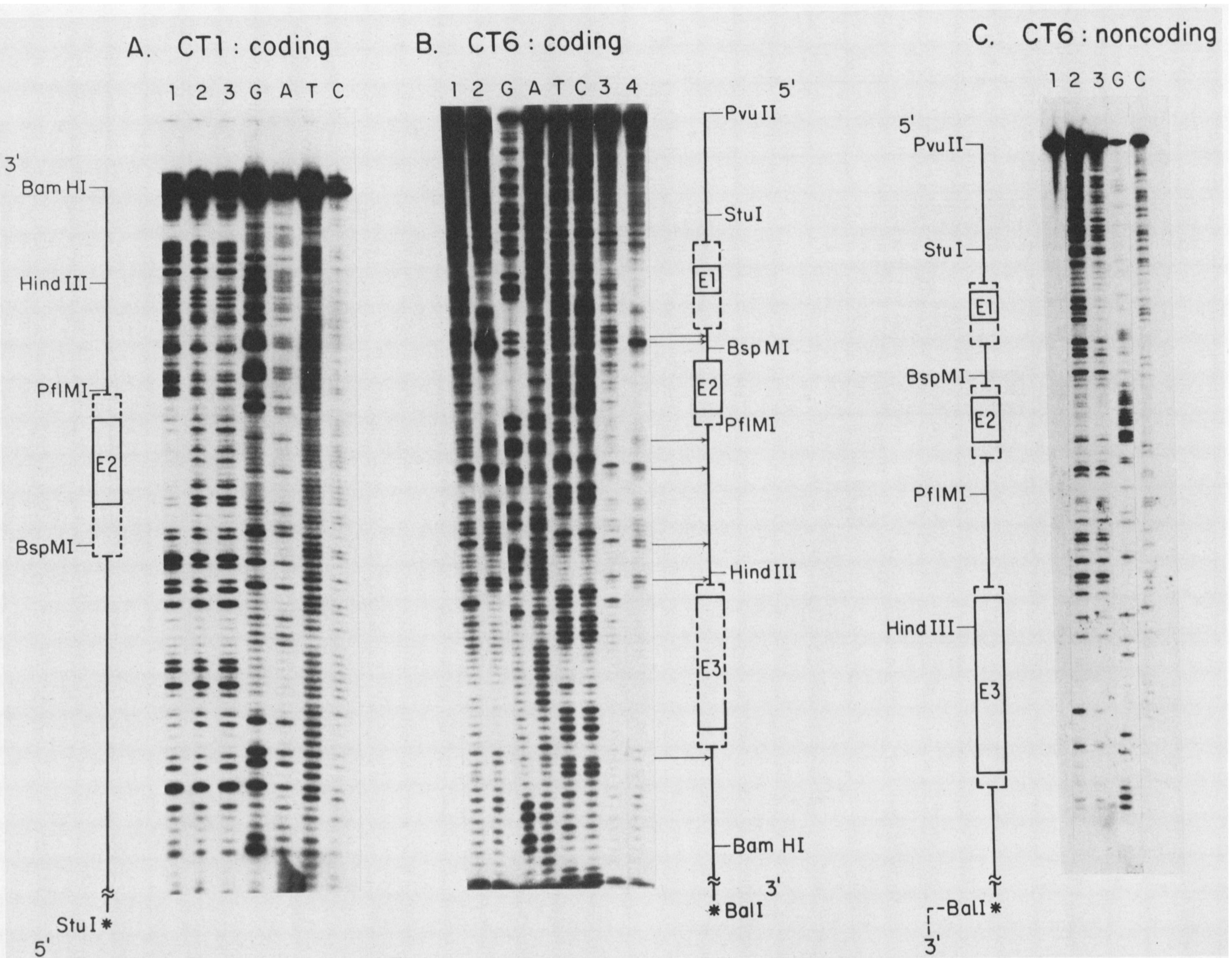

A. CT1 : coding
1 2 3 G A T C
3'
Bam HI
Hind III
PflMI
E2
BspMI
StuI *
5'
B. CT6 : coding
1 2 G A T C 3 4
5'
PvuII
StuI
E1
Bsp MI
E2
PflMI
Hind III
E3
Bam HI
3'
* BalI
C. CT6 : noncoding
1 2 3 G C
5'
PvuII
StuI
E1
BspMI
E2
PflMI
Hind III
E3
BalI *
3'

FIG. 4. Footprinting the *ckm* enhancer region. (A) Footprinting on the coding strand, using CT1, a myotube extract prepared by the procedure of Dignam et al. (17). The fragment used was the *Bam*HI-*Stu*I fragment derived from the *ckm* enhancer region ^{32}P end labeled at the *Stu*I site with polynucleotide kinase. Lanes: 1, protected DNA pattern obtained when DNase I digestion of 0.2 ng of DNA was performed in the presence of 4 μg of poly(dI-dC) and 8 μl of CT1 extract; 2 and 3, DNase I digestion patterns obtained under the same conditions but in the absence of extract. (B) Footprinting on the coding strand, using CT6, a C2 myotube extract prepared by the procedure of Shapiro et al. (65). DNase I digestion of 0.1 ng of DNA was performed in the presence of 3 μg of poly(dI-dC) and 8 μl of extract (lanes 2 and 4) or in the presence of poly(dI-dC) alone (lanes 1 and 3). Lanes 1 and 2 represent a fivefold overexposure of lanes 3 and 4 to show protection of the E3 region more clearly. The substrate fragment used was the *Bal*I-*Pvu*II fragment that includes the *ckm* enhancer region. This was ^{32}P end labeled on the 3′ end at the *Bal*I site by filling in with the Klenow fragment of DNA polymerase I. (C) Footprinting on the noncoding strand at the *Bal*I site as described for panel B, using the same fragment ^{32}P 5′ end labeled at the *Eco*RI site (Materials and Methods) on the noncoding strand with polynucleotide kinase. Lanes: 1, incubation of fragment with extract alone (no endogenous nicking activity was detected under these conditions); 3, digestion of 0.1 ng of fragment in the presence of 3 μg of poly(dI-dC) and 6 μl of CT6 extract; 2, an equivalent digestion in the absence of extract. For all panels, lanes G, A, T, and C are Maxam and Gilbert (40) marker sequencing lanes. Beside each footprint is shown a schematic of the enhancer region sequenced. Landmark restriction sites are indicated. Protected regions are shown by boxed areas; uncertain but possible extents of protection are indicated by dashed lines. These uncertainties result from the absence of DNase I cleavage sites in these regions or from partial band protection. Hypersensitive sites are indicated by arrows extending from the footprint to the schematic. The site of label addition is indicated by an asterisk.

DNase I digestion pattern were observed all the way from −1167 to −1041 and covered a stretch of 127 bp (Fig. 4B). However, the second extract, CT1, prepared as described by Dignam et al. (17), produced a more limited pattern of resistance to DNase I (Fig. 4A). A prominent region of protection centered on a repeat of the motif CACCC, which lies in the middle of the region protected by CT6, was observed. We have designated this region E2. It lies in the center of the enhancer region between −1116 and −1093. This latter result with CT1 was surprising, since CAT transfection assays suggested that sequences both 5′ (5′ to the *Bsp*MI site at −1120) and 3′ (3′ to the *Hin*dIII site at −1063) to this motif were important for enhancer function. Thus, the more extensive pattern of protection observed with extract CT6 is more in keeping with the results of gene transfer experiments (Fig. 2 and 3) that defined the 5′ and 3′ limits of a functional enhancer. However, the results of footprinting with extract CT1 combined with those in which CT6 was used lead us to suggest that the enhancer contains at least three protein-binding (and possibly functional) domains, E1, E2, and E3. These are defined somewhat arbitrarily as follows. Footprinting with the extract CT6 indicates that the area E2 is protected sequence plus sequence both 5′ and 3′ to E2. To the 5′ side of E2 is a region where extensive modification of the DNase I digestion pattern occurs on the coding strand from −1167 to −1124. We have designated this the E1 region. This region potentially covers a 43-bp stretch of sequence that is highly A+T rich at its 5′ end. Within this region is a more completely protected section, termed the E1 core, that extends from −1140 to −1155 on the coding strand. To the 3′ side of E2 is a third area of protection, E3. Again, the sequence protected is highly A+T rich. The E3 segment lies between the *Hin*dIII site at −1063 and the *Bam*HI site at −1031, a region implicated as important for enhancer function by the transient gene transfer experiments shown in Fig. 2 and 3. On the coding strand are observed several hypersensitive sites between E1 and E2 and between E2 and E3. No hypersensitive sites have been observed on the noncoding strand (Fig. 4C). On the coding strand, the modification of the DNase I cutting pattern is observed almost continuously through the enhancer region. On the noncoding strand, however, there appear to be nonprotected regions, particularly between E2 and E3, and a lower level of protection between the E1 core and E2 (Fig. 4C). The results of footprinting experiments are summarized in Fig. 5.

Comparison between the rat and mouse *ckm* enhancer regions. A muscle-specific enhancer element has been previously described for the mouse *ckm* gene (34, 69). This enhancer element is found in the same position relative to the transcript start point as that we have described here for the rat gene. The two enhancer regions are shown aligned in Fig. 6. Jaynes et al. (33) have noted that certain regions of the mouse enhancer sequence show homology with other, more completely characterized enhancers, e.g., the SV40 enhancer and some of the immunoglobulin enhancers. These regions of homology are also indicated in Fig. 6. In addition, Buskin and Hauschka (7a) have described a factor, MEF1, that is myocyte specific and recognizes sequences between −1114 and −1134. This region, which we have called E4, lies in the junction region between E1 and E2. Buskin and Hauschka have concluded that binding of MEF1 to the enhancer region may be important for regulation of *ckm* expression during myogenesis in their system (7a). The regions E1, E2, and E3 that we have defined for the rat *ckm* enhancer are all distinct from the region E4 recognized by

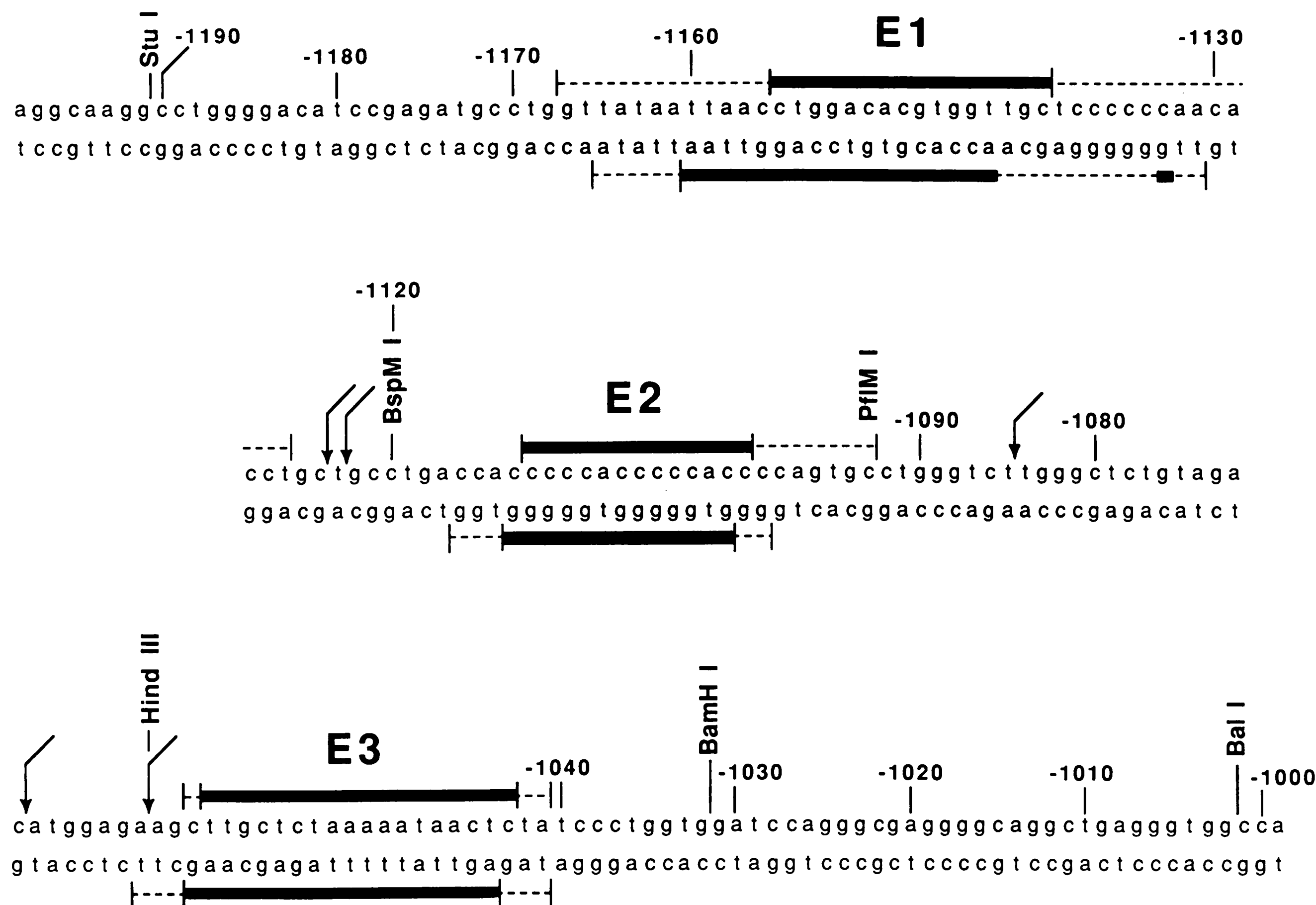


FIG. 5. Summary of footprinting results presented in Fig. 4. Landmark restriction sites are indicated. Sequence is shown from −1198 to −999 and includes the entire rat *ckm* 5′ enhancer region. Footprinted areas on both strands are indicated by bars above and below the sequence. As in Fig. 4, dashed lines indicate less definitive areas of protection. Vertical arrows indicate the positions of DNase I-hypersensitive sites.

MEF1. However, footprinting in the E1 region potentially extends into and possibly includes E4. The E1 core region corresponds to the region that has been described as showing homology with the IgK enhancer (33). However, this core contains several sequence changes between rat and mouse genes, and the homology between rat E1 and IgK is less pronounced than that between mouse E1 and IgK. E2 and E3 fall in regions that have not been described as showing homology with other known enhancers, whereas the MEF1 region (E4) lies in a region reported to show homology with the IgH enhancer. As expected, homology between the rat and mouse *ckm* genes in the enhancer region is high (85%), although gaps must be introduced in three places to achieve maximum alignment. None of the regions E1 to E3 is absolutely conserved between the rat and mouse genes; in fact, the E1 core region falls in one of the less conserved (76%) sections of the sequence. Regions E2 and E3 are about equal in sequence conservation, and region E4 is 100% conserved.

Effects of deletion of enhancer elements. To test further the functional role of the multiple enhancer elements in the rat *ckm* enhancer, each element was deleted individually (Fig. 7). A 5′ deletion to the *Bsp*MI [*Bsp*M 1.1 (ΔE1); Fig. 2] site deleted E1 (and also E4) but left E2 and E3. This deletion resulted in almost complete loss of regulatory activity, although a small degree (1.5-fold) of regulation remained. Deletion of E2 alone with use of a promoter that extended to the *Xba*I site at −1.4 kbp (ΔE2; Fig. 2) was completely able to regulate CAT expression in differentiating C2 myotubes. Similarly, deletion of E3 alone with the same promoter (ΔE3; Fig. 2) had no effect on regulation. A 5′ deletion to the *Hin*dIII site at −1063 [*Hind* 1.0 (ΔE1 + E2); Fig. 2] that deleted E1 and E2 (and E4), however, resulted in complete loss of regulatory activity. We have not tested the effect of deletion of E1 or E4 alone in the context of the *Xba*I (1.4-kbp) promoter. In addition to effects on regulation, some of the constructs tested in Fig. 7 resulted in apparent alterations in absolute levels of expression in myoblasts, e.g., between ΔE1 and ΔE2. The significance of these variations is being investigated further in the presence of control plasmids that will allow us to make comparisons of both levels of expression between constructs and levels of regulation within a construct.

Interaction of the domains of the muscle-specific enhancer element with potential regulatory molecules. The gel retardation assay was used as a complement to footprinting assays to examine the interaction of the rat *ckm* enhancer with potential regulatory proteins. A *Bam*HI-*Stu*I fragment was used as a probe (Fig. 8). This probe contains the entire minimal enhancer region. The enhancer fragment was incubated with extracts derived from well-differentiated C2 myotubes, C2 myoblasts, HeLa cells, L cells, and liver and

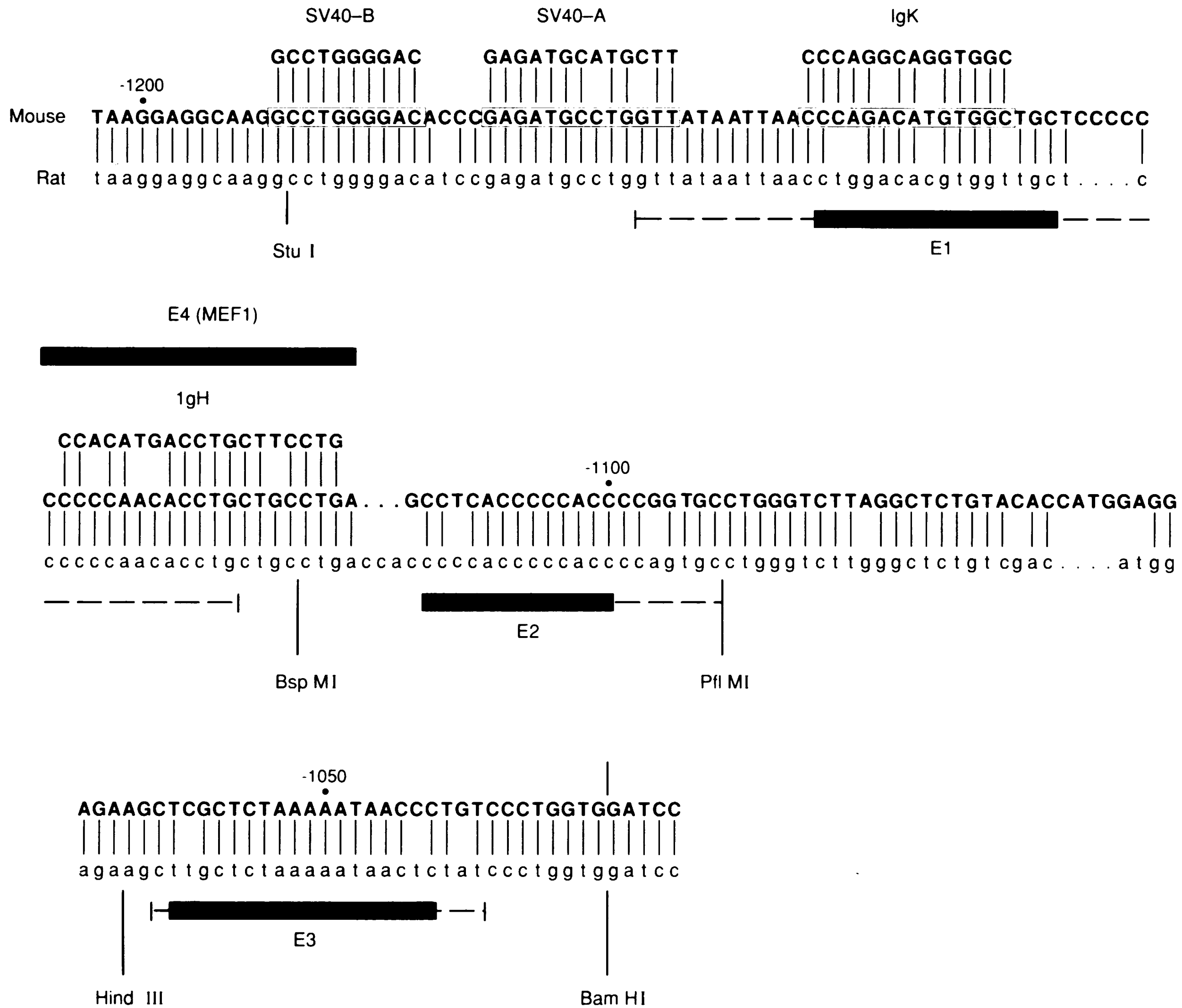


FIG. 6. Comparison of the 5′ muscle-specific enhancers for the rat and mouse *ckm* genes between −1203 and −1027 (rat numbering system). The mouse sequence, shown on top, is taken from Jaynes et al. (34). Footprinted regions E1, E2, and E3 for the rat *ckm* gene are shown as a combination of bars and dashed lines below each line, using the convention described for Fig. 5. The location of the MEF1-binding region E4 (Buskin and Hauschka [7a]) is shown above the line, as are regions of homology with the SV40 enhancer, the AP-2 binding region, and the IgK enhancer (34, 69). Regions of SV40 and IgK homology are boxed, and landmark restriction sites are indicated.

brain extracts. A major retarded band, B_{E1}, was observed with all extracts tested (Fig. 8). This band was specifically absent when a synthetic oligodeoxynucleotide that matched the E1 core sequence (5′-CCTGGTTATAATTAACCT GGACACGTGGTTGCTGCC-3′) was included in the binding reaction, but not when an unrelated oligodeoxynucleotide was included. This result confirms the footprinting analysis data in that it suggests the existence in these nuclear extracts of an activity that specifically recognizes the E1 sequence region. This binding activity was present in all sources examined, including C2 myotubes, HeLa cells, rat liver and brain, and C2 myoblasts (Fig. 8).

In addition, a second retarded band, B_{E2a}, B_{E2b}, or B_{E2c}, was observed with extracts derived from C2 cells, HeLa cells, L cells, and brain. This band appeared to be absent from liver nuclear extracts. Band B_{E2a} or B_{E2b} was specifically absent when an oligodeoxynucleotide that contained the E2 sequence, 5′-GATCCTGACCACCCCCACCCCCAC CCCAGTGCCA-3′, was included in the binding reaction, but not when an unrelated oligodeoxynucleotide or an oligodeoxynucleotide that matched the E1 core or E3 sequence was included (Fig. 8). This result too is consistent with footprinting results (Fig. 4 and 5) indicating binding to the E2 region. The activity that competed with the E2 oligodeoxynucleotide appeared to be present in C2 cells (myoblasts and myotubes), brain extract, and HeLa cells but absent from liver extract. There was a tendency for the intensity of B_{E2a} and B_{E2b} to increase in the presence of E1-competing oligodeoxynucleotide, particularly in brain extract. This slowly migrating band was also absent from L-cell extracts, although B_{E2c} had some of the properties of B_{E2a} and B_{E2b} but migrated much more slowly.

Under the conditions described, the gel retardation assay provides no support for binding to the E3 region when the entire *Stu*I-*Bam*HI enhancer fragment is used as a probe in that no retarded band was specifically absent when a competing E3 oligodeoxynucleotide was included in the binding reaction. However, footprinting experiments indicated

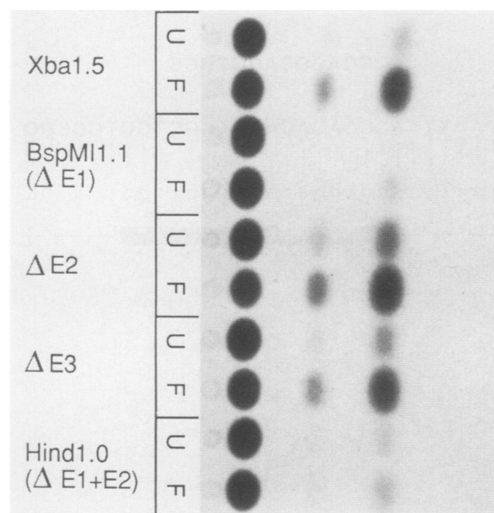


FIG. 7. Analysis of CAT constructs deleted in enhancer elements. Constructs containing the wild-type enhancer (*Xba* 1.5 kbp) or deleted in E1, E2, E3, or E1 plus E2 were transfected into C2 myoblasts as described in Materials and Methods. CAT assays were performed on equivalent amounts of protein. Chloramphenicol and its acetylated forms were separated by thin-layer chromatography. After autoradiography, ^{14}C-labeled spots were excised and assayed by scintillation counting. Relative ratios of CAT activity in myoblasts and myotubes are given in Fig. 2. U, Unfused cells (myoblasts); F, fused cells (myotubes).

protection in this region. Therefore, to test for binding to this se-quence, a synthetic E3 oligodeoxynucleotide sequence, 5'-AAGCTTGCTCTAAAAATAACTCTATCCC-3', was used as a probe. In this situation, a highly retarded smear, B_1 (Fig. 9), was generated that was removed in the presence of the same E3 oligodeoxynucleotide but not in the presence of a second nonspecific oligodeoxynucleotide. Similar highly retarded species were observed with C2 myotube, HeLa, and brain extracts but not with extracts derived from liver tissue (Fig. 9). In addition, HeLa extracts resulted in a second retarded band, B_2, that was also absent when an E3 oligodeoxynucleotide competitor was included. Therefore, we conclude that an E3-binding activity was observed in nuclear extracts derived from C2C12 cell, HeLa, and brain extracts but not liver extracts. Additional bands were observed in C2C12, HeLa, and brain extracts. These bands were not effectively removed in the presence of either the E3 oligodeoxynucleotide or the nonspecific oligodeoxynucleotide. The origin of these retarded species is unclear, but they may have originated from nonspecific binding to the kinase-labeled probe that was not competed against by the non-kinase-labeled competitors.

DISCUSSION

The *ckm* muscle-specific enhancer element. In this study, we localized a muscle-specific enhancer element in the 5' region of the rat *ckm* gene. This enhancer element was found approximately 1 kbp upstream of the transcription start point, a location equivalent to that of the enhancer element described previously for the mouse *ckm* gene (34, 69). We investigated the minimal sequence requirements for this enhancer to function in the context of a particular rat *ckm* promoter unit. This promoter unit is defined by the sequence from the transcript start point to an *Nhe*I site at −480. With this promoter unit, we defined a minimal enhancer segment that covers a stretch of 159 bp between the *Stu*I site at −1190 and the distal *Bam*HI site at −1031. Functional tests on deletion mutants indicate the presence of multiple domains within this enhancer region that contribute to the overall up-regulated expression observed for the *ckm* gene during C2 differentiation. Deletion of sequences included within either the 5' or 3' region of this minimal enhancer element impairs the ability of the enhancer to confer up-regulation of CAT expression driven by the *ckm* (*Nhe* 0.48 kbp) promoter.

Using our transfection protocol, we observed a four- to fivefold induction of rat *ckm* promoter-driven CAT expression upon C2 cell differentiation. This finding contrasts with the results of previous workers (34, 69), who observed a 10- to 100-fold induction of mouse *ckm* promoter CAT constructs in MM14 cells (34) and C2 cells (69). In our study, the degree of up-regulation of *ckm* promoter CAT constructs is sensitive to transfection conditions. We find 10- to 100-fold regulation by using different recipient cell densities in our transfection protocol. For the skeletal muscle α-actin gene, it has been shown that although the endogenous gene is regulated during myogenesis, introduced α-actin promoter-driven CAT constructs are not (41, 63). This has led to proposal of a two-step model for α-actin gene regulation which suggests that factors necessary for α-actin promoter up-regulation are already present in undifferentiated C2 cells but that the endogenous gene is inaccessible (41). Nevertheless, these factors can stimulate expression of introduced α-actin promoter constructs. Clearly, the rat and mouse *ckm* promoters can be regulated in C2 cells with use of an introduced CAT reporter gene construct. Whatever conditions are used, these gene transfer experiments allow us to study sequences in the rat *ckm* promoter that are able to confer myogenic regulation to a heterologous reporter gene and are in qualitative agreement with the behavior of the endogenous gene and with the results of previous authors who used similar mouse *ckm* promoter constructs.

Footprinting the *ckm* enhancer element. Footprinting experiments on the minimal enhancer element indicate an extensive region of protection, particularly on the coding strand (Fig. 5). Modulation of the DNase I digestion pattern extends all the way from −1167 to −1041. Certain nuclear extracts prepared by the procedure of Dignam et al. (17) protect a much less extensive region that includes a CACCC repeat and runs from −1116 to −1093. On the basis of the combined results of these two experiments, we have defined at least three footprintable regions within the enhancer: E1, which extends from −1167 to −1124, with a core region from −1160 to −1139; E2, which extends from −1116 to −1093; and E3, which extends from −1064 to −1041. Deletion of sequences including either the E1 or E3 region impairs minimal enhancer function. Deletion of E2 has not been tested by using the minimal enhancer linked to the *Nhe* 0.48-kbp promoter. Therefore, the functional significance of E2 remains to be determined. However, the E1 and E3 regions define the 5' and 3' limits, respectively, of the minimal enhancer element. We are currently testing the function of internal sequences such as E2 on minimal enhancer function.

The differences in the footprinting activities of extracts prepared by different methods can be explained in several ways. The principal differences between the method of Dignam et al. (17) and that of Shapiro et al. (65) lie in the buffers used for nuclear preparation, the speed of isolation of nuclei once cell lysis has occurred, and the final method of

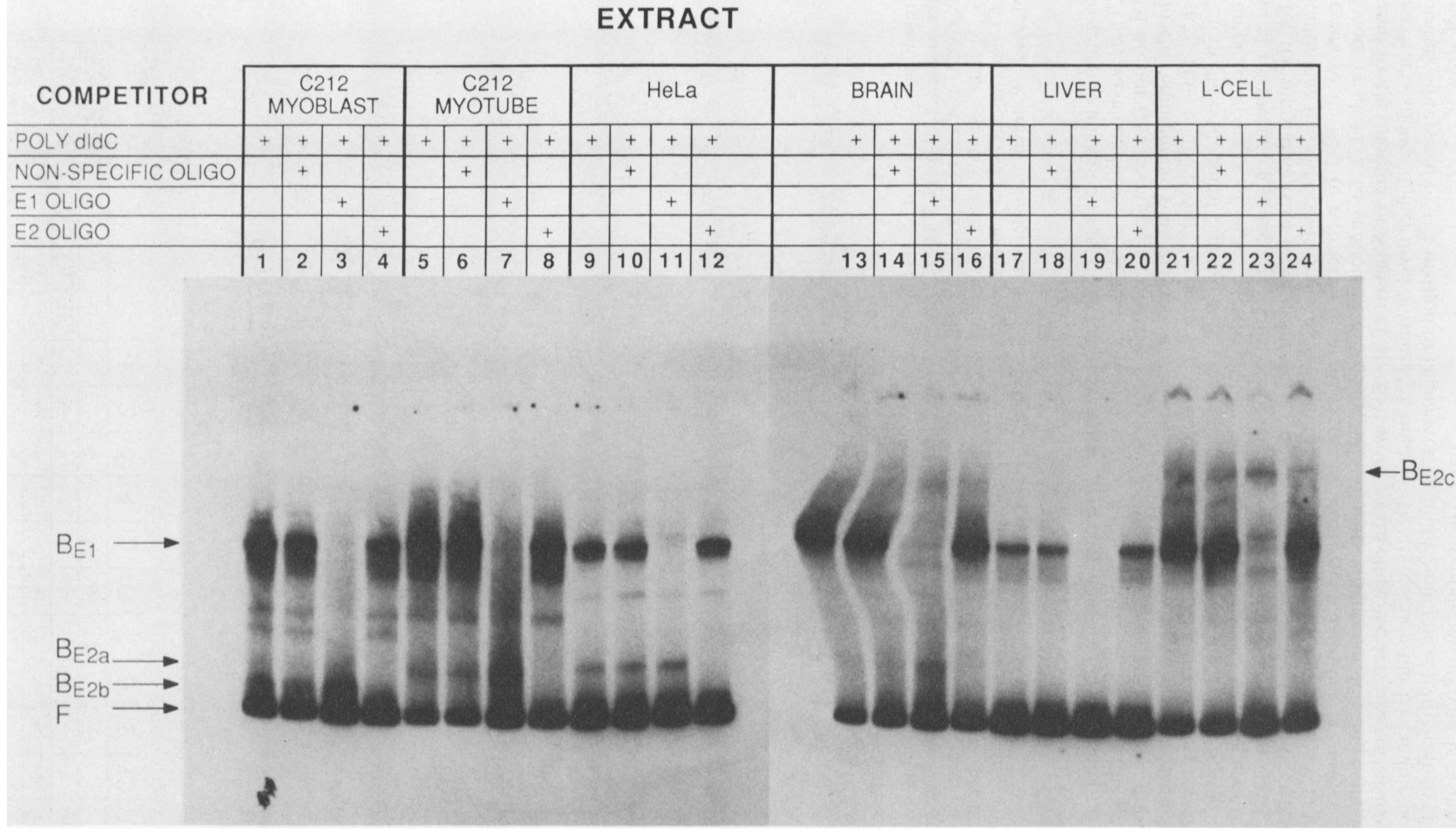


FIG. 8. Tissue distribution of E1- and E2-binding activities as determined by gel retardation assay. A 180-bp *Stu*I-*Bam*HI fragment containing the entire muscle enhancer region was 5′ end labeled by the kinase exchange reaction (38). Then 0.2 ng of labeled probe and 3 μg of nonspecific poly(dI-dC) competitor were added to 3 to 10 μg of nuclear extracts from six different tissue sources. Each sample was challenged with a double-stranded nonspecific oligodeoxynucleotide (5′-TCACGCCCTGGGGCAGCCCATACAAGG-3′) or with a double-stranded synthetic version of enhancer element E1 (5′-CCTGGTTATAATTAACCTGGACACGTG GTTGCTGCC-3′) or E2 (5′-GATCCTGACCACCCCCACCCCCACCCCAGTGCCA-3′), as indicated. F, Free probe; B_{E1}, a retarded band specifically absent when oligonucleotide E1 was included; B_{E2a}, B_{E2b}, and B_{E2c}, retarded bands specifically absent when oligonucleotide E2 was included.

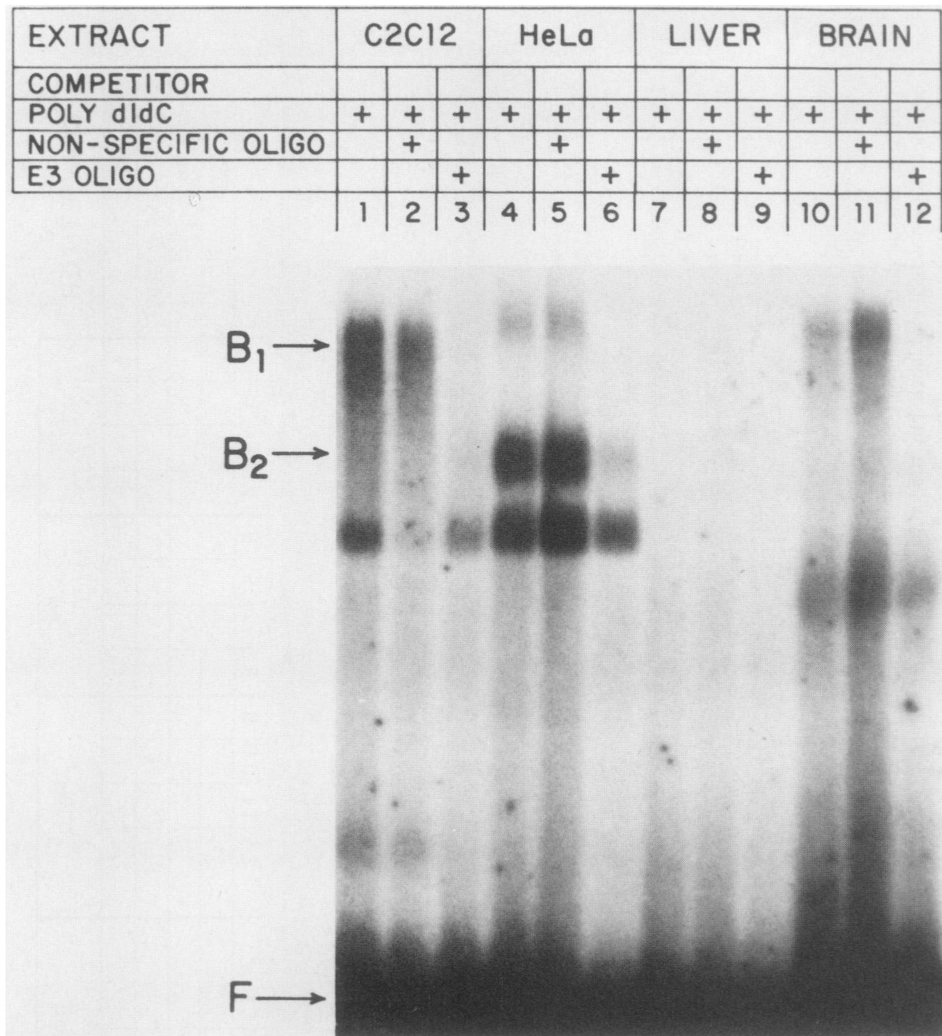


FIG. 9. Gel retardation assays to study binding to enhancer element E3. The probe used was a double-stranded synthetic oligodeoxynucleotide with the sequence 5'-AAGCTTCTCTAAAAATAACTCTATCCC-3'. The nonspecific oligodeoxynucleotide had the sequence 5'-CACGCCCTGGGGCAGCCCATACAAGC-3'. The E3-specific oligodeoxynucleotide competitor was the same as the probe. F, Free probe; B_1 and B_2, specific retarded bands. Each reaction contained 0.4 ng of 5'-^{32}P-labeled probe at a specific activity of 2×10^7 cpm/μg, 4 μg of poly(dI-dC) (Pharmacia), and a 150-fold excess of specific or nonspecific competitor. Otherwise, conditions were as described in Materials and Methods. Extracts were derived from C2 myotubes, HeLa cells, liver, or brain.

nuclear lysis to generate nuclear extracts. We find that under the 0.42 M KCl salt wash conditions of Dignam et al. (17), C2 myotube nuclei do not lyse completely, whereas complete lysis occurs in the ammonium sulfate lysis procedure of Shapiro et al. (65). This, coupled with the increased speed of nuclear isolation in the protocol of Shapiro et al., may have led to more complete recovery of intact nuclear proteins in extracts prepared by the latter procedure. This possibility may account for the more extensive footprinting seen with these extracts, although it is interesting that protection of the E2 region appears to be best in extracts prepared by the method of Dignam et al. (17). In support of the quality of extracts prepared by the method of Shapiro et al. (65), we find that these extracts show a higher level of transcriptional activity than do those prepared by the method of Dignam et al. (17) (data not shown).

Tissue specificity of protein binding to the *ckm* enhancer element. Each of the footprintable domains within the minimal enhancer unit appears to be a binding site for a sequence-specific DNA-binding protein or proteins. This can be demonstrated by using the gel retardation assay, which also allows us to examine more conveniently the tissue distribution of each of these binding activities. The activity that recognizes the E1 core region appears to be found in all sources examined. This is true also for the activities that recognize E2 and E3, although these latter activities appear to be absent from liver extracts. The absence from liver could be an artifact of extract preparation. This is unlikely, however, since these liver extracts are transcriptionally competent for other promoters, including the rat *ckb* and adenovirus promoters and compare favorably in this respect with other extracts used in the assay. Also, the liver extracts clearly contain the E1-binding activity. The absence of E2- and E3-binding activity from liver is of potential significance. The E3-binding activity is also able to bind sequences that are believed to be important for function of the rat *ckb* promoter (R. A. Horlick et al., manuscript in preparation). We postulate that the E3-binding activity plays a role in *ckm*

and *ckb* expression. Liver is one of the few tissues in which neither *ckm* or *ckb* appears to be expressed.

The E2 region contains a CACCC repeat motif. This sequence motif has been shown to have regulatory significance for the β-globin gene (46) and the tryptophan oxidase gene (62). In addition, the E2 region shows an 18-of-25 match with a sequence important for α-crystallin expression (9). The latter observation is of interest since it has been shown that CKM is expressed in the lens of the eye (Perryman, personal communication). The possible E2-binding activity from L cells appears to migrate differently from that found in C2 cells and HeLa cells (Fig. 8). This phenomenon is currently being investigated in more detail. In conclusion, binding to the multiple sequences E1 core, E2, and E3 that we have defined within the muscle-specific enhancer does not appear to be a muscle- or a myotube-specific phenomenon, although the enhancer displays muscle-specific activity.

An anomalous observation is our inability to demonstrate an activity that competes with the E3 sequence by using the *Stu*I-*Bam*HI fragment in a gel retardation assay. This result is in contrast to the demonstration of sequence-specific binding to this motif by using a synthetic oligodeoxynucleotide probe. Nevertheless, footprinting experiments demonstrate that binding can probably be observed simultaneously to the E1, E2, and E3 regions of the enhancer. This binding appears to be a function of conditions used in the gel shift assay, and we have recently determined conditions in which E3 binding can be observed on the enhancer fragment by using this assay. These conditions require the use of *Escherichia coli* DNA instead of poly(dI-dC) as a nonspecific DNA competitor and have led to the observation that the E3 domain is homologous to the A+T-rich region at the 5' end of the E1 core sequence (Horlick et al., in preparation).

When assayed with the *Nhe* 0.48-kbp promoter, E1 and E3 appear to be potential functional elements. Deletions that include either of these regions impair enhancer activity. Deletions of regions internal to the enhancer have not been made in this promoter context. However, internal deletion of either E2 or E3 had no effect on enhancer function with use of the wild-type, noninternally deleted promoter. Since E3 appears to be important for function in shorter promoter constructs, the simplest explanation for this result is that E3 is redundant and that additional E3 sequences, or sequences that can substitute for E3, are found within the *ckm* promoter-enhancer region. This hypothesis is currently being investigated. There are at least four possible reasons for the lack of effect of deletion of E2 (ΔE2; Fig. 2): (i) E2 is functionless, (ii) E2 is redundant in a manner similar to that proposed for E3, (iii) the function of E2 is not assayable by using transient transfer into differentiating C2 cells, and (iv) E2 functions to regulate the gene in another context, e.g., lens.

Buskin and Hauschka (7a) have described studies of the interaction of the mouse *ckm* muscle-specific enhancer with potential regulatory proteins. They found binding of a myocyte-specific factor, MEF1, to a region within the enhancer that we have designated E4. E4 lies at the junction between E1 and E2. In our hands, rat *ckm* enhancer activity is lost with removal of sequences between the *Stu*I site (−1190) and the *Bsp*MI site (−1120). This region contains our strongly footprinted E1 segment, region E4, and the region showing homology to the SV40 enhancer and potential Ap-2-binding site (32, 33, 43, 69). We have no data on the effect of deletion of E1 alone. Therefore, loss of enhancer activity could result from loss of any of these elements, singly or in combination. Our footprinting and gel shift experiments clearly indicate binding to the E1 region, and the footprinting data are at least consistent with binding in the E4 region, although this is not as prominent in our study as is binding elsewhere. Footprinting in the E1 region extends into an A+T-rich segment at the 5' end, although the most highly protected area is not A+T rich. It is possible that part of the binding we observe in the E1 region represents a topoisomeraselike activity or one of the other activities that have been reported to interact with A+T-rich DNA (21, 67). At the 3' end of E1, our footprinting data (Fig. 4 and 5) on the coding strand suggest extensive protection that would include the E4 region, where several DNase I-hypersensitive sites are observed. However, little protection is seen in this region on the noncoding strand. This observation does not rule out the possibility of binding to the E4 region. First, some well-characterized DNA-binding proteins show protection on only one strand in a DNase I protection assay (55). Second, since we observe that the mode of extract preparation can profoundly affect patterns of protection, it is possible that E4-binding activity is deficient in our extracts. We have tried competition with an E4 oligodeoxynucleotide in gel retardation assays and seen no evidence for any binding activity to the enhancer that competes with E4 (data not shown), although Buskin and Hauschka (7a) also report poor competition by their MEF1-binding oligodeoxynucleotide in gel retardation assays. However, we can demonstrate weak specific binding to E4 by using the E4 oligodeoxynucleotide (5'-TGCCCCCAACACCTGCTGCCTGACCAC-3') as a probe in a gel retardation assay (data not shown). Taken together, this result and those of Buskin and Hauschka (7a) suggest that the enhancer may contain at least one other domain (E4).

Buskin and Hauschka (7a) also report footprinting of an A+T-rich region to the 3' side of their MEF1-binding site. This region appears to be equivalent to E3. These authors dismiss this region as having no functional significance in their hands. We present evidence here that E3 is indeed functional when assayed with the appropriate promoter and that individual enhancer elements or equivalents may be repeated elsewhere in the promoter. This has been shown to be the case for several enhancer-promoter combinations. For example, the octamer-binding motif is found in both the enhancer and promoter regions of the immunoglobulin heavy-chain gene (66, 68).

Comparison of the enhancer with other muscle-regulatory signals. How does this muscle-specific enhancer element compare with other muscle-specific regulatory sequences? One muscle-specific regulatory element that has been described is the CArG box (42), C-bar element (5), or CAAT-like sequence (63). This sequence has been shown to be important for muscle-specific expression of the actin genes. Like E3 and part of E1, this is an A+T-rich element with the consensus sequence CC(A/T)$_6$GG. Since E1 and E3 are both A+T-rich, we have asked whether binding to these sequences can be inhibited by an oligodeoxynucleotide (5'-CCTTGGCTCCATGAATGGCCTCGG-3' or 5'-GCGAAGG GGACCAAATAAGGCAAGGTGGC-3') prepared to the functional CArG boxes in the α-actin gene. Neither E1- nor E3-binding activity can be inhibited by a CArG box sequence (data not shown). Thus, E1- and E3-binding activities appear to be distinct from the CArG box recognition activities. As pointed out previously, the *ckm* promoter-enhancer contains two CArG-like sequences, one close to the enhancer and one close to the promoter (69). Deletion 5' to the *Stu*I site at −1190 deletes the upstream CArG-like sequence and yet has no effect on regulation. Therefore, we argue that this element cannot be necessary for up-regulation

driven by the *ckm* promoter in this construct. Similarly, the downstream CArG element alone is unable to support muscle-specific expression of the *ckm* promoter in C2 cells. Also, the promoter alone from +14 to −480 (*Nhe* 0.48 kbp; Fig. 2) appears to show no myotube specificity in C2 cells. In addition, removal of this element (Δ*Nco-Nhe*; Fig. 2) has no effect on regulation, although we have not tested the effect of removing both CArG elements. Therefore, we cannot rule out the possibility that these CArG-like sequences function in other contexts or cellular backgrounds. For example, it has been shown that the CArG sequences in α-actin constructs do not support up-regulation of these constructs in C2 cells (41). These results are in agreement with those of Sternberg et al. (69), who demonstrate that the mouse *ckm* promoter alone is not muscle specific in C2 cells, although Jaynes et al. (34) indicate that the promoter alone does show residual muscle specificity in MM14 cells. It is possible that the multiple regulatory elements of the *ckm* promoter-enhancer region function differently in myogenic cells of different origins and in response to different differentiation signals.

Another muscle-specific enhancer element has been presented for the rat myosin light-chain 1/3 gene; this element covers a 500-bp region (17a) and contains A+T-rich motifs that might be related to E1 and E3. So far, we have no evidence that any of these elements competes efficiently for binding to the *ckm* E1 and E3 elements (data not shown). However, Arnold et al. (1) have described a distal promoter element for the chicken cardiac myosin light-chain 2 gene. This A+T-rich element is important for up-regulation of this gene in primary chicken cardiac cells in culture. Binding to the E3 *ckm* enhancer element is competed against by this sequence (Horlick et al., in preparation). Therefore, E3 may be related to other muscle-specific elements that are important for regulation in cardiac tissue. This may be of significance, since *ckm* is coexpressed with *ckb* in cardiac tissue (71).

How is muscle-specific expression generated by the *ckm* enhancer? None of the factors (E1, E2, and E3) that we have identified that recognize the enhancer element appears to be muscle specific. Similarly, the CArG box binding factor that is important for α-actin regulation appears not to be muscle specific, although this result is in dispute (74, 75). Factor MEF1, described by Buskin and Hauschka (7a) to recognize E4, is reported to be myocyte specific. Thus, muscle specificity may be obtained by a combination of muscle-specific and non-muscle-specific regulators similar to that described for the prealbumin enhancer (13). Since deletion of E3 impairs enhancer activity in certain constructs, E1, E4, and E2 are clearly not sufficient for full muscle-specific regulation. This would imply that MEF1, if myocyte specific, is not alone sufficient for complete myogenic regulation and must interact with non-muscle-specific regulators. It is also possible that muscle specificity is generated by appropriate combinations of non-muscle-specific factors. Multiple interacting elements have been described for several enhancers (14, 25, 29, 52, 60, 61, 79) and may act to regulate both enhancer specificity and enhancer strength. Finally, we do not know whether the binding activities that recognize the different motifs (E1, E3, etc.) within the *ckm* enhancer are the same in different tissues. The activities that we have identified may result from different factors in each tissue that bind the same sequence motif and generate the same gel shift pattern but have different effects on transcriptional regulation.

The demonstration of binding activity in a nuclear extract does not mean that the protein is bound to the regulatory region in the in vivo nucleus. Genomic footprinting techniques (3, 18) should be important in addressing this problem. Interaction of regulators with their target sequences or their activities once bound could be controlled at the biochemical level, e.g., by availability of recognition sequences for binding, modification of regulatory proteins (56), or interaction of regulatory proteins with each other (20), such as that shown between Fos and AP-1 (10) and between AP-2 and T antigen (43), or with auxiliary proteins, such as COUP-1 (58), that do not bind DNA directly. For example, the adenovirus E1a protein regulates gene expression but does not itself interact directly with DNA (19). E1a has been shown to influence muscle differentiation in C2 cells (77). Second, factor binding could be controlled by changes in DNA structure or modification, e.g., methylation. Finally, tissue specificity may be generated by tissue-specific combinations of regulatory proteins, none of which is itself tissue specific. This latter mechanism might allow for coordination of gene-regulatory patterns. Thus, we would argue that the apparent lack of tissue specificity of these enhancer-binding proteins does not necessarily argue against their importance in determining enhancer function.

Comparison with other tissue-specific enhancers. Tissue-specific enhancers have been described for several systems (12, 25, 27, 53). The mechanistic details of enhancer function are not known for any enhancer, but one possibility is that enhancers activate transcription by interaction with promoter elements mediated by protein-protein interactions and DNA bending. It will be interesting to see whether any of the proteins that interact with the *ckm* enhancer interact directly with the *ckm* promoter region or with proteins known to recognize this promoter (C. Earhart-Ohlendorf et al., manuscript in preparation). Multiple functional domains are a common feature of many enhancers (25, 29, 51, 52, 60, 61, 79). Similarly, enhancer elements may show regulatory motifs shared in common with promoter elements. For example, the immunoglobulin octamer motif shown to be important for tissue-specific function of the immunoglobulin promoters is found also within the tissue-specific enhancer located internally to the gene (64). It will be interesting to see how the multiple enhancer domains for the upstream *ckm* enhancer relate to *ckm* promoter function and also how the upstream enhancer is related to that found in the first intron (69). Combined action of enhancer elements has been shown to generate diversity of expression for the α-fetoprotein gene (27). Of further interest will be the relationship of proteins binding this enhancer to gene products now known to specify the myogenic lineage, e.g., *myoD* (15) and *myd* (54).

In conclusion, we have demonstrated that like the mouse *ckm* gene, the rat *ckm* gene contains a muscle-specific enhancer element in its 5′ region. This enhancer element contains multiple protein-binding motifs and multiple functional elements. These elements probably represent a simplified picture of the total regulatory apparatus for this gene. Our current experiments indicate that a much more complex array of both positive and negative *cis*-acting elements exists for this gene. As has been pointed out previously (69), this pattern is distinct from that described for any other muscle-specific gene. This finding may be in part a reflection of the fact that this gene codes for a muscle metabolic function rather than for a major structural component of the contractile apparatus, although CKM has been proposed to serve a structural role in filament assembly (72). This gene may need to respond to differentiation signals as well as signals that monitor the metabolic state of the cell, e.g., rate of ATP

turnover. Indeed, the homologous *ckb* gene is known to respond to hormonal stimuli (57). Studies on the differential regulation of these two genes should lead to insights into gene-regulatory mechanisms and coregulation of gene sets during development and in response to multiple external stimuli.

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ADDENDUM

After submission of the manuscript, the human *ckm* promoter-enhancer sequence was published (70). This sequence shows extensive conservation with the rat and mouse *ckm* sequences in the promoter and 5′ enhancer regions of the gene. Sequence conservation in the enhancer falls markedly 3′ to our E3 region, in support of our conclusion that the E3 region represents a 3′ boundary of functionally important sequences.

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