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Identification of a Myocyte Nuclear Factor That Binds to the Muscle-Specific Enhancer of the Mouse Muscle Creatine Kinase Gene

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The muscle creatine kinase (MCK) gene is transcriptionally induced when skeletal muscle myoblasts differentiate into myocytes. The gene contains two muscle-specific enhancer elements, one located 1,100 nucleotides (nt) 5′ of the transcriptional start site and one located in the first intron. We have used gel mobility shift assays to characterize the *trans*-acting factors that interact with a region of the MCK gene containing the 5′ enhancer. MM14 and C2C12 myocyte nuclear extracts contain a sequence-specific DNA-binding factor which recognizes a site within a 110-nt fragment of the MCK enhancer region shown to be sufficient for enhancer function. Preparative mobility shift gels were combined with DNase I footprinting to determine the site of binding within the 110-nt fragment. Site-directed mutagenesis within the footprinted region produced a 110-nt fragment which did not bind the myocyte factor in vitro. The mutant fragment had about 25-fold-less activity as a transcriptional enhancer in myocytes than did the wild-type fragment. Complementary oligomers containing 21 base pairs spanning the region protected from DNase degradation were also specifically bound by MM14 and C2C12 myocyte nuclear factors. The oligomer-binding activity was not found in nuclear extracts from the corresponding myoblasts, in nuclear extracts from a variety of nonmuscle cell types (including differentiation-defective MM14-DD1 cells and 10T1/2 mesodermal stem cells), or in cytoplasmic extracts. Both the 5′ and intron 1 enhancer-containing fragments competed for factors that bind the oligomer probe, while total mouse genomic DNA and several DNA fragments containing viral and cellular enhancers did not. Interestingly, a 5′ MCK proximal promoter fragment that also contains muscle-specific positive regulatory elements did not compete for factor binding to the oligomer. We have designated the factor which interacts with the two MCK enhancers myocyte-specific enhancer-binding nuclear factor 1 (MEF 1). A consensus for binding sites in muscle-specific regulatory regions is proposed.

Muscle creatine kinase (MCK) gene expression is developmentally controlled and restricted to certain tissues. High levels of MCK protein are found in vertebrate cardiac and skeletal myocytes but not in the myoblast precursors of these cells. The regulation of muscle-specific genes, such as *MCK*, can be conveniently studied in several permanent cell lines, including the mouse skeletal muscle satellite cell line MM14 (35). MM14 myoblasts do not express the muscle-specific genes associated with terminal differentiation when fibroblast growth factor (FGF) is present in the medium, but they rapidly commit to the myocyte phenotype and express these genes upon FGF withdrawal (9).

Our analysis of MCK expression in MM14 cells identified a muscle-specific transcriptional enhancer within a 206-nucleotide (nt) DNA fragment, position −1256 to −1050 with respect to the transcription start site, which is inactive in myoblasts but which increases transcription from either the MCK or herpes simplex virus thymidine kinase promoters 20- to 40-fold in myocytes (29). This region is also important for transcriptional activation in other muscle cell lines and in transgenic mice (54; J. E. Johnson, B. J. Wold, and S. D. Hauschka, submitted for publication), and a corresponding region exists in the rat MCK gene (25). Additional positive elements are located more proximally, in the region from nt −776 to the transcription start site (29). Besides these 5′ elements, a fragment from the MCK first intron also serves as a transcriptional enhancer (54; J. B. Jaynes, Ph.D. thesis, University of Washington, Seattle, 1987; Johnson et al., submitted). *cis*-Acting regulatory elements of other cloned muscle-specific genes, such as α-skeletal actin (4, 44, 59), α-cardiac actin (39, 41, 42), troponin I (33), myosin light chain 1/3 (5, 13), myosin light chain 2 (1), acetylcholine receptor α subunit (30, 60a), and myosin heavy chain (7), have also been mapped. Sequence comparisons between muscle-specific genes have revealed similarities (28, 32, 39), but no consensus sequence has yet been identified as critical for the coordinated expression of muscle-specific genes.

Little is known about the *trans*-acting regulatory factors involved in muscle-specific gene regulation. In one study, the binding of nuclear factors to a chicken skeletal α-actin promoter fragment indicated that the predominant binding factors from rat L6 myogenic cells were different from the predominant factors in nonmyogenic cells (58). However, the binding factors from L6 myoblasts, which did not express the α-actin gene product, were indistinguishable from those of myocytes expressing the gene. In addition, non-cell-type-specific factors which interact with regulatory regions of the human cardiac and skeletal α-actin genes and a rat myosin heavy-chain gene have been identified (22, 37, 43).

We have begun investigating the binding of mouse cell-derived factors to the enhancer regions of the mouse MCK gene. One factor identified by gel shift assays and DNase I footprinting binds to a site important for enhancer function. In contrast to the results of the studies mentioned above, this factor appears to occur only in myocytes, and we have accordingly termed it myocyte-specific enhancer-binding nuclear factor 1 (MEF 1).

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In addition to the delineation of muscle-specific *cis*-acting elements and their corresponding binding factors, the recent discovery of four myogenic determination genes provides a new approach to studying muscle-specific gene regulation. Expression in mesodermal stem cells of MyoD1 (11), *myd* (47), myogenin (60b), or *myf5* (7a) causes their conversion to myoblasts. This conversion establishes a responsiveness of muscle-specific genes, including *MCK*, to environmental signals such as FGF. The newly established gene responsiveness suggests that one or more of the determination gene products may be directly involved in muscle-specific gene activation. We have investigated this possibility, and preliminary results indicate that MEF 1 is identical or closely related to MyoD1 (J. Buskin, A. B. Lassar, R. L. Davis, H. Weintraub, and S. D. Hauschka, J. Cell Biol. **107**:98a, 1988).

MATERIALS AND METHODS

Cell culture. MM14 cells were grown in Ham F10C containing 15% horse serum and 2 ng of FGF per ml on collagen-coated plates as previously described (9, 46) or in Spinner flasks on collagen-coated beads (Cytodex-3; Pharmacia) (E. Cheung and S. D. Hauschka, unpublished). MM14 myoblasts were maintained in exponential growth by feeding them at 12-h intervals with the FGF-containing medium, while myocytes were obtained 20 to 38 h after switching log-phase cultures to medium containing 1 μM insulin and no added FGF.

Two hybridoma cell lines were used. Cell line 1 (CAT-2, a gift from C. Gorman, Genentech) produced antibodies against the bacterial protein chloramphenicol acetyltransferase (CAT). Cell line 2 (MF20, a gift from D. Bader and D. Fischman, Department of Cell Biology, Cornell Medical School, Ithaca, N.Y.) produced antibodies against myosin heavy chain (3).

The C2C12 (6) subline of C2 (61) cells were grown similarly to MM14 cells, except that feeding of myoblasts was daily and harvesting of myocytes was 48 h after a switch to F10C medium containing 5% horse serum and 1 μM insulin.

Cells from a differentiation-defective clonal cell line derived from MM14 cells (34), MM14-DD1, were grown in F10C plus 15% horse serum and FGF until about confluent and were harvested 6 days later, 8 days after the last feeding. At this point the cultures are quiescent (34).

L cells were a standard thymidine kinase-minus laboratory stock. These were grown in Dulbecco modified Eagle medium plus 10% supplemented bovine calf serum (Hyclone).

10T1/2 cells (56) were grown in F10C plus 15% horse serum and FGF.

HeLa-S cells and the first HeLa nuclear extract used were gifts from Jacques Peschon (Department of Biochemistry, University of Washington). The cells were grown in suspension in Joklik minimal essential medium.

FGF was a mixture of the acidic and basic forms purified from bovine brains (46).

Cells were harvested by scraping monolayers (MM14, C2C12, L, DD1, and 10T1/2 cells) with a rubber policeman, by gentle pipetting across loosely adherent cells (hybridoma cells), by collagenase dissociation of cells on beads (MM14 cells), or by centrifugation of cells in suspension (HeLa-S cells).

Growth of cells was assessed in some cases by the incorporation of [^{3}H]thymidine (3 μCi/ml) in a 30-min pulse followed by fixing and autoradiography. The differentiation status of myogenic cell lines growing in monolayers was assessed in some cases by immunohistochemical staining for myosin heavy chain as described previously (9).

Protein extracts. Nuclear and cytoplasmic extracts were made essentially by the method of Dignam et al. (12) with additional protease inhibitors. Leupeptin (1 μg/ml; Sigma Chemical Co.) and pepstatin (1 μg/ml; Sigma) were added to solutions A, C, and D; aprotinin (20 Kallikrein inhibitor units per ml; Sigma) was added to solutions A and C. Dialysis time and solution D volume were increased, generally to >12 h and greater than 500-fold excess over extract. Extracts were stored at −70°C and were always quick-frozen in ethanol-dry ice.

Tissue extracts were made from mouse organs by a variation of the procedure of Dignam et al. (12). Skeletal muscle was minced and then dissociated in Dignam solution A by using a Tekmar homogenizer (SDT-100EN generator, 50% setting). Nuclei were separated from larger myofibrillar fragments by filtration (42.5 gauge, Nitex), concentrated by centrifugation, and extracted with Dignam solution C similarly to nuclei of cultured cells. Nuclei of brain and liver were collected by mincing, Dounce homogenizing, and centrifugation.

DNA used as probes and competitors. MCK fragments used as probes or competitors were derived from mouse genomic clones (28) as indicated in Fig. 1. The 206-nt upstream enhancer-containing fragment E extended from an exonuclease III deletion endpoint at nt −1256 relative to the transcription start site (29) to the *Bam*HI site at nt −1050. This fragment was subcloned in plasmid pUC-E by deleting remaining MCK, CAT, and simian virus 40 (SV40) sequences from plasmid −1256MCKCAT (29); the fragment was excised by using polylinker sites at either end or the MCK *Bam*HI site at the 3′ end. Smaller fragments within E, as indicated in Fig. 1, were f1, from the internal *Nco*I site through the 3′ end; f2, from the 5′ end to the internal *Ava*I site; f3, from the 5′ end to the internal *Bst*XI site; f4, the *Ava*I-to-*Nco*I fragment; and f5, from the *Bst*XI site to the 3′ end of E.

The MCK upstream fragment B is a 247-nt *Bam*HI fragment adjacent to fragment E, extending from nt −1050 to −803; the fragment P contains proximal MCK promoter sequences from nt −803 (*Bam*HI site) to +7 (*Bst*EII site); and fragment H is a ~900-nt *Hin*dIII fragment located in intron 1.

A mutant form of pUC-E was produced by using the single-stranded form of pUC-E and the DNA oligomer CCCCCCAACACGGTAACCCTGAGCCTCA (changed sequence is underlined) by standard methods (63). The mutant 110-nt equivalent of f4 was used as the probe, and the mutant equivalent of E was used as the competitor.

Other fragments used were a 115-nt *Ava*II-*Pvu*II fragment from the rat myosin light-chain-1/3 gene enhancer (13), kindly provided by N. Rosenthal and B. M. Wentworth, Boston University Medical School, Boston, Mass.; the 473-nt mouse immunoglobulin light-chain-κ enhancer *Alu*I fragment (2) (contained in plasmid pE_k473, kindly provided by R. Perry and M. Atchison, Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pa.); the 365-nt mouse immunoglobulin heavy-chain enhancer *Dra*I-to-*Eco*RI fragment (nt 320 to 685 of sequence reported by Ephrussi et al. [15], contained in plasmid pUC 2084-4, kindly provided by R. Palmiter, Department of Biochemistry, University of Washington); the ~420-nt *Acc*I-to-*Nco*I fragment derived from pSV2CAT (19) including the SV40 72-nt enhancer repeats and the 21-nt repeats containing Sp1-binding sites (14) (fragment excised from a fusion gene constructed by J. E. Johnson, unpublished); and the ~550-nt Rous sarcoma virus long terminal repeat promoter-enhancer frag-

ment derived from pRSVgpt (20) and excised from pRSV-lacZ (W. Albert, unpublished) with *Nde*I and *Hin*dIII.

Other DNAs used were the bacterial plasmid pUC118 (57), provided by J. Vieira (Waksman Institute, Rutgers University, New Brunswick, N.J.); mouse genomic DNA; and poly[d(I-C) · d(I-C)] (Sigma).

Synthetic DNA oligomers were prepared by the Howard Hughes Medical Institute facility at the University of Washington. The sequence was confirmed by Maxam-Gilbert sequencing (38) as modified for oligomers (63).

Fragments used as competitors were purified from agarose gels by electrophoresis into, and salt elution from, Whatman DE81 filter paper.

Preparation of probes. Standard methods (Maniatis et al. [36] or instructions of manufacturers) for DNA restriction or modification were used to prepare DNA probes radiolabeled at one end of one strand. Typically, plasmids were cut at a restriction enzyme site at one end of the desired fragment. ^{32}P was then incorporated by using *Escherichia coli* DNA polymerase Klenow fragment fill in or T4 polynucleotide kinase in an exchange reaction. After the other end of the probe fragment was cut, DNA was spermine precipitated to remove free nucleotides (24) and fragments were purified by using thin acrylamide gels.

Oligonucleotide probes were end labeled by using T4 kinase, after which complementary strands were hybridized in appropriate salt concentrations before purification on thin acrylamide gels.

Gel mobility shift assay. Probe, buffer, and unlabeled DNA were mixed in 5 μl and placed in a 0°C bath. Protein extracts diluted in Dignam solution D (12) were mixed gently by pipetting. The binding-mixture composition, including components from the extracts, was 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH adjusted to 7.9 at room temperature as 1 M stock)–0.5 mM EDTA–0.5 mM dithiothreitol–50 mM KCl–10% glycerol. The mixture included 1.0 ng of probe DNA (0.1 ng for oligomer probe in the experiment shown in Fig. 7), 400 ng of unlabeled DNA, and 1 μg of protein. Incubation was for 20 min at 0°C followed by 5 min at 22°C. A total of 6 or 8 μl of the samples was loaded on nondenaturing gels.

Gels consisted of 4% acrylamide (6% for oligomer probe), 1/30 *bis*-acrylamide cross-link, 50 mM Tris hydrochloride, and 1 mM EDTA, with pH adjusted to 8.5 at room temperature in a 10× Tris-EDTA stock solution. Gels were preelectrophoresed for 30 min and run for 90 min at 225 V and 4°C with buffer recirculation between reservoirs. Dimensions were 0.75 mm by 16 cm by 20 cm, with 7-mm-wide wells. Narrower wells greatly diminished band resolution.

After electrophoresis, gels were dried and the position of the probe was determined by autoradiography.

DNase I footprinting. Under the conditions used to detect factors binding to the MCK probes, most of the probe remained unbound (Fig. 2) and thus unprotected from DNase cleavage. Therefore, footprinting methods needed to be combined with preparative mobility shift gels to see a signal from the small portion of probe to which factor bound. Binding was scaled up by using 9 μg of protein, 1,200 ng of unlabeled DNA, and 3 ng of probe in 30 μl. DNase I treatment was initiated by adding 30 μl of DNase I (10 μg/ml)–18 mM $CaCl_2$–5% glycerol and incubating for 1 min at 22°C; the reaction was stopped by the addition of 8 μl of 125 mM EDTA–50% glycerol. After nondenaturing electrophoresis as described above, autoradiography was performed on wet gels at 4°C. DNA was extracted from the bands corresponding to free and shifted probe by a simplified version of the method of Maxam and Gilbert (38) by using four freeze-thaw cycles to break up the polyacrylamide matrix. Samples were run on denaturing gels along with Maxam and Gilbert A-plus-G reactions (38).

DNA used in transfections. Plasmids p118CAT, −80MCKCAT, and −3300MCKCAT have been described previously (28, 29); these contain 0, 80, and 3,300 nt of MCK 5′-flanking sequences, respectively, linked to a reporter gene, *CAT*. Plasmids p(+enh110)80MCKCAT and p(−enh110)80MCKCAT were constructed by J. E. Johnson from −80MCKCAT by insertion of fragment f4 into the *Sal*I site of the polylinker immediately upstream of the truncated MCK promoter. p(+mut110)80MCKCAT and p(−mut110)80MCKCAT were similarly constructed by using the mutant 110-nt fragment described above. (Plus or minus in the designations of these plasmids indicate the orientation of the f4 fragments.) The reference gene plasmid was pSV_2Apap (23), a gift of P. Henthorn and T. Kadesch.

Transfections. Transfections were performed as previously described (28, 29), with the following modifications. A total of 8 μg of test gene and 2 μg of reference gene were used per 100-mm plate. Myocytes were harvested 30 h after being switched to differentiation medium. Cells were collected by centrifugation, and the NaCl-Tris-EDTA supernatant was aspirated to 100 μl; cells were disrupted by adding 50 μl of butanol, vortexing them in 1-s bursts five times, and incubating them for 30 min at 37°C. After centrifugation at 4°C, the aqueous phase was transferred to a new tube and frozen.

CAT assays were performed by a modification of the method of Neumann et al. (45); 0.05 μCi of [^{14}C]acetyl coenzyme A was used in a total volume of 50 μl. After a suitable incubation period, acetylated products were extracted with 200 μl of ethyl acetate. A total of 180 μl of the organic phase was transferred to a scintillation vial, dried, and counted with a water-miscible scintillation fluid. Placental alkaline phosphatase assays were performed essentially by the method of Henthorn et al. (23). CAT activity was corrected for transfection efficiency by dividing by placental alkaline phosphatase activity; values were then normalized to set −80MCKCAT equal to 1. Values reported are the averages of four individual transfections from two experiments, each using two different plasmid preparations for each construct.

RESULTS

Binding of myocyte factors to an MCK enhancer fragment. To characterize *trans*-acting factors which interact with the MCK enhancer (29), nuclear and cytoplasmic extracts were prepared from myoblasts, myocytes, and nonmuscle cells (12). The extracts were tested for the presence of specific DNA-binding factors using a gel mobility shift assay (16). The first probe tested was a 206-nt fragment from the MCK 5′ region containing a muscle-specific enhancer; the position of this probe within the MCK gene is indicated in Fig. 1 by E. An MM14 myocyte nuclear extract was incubated with the probe in the presence of a large excess of unlabeled DNA (unlabeled DNA is also referred to as the competitor). When the linearized bacterial vector pUC118 (pUC) was used as the unlabeled DNA, three bands were observed (Fig. 2a, lane 1); these are indicated by f (free probe) and by m and n (myocyte specific and non-cell type specific, respectively; see below). m and n have decreased mobilities relative to that of free probe, due to the binding of myocyte nuclear factors.

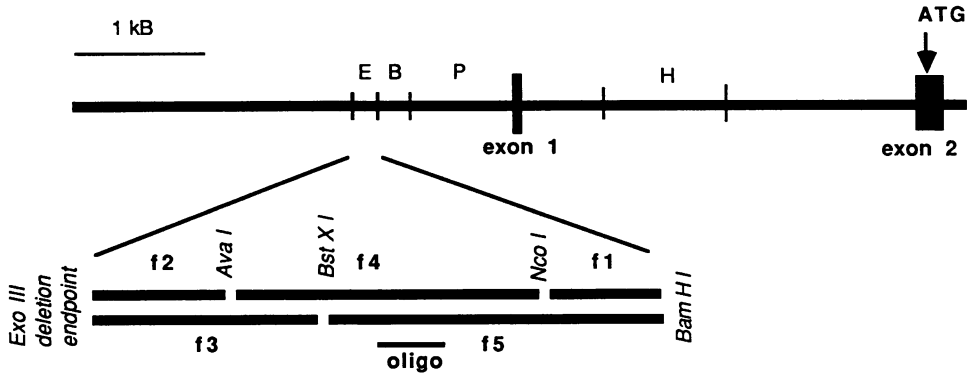

FIG. 1. Map of the MCK gene 5′ region showing probes and competitor fragments used in this study. The portion of the gene shown extends from the 5′ border of upstream sequences used for deletion analysis in our previous studies (29) to exon 2, which contains the translation start site. Exons are indicated (■). Regions of the gene which were used as probes or competitors in this study are indicated by E, B, P, and H. The expanded map at the bottom shows smaller fragments f1 to f5 of region E which were used as probes as well as the oligo region containing sequences used in synthetic oligomers. kb, Kilobase.

Sequence specificity of binding to MCK 5′-enhancer region. Since unfractionated nuclear extracts have large numbers of nonspecific DNA-binding proteins, it was necessary to test the binding for its specificity with respect to sequence recognition. This was achieved by comparison of the ability of MCK 5′-enhancer sequences versus other heterogeneous sequences to act as competitors for factor binding. One such comparison can be seen in Fig. 2a, in which an unlabeled linearized plasmid containing the 206-nt MCK 5′-enhancer fragment in the vector pUC118 (pUC-E, lane 2) reduced the intensity of the shifted bands m and n relative to that observed when linearized pUC was used as the unlabeled DNA (lane 1). Since any heterogeneous DNA may reduce factor binding to specific sites, competition was also tested by using a mixture of the simple repeating heteropolymer poly[d(I-C) · d(I-C)] plus either the isolated 206-nt MCK enhancer fragment E (IC + E) or the isolated MCK fragment B (IC + B) shown in Fig. 1. Fragment B is not known to have any regulatory function and is slightly larger (247 nt) than the enhancer fragment. Each fragment was used in a 200-fold molar excess over the probe. The intensities of shifted bands, especially that of m, were greater with fragment B plus poly[d(I-C)] (Fig. 2a, lane 3) than with the heterogeneous competitor pUC (lane 1); however, inclusion of unlabeled MCK 5′-enhancer fragment (lane 4) greatly reduced the intensities of both shifted bands. These comparisons show that the factors in MM14 myocyte nuclear extracts which yielded bands m and n recognized specific sequences contained in the MCK 5′-enhancer fragment.

The sequence specificity of myocyte factor binding was also tested by comparing binding to the enhancer-containing E fragment with binding to the B nonenhancer fragment (Fig. 1). The MM14 myocyte extract again generated two shifted bands with the E probe; the intensities of both were reduced by the use of unlabeled plasmid containing the enhancer sequence (pUC-E; Fig. 2b, lane 6) compared with plasmid without enhancer (pUC, lane 5). However, no shifted bands were observed under identical conditions using the B fragment as the probe (lanes 1 and 2).

Cell-type and differentiation-state occurrence of factors which bind the MCK 5′ enhancer. An initial investigation of the cell-type occurrence of the MCK 5′-enhancer-binding factors is shown in Fig. 2b; lanes 7 and 8 demonstrate the pattern obtained when a hybridoma nuclear extract is used with the MCK 5′ enhancer as the probe. The upper band seen with the myocyte nuclear extract was not present, but the lower band was present with either extract. The hybridoma extract, like the MM14 myocyte extract, failed to produce shifted bands when incubated with the B fragment as the probe (Fig. 2b, lanes 3 and 4).

The cell-type occurrence of MCK 5′-enhancer-binding factors was further investigated by comparing nuclear factors from proliferating MM14 myoblasts with those from terminally differentiated MM14 myocytes (Fig. 2c). The myocyte extract again produced two shifted bands (m and n) whose binding specificity could be demonstrated by comparison of a binding mixture containing unlabeled fragment B (lane 5) with a mixture containing the enhancer fragment E (lane 6). In contrast, the myoblast extract yielded only n, the lower of the two shifted, "competable" bands (lanes 2 versus 3). ("Competable" is used to indicate the property of being able to be reduced by the inclusion of specific unlabeled DNA sequences.)

The upper band was not unique to differentiated MM14 cells; a band in the same position was observed when an extract from myocytes of the independently derived mouse myogenic C2C12 cell line was used. The C2C12 pattern, however, was more complex; besides competable bands (Fig. 2c, lane 11 versus 12) in the same positions as the two MM14 myocyte competable bands, it contained additional bands. Other MM14 myocyte extracts tested also contained additional minor bands (data not shown).

Two nonmuscle extracts were also examined in this experiment; these were from a mouse hybridoma cell line (as in Fig. 2b) and the human cervical carcinoma cell line HeLa. The hybridoma extract again yielded a shifted, competable band in the position of myocyte band n but no band in the position of myocyte band m (Fig. 2c, lanes 14 and 15). A band with mobility slightly greater than that of m was seen when the HeLa extract was used (lanes 8 and 9), but it does not appear to be due to sequence-specific binding, as its intensity is not decreased by substituting fragment E for B as a portion of the unlabeled competitor. A prominent band of lesser mobility than m which was subject to competition by fragment E was also observed in the HeLa extract (lane 8); this coincides with a band seen with the C2C12 myocyte extract (lane 11).

With any of these extracts, the omission of unlabeled, heterogeneous DNA correlated with numerous strong bands, many of which may be due to binding factors with

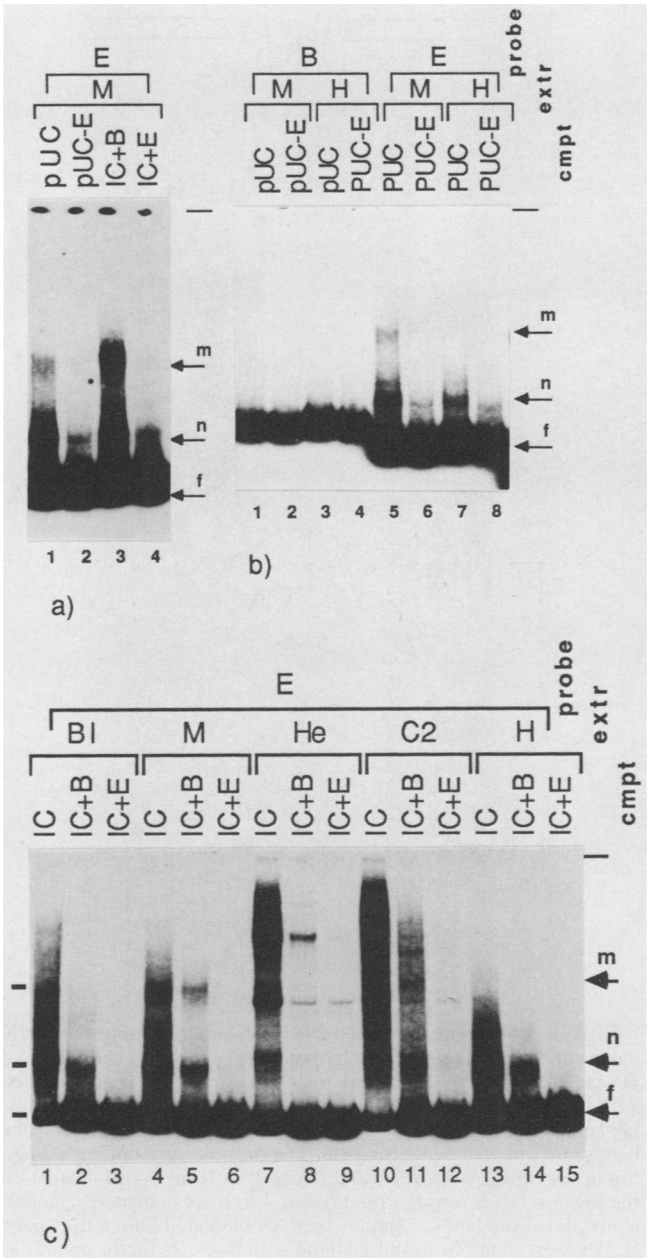

FIG. 2. Myoblast, myocyte, and nonmuscle factors which bind specifically to the MCK enhancer-containing fragment as revealed by mobility shift assays. The arrows indicate the positions of free probe, f, and two competable shifted-mobility bands, m and n. (a) MM14 myocyte extract with MCK enhancer probe and various competitors. A nuclear extract derived from differentiated MM14 myocytes (M) harvested 24 h after a switch to FGF-deficient medium was incubated with the MCK enhancer-containing labeled E fragment (Fig. 1). The unlabeled competitor DNAs were as follows (indicated above the lanes): pUC, linearized bacterial plasmid; pUC-E, linearized plasmid containing the 206-nt MCK enhancer-containing E fragment; IC + B, poly[d(I-C) · d(I-C)] plus isolated MCK fragment B (Fig. 1), which does not contain known regulatory sequences; IC + E, poly[d(I-C) · d(I-C)] plus isolated fragment E. (b) Myocyte versus hybridoma extracts and enhancer versus nonenhancer probes. Either MM14 myocyte (M) or hybridoma (H) nuclear extract was incubated with probe B or E (Fig. 1) in the presence of unlabeled competitor DNAs pUC or pUC-E, as indicated above the lanes. Note that probe B is slightly larger than probe E so that free probe in lanes 1 to 4 is in a different position than free probe in lanes 5 to 8; the arrow refers to probe E. (c) Comparison of factors in myoblasts, myocytes, and nonmuscle cells as revealed by a mobility shift assay. Nuclear extracts from MM14 myoblasts (Bl), MM14 myocytes (M), HeLa cells (He), C2C12 myocytes (C2), or hybridoma cells (H) were incubated with the enhancer-containing E fragment of MCK (Fig. 1). Unlabeled DNA was poly[d(I-C) · d(I-C)] (IC), IC + B, or IC + E as indicated above the lanes. (See Materials and Methods section for details about probe and competitor DNAs, extracts, binding, and electrophoresis.)

TABLE 1. Binding of myocyte and hybridoma factors to smaller fragments within 206-nt MCK enhancer fragment E

Fragment[a]	Common[b]	Unique[c]
f1	+	−
f2	−	−
f3	−	−
f4	−	+
f5	+	+

[a] Positions of fragments within the gene are shown in Fig. 1.
[b] +, Presence of a predominant shifted band, subject to competition by fragment E seen in both myocyte and hybridoma lanes when the fragment is used as a probe in the standard gel shift assay; −, no common shifted band.
[c] +, Presence of a shifted band present in the myocyte lane but not present in the hybridoma lane; these bands were not as intense as the common bands; −, no shifted band unique to myocytes.

little specificity for the probe (Fig. 2c, lanes 1, 4, 7, 10, and 13). When no unlabeled DNA was included, the probe remained at the top of the gel (not shown), presumably due to binding of multiple, mostly nonspecific factors. This confirmed the need for carefully controlled binding mixtures when testing the binding specificity of crude extracts; for example, if comparisons were made of lanes with poly[d(I-C) · d(I-C)] alone versus poly[d(I-C) · d(I-C)] plus fragment E, entirely different conclusions would be drawn than those drawn as a result of using the more parallel comparisons of poly[d(I-C) · d(I-C)] plus fragments B versus E.

Comparison of the various extracts suggests the designation of n or non-cell type specific for the lower band since it is present in nonmyogenic hybridoma cells as well as in myoblasts and myocytes. In contrast, the band designated m was observed in myocyte extracts from two independently derived myogenic cell lines but not in extracts from myoblasts or from two nonmyogenic cell types; these and subsequent observations (see below) justify the designation of the factor(s) producing band m as myocyte-specific enhancer-binding nuclear factor 1 (MEF 1).

Mapping the sites of MEF 1 binding. To determine binding sites of factors within the 206-nt MCK 5′-enhancer fragment, overlapping subfragments (f1 through f5 in Fig. 1) were used as probes in the gel mobility assay. The pattern of MM14 myocyte and hybridoma nuclear factor binding to these probes is described in Table 1. On the basis of the pattern observed with the 206-nt fragment, we predicted that the factor yielding band n in Fig. 2b (lanes 5 and 7) would generate a predominant competable band with both extracts when incubated with one or more of the smaller fragments. Bands common to both extracts were observed with fragments f1 and f5. It thus seems likely that the factor binding to the 206-nt 5′-enhancer fragment yielding band n in Fig. 2 is a non-cell-type-specific factor that recognizes sequences within f1 which are also included in the larger fragment f5.

Since band m in Fig. 2 was less intense than band n, and since it was present only in myocyte extracts, we predicted that the factor(s) yielding band m would produce a compet-

able lower-intensity band with one or more of the smaller fragments tested and that this would be seen with the myocyte extract but not with the hybridoma extract. This was the case for fragment f4. Since fragment f4 overlaps f3 and f5, we would also expect to find a myocyte-specific factor binding to one of these unless the recognition site is at the junction between f3 and f5. Fragment f5 plus the myocyte extract exhibited two pUC-E competable bands with no counterparts when the hybridoma extracts were used, while fragment f3 exhibited no bands whose intensities were reduced by competition with unlabeled enhancer. This suggests that MEF 1 binds within the region of fragment f4 3′ of the *Bst*XI site (Fig. 1).

DNase I footprinting. To define the factor-binding sites more precisely, preparative mobility shift gels were combined with DNase I footprinting (17, 50). In this method, a brief DNase treatment was used to nick the probe subsequent to binding and before nondenaturing electrophoresis. After autoradiography, portions of the gel corresponding to shifted and free probe were extracted and the DNA was run on a denaturing sequencing gel. This permitted detection of a footprint from the relatively small proportion of the probe which had factor bound under conditions such as those in Fig. 2, independent of the far larger amount of free probe present in the binding mixture.

An MM14 myocyte extract produced footprints on both strands of fragment f4 (Fig. 3, lanes 1 and 4 versus lanes 2 and 5, free probe). Consistent with our predictions, the location of the protected region was within the overlap between f4 and f5 (Fig. 1). The footprints also exhibited DNase I-hypersensitive sites just outside of the area protected from DNase degradation, as has been observed with other DNA-binding proteins. While this work was in progress, further transfection data (J. E. Johnson, J. N. Buskin, and S. D. Hauschka, unpublished data) showed that the 110-nt fragment f4 causes an enhancement of transcription in myocytes similar to that caused by the 206-nt 5′-enhancer-containing fragment (29) used in Fig. 2.

The site of interaction of the non-cell-type-specific factor binding within fragment f1 was also determined; both myocyte and hybridoma extracts protected an adenine-thymine-rich region within f1 (data not shown). Because this site is outside of fragment f4 (which is sufficient for transcriptional activation) and because the binding was also found in a nonmuscle extract, it seems less likely that the factor responsible for the footprint in f1 is critical for the function of the MCK enhancer. However, the binding site of this factor has not yet been tested in a functional assay. Because f4 was the smallest fragment tested which activated myocyte transcription similarly to the 206-nt MCK 5′-enhancer fragment and because the f4-binding factor, MEF 1, appears to be myocyte specific, we have concentrated on further characterization of this factor.

Function of the MEF 1-binding site. We tested the role of the MEF 1-binding site in transcriptional activation by using a mutant with its sequence changed in six contiguous positions in the center of the footprinted region. The mutant and wild-type sequences are shown in Fig. 4a. We first tested the mutant for its binding to MEF 1 in vitro; Fig. 4b shows that the mutant E fragment did not compete for binding of MEF 1 to the wild-type f4 fragment (compare lane 4 [mutant competitor] with lane 3 [wild-type enhancer fragment E competitor]); in addition, when the mutant f4 was used as the probe, no shifted band corresponding to MEF 1 binding was observed (compare lane 7 [mutant probe] with lane 2 [wild-type f4 probe]). Thus, the mutation was sufficient to abolish MEF 1 binding in vitro.

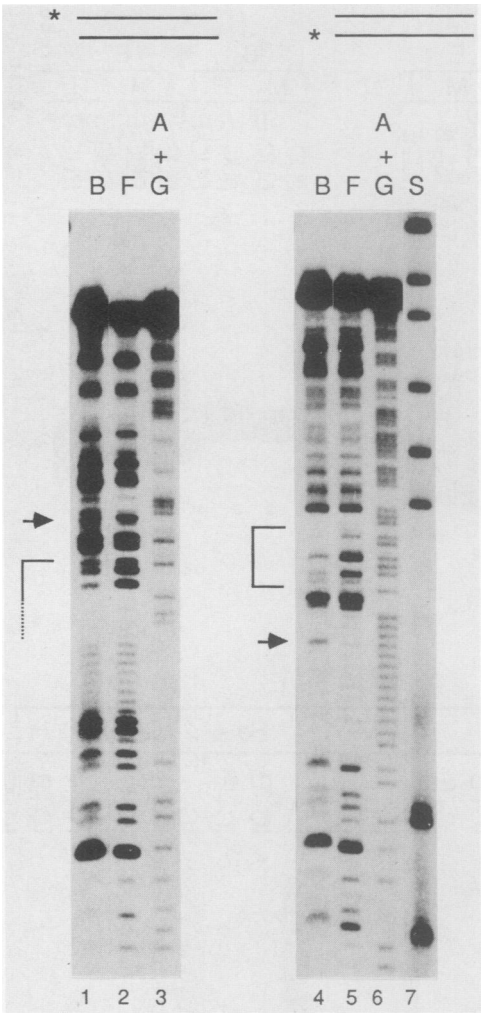

FIG. 3. Location of the site of MEF 1 binding within the MCK enhancer fragment as revealed by preparative mobility shift gels and DNase I footprinting. MM14 myocyte nuclear extract was incubated with probe f4 (Fig. 1); after a brief DNase I treatment, nondenaturing gels were run. DNA was extracted from shifted and free-probe bands and run on a denaturing gel. The probe was 5′-end-labeled on the upper (mRNA-sense) strand (lanes 1 to 3) or 3′-end-labeled on the lower (cDNA sense) strand (lanes 4 to 6) as indicated schematically above the lanes. Samples were as indicated above the lanes: B, DNA extracted from shifted band with myocyte factor bound; F, DNA extracted from free-probe band; A + G, Maxam and Gilbert A-plus-G sequencing reaction of the probe; S, size standards (end-labeled *Msp*I fragments of pBR322). Arrows indicate DNase I-hypersensitive sites produced by myocyte factor binding, while brackets indicate protected regions. The extent of the protected region for the upper strand cannot be determined precisely because it overlaps an oligo(dC) region refractory to DNase I cleavage.

Interestingly, a fragment of the rat myosin light-chain-1/3 enhancer region (13) also competed effectively for MEF 1 binding (Fig. 4b, lane 5). When the myosin light-chain fragment was used as a probe, a band in a similar position to the MCK MEF 1 band was observed; this band was subject to competition by wild-type but not mutant MCK (lanes 12 to 14). Thus, it is likely that MEF 1 binds to the enhancer region of other muscle-specific genes. Complementary experiments are under way to test whether the MCK enhancer

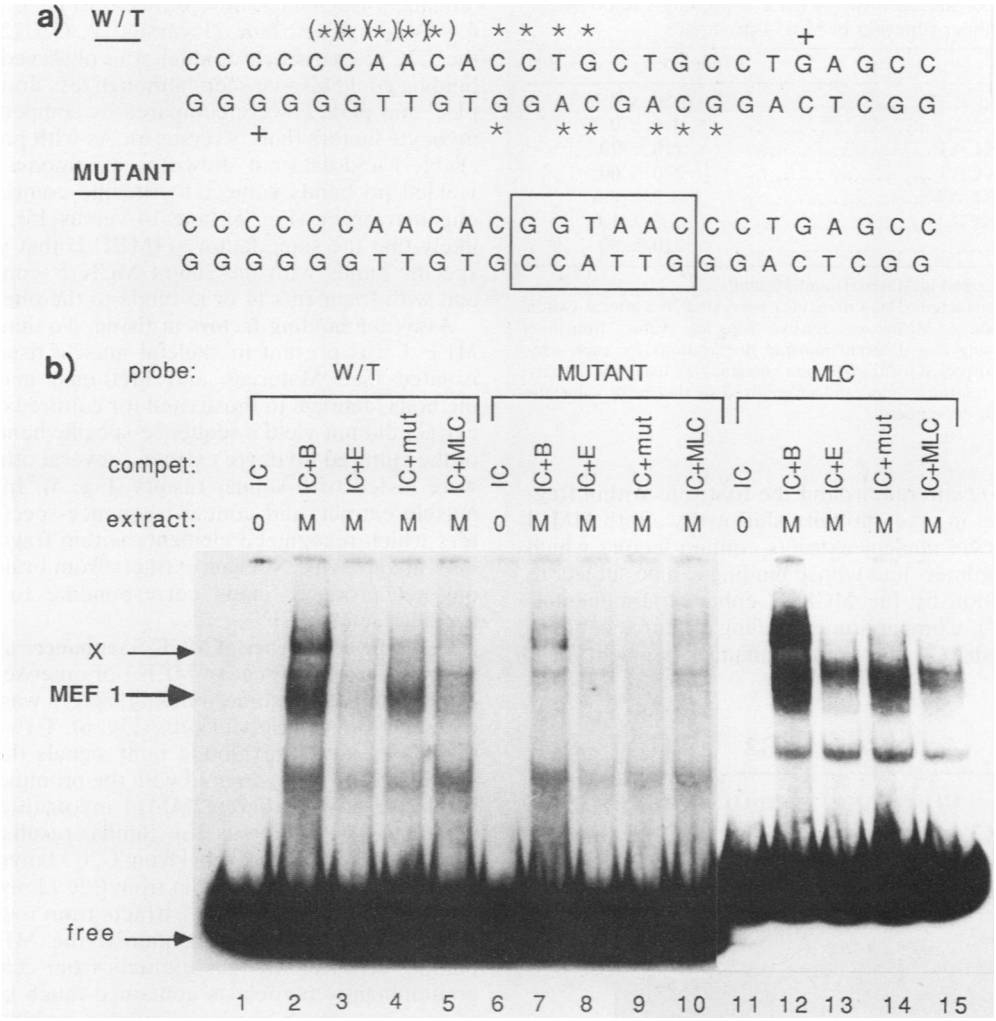

FIG. 4. Mutated enhancer does not bind MEF 1 in vitro. (a) Sequences of the wild-type (W/T) and mutant probes. The sequence surrounding the MEF 1 DNase I footprint (29) is shown. The protected sites within the wild-type sequence are indicated (*), with lesser protection indicated in parentheses. DNAse I-hypersensitive sites are also indicated (+). (b) Wild-type (lanes 1 to 5) or mutant (lanes 6 to 10) 110-nt MCK f4 fragments or a 115-nt fragment from the rat myosin light-chain-1/3 enhancer (13; lanes 11 to 15) were used as probes in the gel shift assay. Lanes 1, 6, and 11 contain no nuclear extract; all other lanes contain MM14 myocyte nuclear extract. Unlabeled competitors are as indicated above the lanes: IC, poly[d(I-C) · d(I-C)]; IC + B, IC plus isolated fragment B (Fig. 1) which does not contain known regulatory sequences; IC + E, poly[d(I-C) · d(I-C)] plus isolated enhancer fragment E; IC + mut, IC plus the mutant equivalent of E; IC + MLC, IC plus the myosin light-chain fragment. The molar ratio of unlabeled fragment to probe was 30, with the addition of fragment B to IC + MLC mixtures to bring total heterogeneous DNA to a constant level. Arrows indicate free probe, MEF 1, and a higher competable band seen in most MM14 myocyte extracts (X).

region competes for other factors that bind to the myosin light-chain enhancer region (N. Rosenthal and B. M. Wentworth, personal communication).

Figure 4b also shows a minor band (X) produced by the MM14 myocyte extract with the MCK probe; this band was subject to competition about equally by the mutant and wild-type 206-nt fragments. Therefore, the factor responsible for this band may be a transcription factor which binds to a site distinct from the MEF 1-binding site (see Discussion).

The mutant f4 fragment was then examined for its ability to enhance myocyte transcription. We tested plasmids containing either the wild-type or mutant f4 fragment inserted in either orientation upstream of the truncated MCK promoter containing 80 nt of 5′-flanking sequence and fused to the CAT reporter gene at MCK position +7 relative to the transcription start site. The wild-type 110-nt fragment, in the plus or minus orientation, caused a 110- or 220-fold increase in expression over that of parental plasmid −80MCKCAT (Table 2). While the mutant 110-nt fragments also caused a significant increase over the parental plasmid (four- and eightfold), these constructs were about 25 times less active than their wild-type counterparts. We therefore conclude that the MEF 1-binding site is an important component of the MCK upstream enhancer. It seems likely that the myocyte-specific binding factor MEF 1 which we have identified by in vitro binding assays plays a part in the transcriptional activation of MCK via this site.

Binding of myocyte factors to synthetic oligomers. To further characterize MEF 1, we have used complementary synthetic oligomers containing the 21 base pairs of MCK

TABLE 2. Effect of mutagenesis of MEF 1-binding site on MCK enhancer function in MM14 myocytes

Plasmid[a]	Expression[b] (mean ± SD)
p118CAT	0.50 ± 0.13
−80MCKCAT	1.0 ± 0.2
p(+enh110)80MCKCAT	110 ± 20
p(−enh110)80MCKCAT	220 ± 60
p(+mut110)80MCKCAT	4.3 ± 0.6
p(−mut110)80MCKCAT	8.3 ± 1.6
−3300MCKCAT	210 ± 80

[a] Plasmids are described in Materials and Methods.

[b] Expression in transfected MM14 myocytes harvested 30 h after a switch to differentiation medium. Means are derived from four values from two experiments, each using two different plasmid preparations for each construct. Values were corrected for transfection efficiency by using the activity of the cotransfected reference gene and normalized to the mean value for −80MCKCAT in each experiment.

sequence (Fig. 5) centered around the footprint within fragment f4 as probes in a gel mobility shift assay. Both MM14 and C2C12 myocyte nuclear extracts contain factors which recognize the oligomers and whose binding can be subject to specific competition by the MCK 5′-enhancer fragment E (Fig. 5, bands m°). Comparison of binding mixtures containing poly[d(I-C) · d(I-C)] plus B (nonenhancer fragment) or E (enhancer fragment) showed this most clearly (lane 3 versus 4, MM14 extract; lane 11 versus 12, C2C12 extract). With the C2C12 extract, a doublet was observed. Specificity of binding could also be seen, although less dramatically, when pUC and pUC-E were compared as competitors for MM14 myocyte factors (lane 5 versus 6). As with probe fragment f4 (Table 1 and data not shown), a hybridoma nuclear extract yielded no bands subject to specific competition with the oligomer probe (Fig. 5, lane 14 versus 15). Thus, it seems likely that the same factor(s) (MEF 1) that yields myocyte-specific bands with the 206-nt MCK 5′-enhancer fragment and with fragments f4 or f5 binds to the oligomer probe.

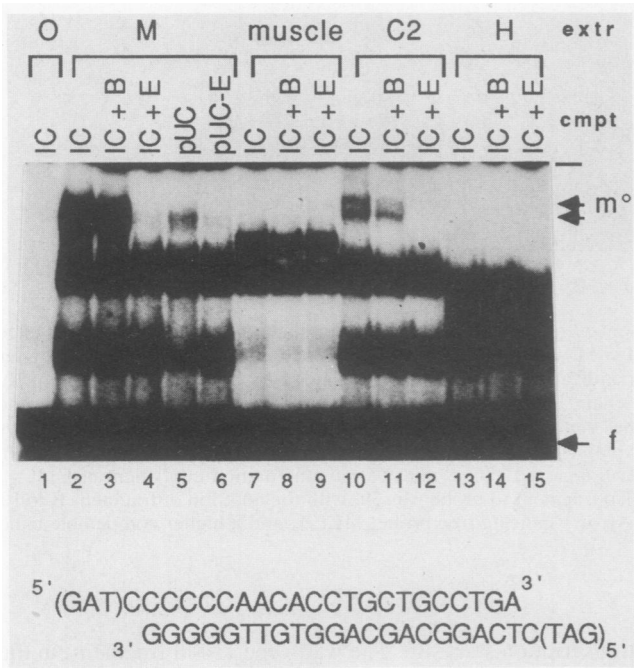

FIG. 5. Sequence-specific binding of MEF 1 to synthetic DNA oligomers as revealed by a mobility shift assay. Complementary DNA oligomers containing the region of the MCK sequence centered on the footprint produced by binding of a myocyte factor (Fig. 3) were end labeled and hybridized. Nuclear extracts were derived from MM14 myocytes (M), from adult mouse muscle (muscle), from C2C12 myocytes (C2), or from hybridoma cells (H). Lane 1 contains no extract. Unlabeled DNAs included in the mixture were as follows: IC, poly[d(I-C) · d(I-C)]; IC + B, nonenhancer MCK fragment (Fig. 1); IC + E, enhancer-containing MCK fragment; pUC, linearized bacterial plasmid; and pUC-E, linearized plasmid containing enhancer fragment E. Arrows indicate free probe (f) or shifted, competable MEF 1 bands (m°) in the myocyte lanes. The sequences of the oligomers are shown at the bottom; bases shown in parentheses were not derived from the MCK sequence.

Assay for binding factors in tissue. To determine whether MEF 1 was present in skeletal muscle tissue, nuclei were isolated (see Materials and Methods) and extracted by methods identical to those used for cultured cells. The tissue extract did not yield a sequence-specific band similar to that of the cultured myocyte extracts; several other preparations were tested with similar results (Fig. 5). Interestingly, the muscle extracts did contain sequence-specific binding factors which recognized elements within fragment f2 (Fig. 1; data not shown). Nuclear extracts from brain and liver also did not produce bands corresponding to the sequence-specific factor MEF 1.

Cell-type occurrence of MCK 5′-enhancer oligomer-binding factors. The occurrence of MEF 1 or other sequence-specific MCK enhancer oligomer-binding factors was investigated in extracts from a variety of cells (Fig. 6). Two different MM14 myoblast extracts exhibited faint signals (lanes 4 versus 5 and 13 versus 14) compared with the prominent, competable bands from two different MM14 myocyte extracts (lane 8 versus 9; lane 15 versus 16). Similar results were obtained with C2C12 cells. The signal from C2C12 myoblasts (lane 28) was faint compared with that from C2C12 myocytes (lane 32 versus 33). Thus, myocyte extracts from two independently derived myogenic lines contained the MEF 1 oligomer-binding activity, whereas extracts from cultures that were predominantly myoblasts contained much smaller amounts of factor(s) which produced similar mobility shifts. While these results would be consistent with the hypothesis that myoblast nuclei contain low levels of MEF 1 which increase upon differentiation, we consider it more likely that myoblasts contain no MEF 1 and that the bands seen in the myoblast extracts were actually due to a small fraction of myocytes which contaminated the myoblast cultures. This was supported by comparison of the faint band in lane 13 resulting from cultures with <0.5% of nuclei in myosin-positive cells with the more pronounced bands in lane 28 derived from cultures with 6% of nuclei in myosin-positive cells.

A variety of other cell types were tested for MEF 1; this factor was not evident in nuclear extracts from any of the nonmyogenic cell lines tested. These included mouse L cells, which are fibroblast-like cells (Fig. 6, lanes 21 and 22); mouse 10T1/2 cells, thought to be multipotent mesodermal stem cells (lanes 19 and 20); two different lymphoid hybridoma cell lines (lanes 10 and 11 and lanes 23 and 24); and two different transcriptionally active HeLa cell extracts (lanes 25 and 26 and lanes 38 and 39). Of these nonmyogenic cell lines, 10T1/2 was particularly interesting because these cells can be induced at high frequency to become myogenic by treatment with 5-azacytidine (32, 56). We also tested another muscle-related cell type, the DD1 line derived clonally from MM14 cells (34). These differentiation-defective cells have lost their FGF dependence, and unlike MM14 cells, they do not rapidly differentiate when FGF is withdrawn. At high

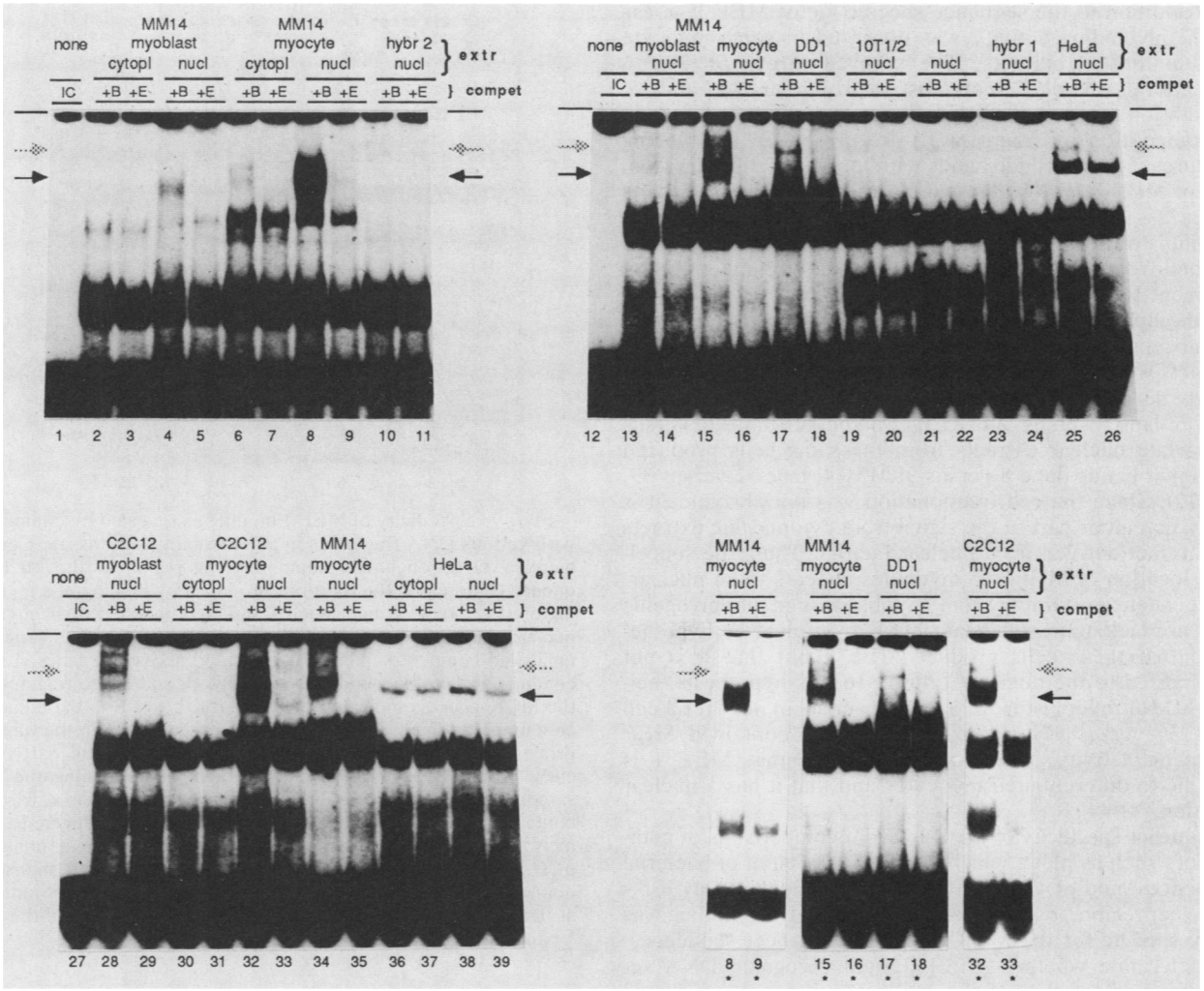

FIG. 6. Survey of cell types for MEF 1. Nuclear or cytoplasmic extracts were tested for binding activity by using the gel mobility shift assay. Probes were the complementary oligomers shown in Fig. 5. Unlabeled DNA was as indicated above the lanes: IC, poly[d(I-C) · d(I-C)]; +B, IC plus MCK fragment B (Fig. 1); +E, IC plus enhancer-containing MCK fragment E. Lanes 1 to 39 were exposed to best show minor bands; lanes at the lower right (*) are lighter exposures of the corresponding numbered lanes to more clearly show the major bands. Extracts were as indicated. Lanes 1, 12, and 27: No extract. Myoblast extracts: MM14 myoblast cytoplasmic (cytopl) extract (lanes 2 and 3) and nuclear (nucl) extract from the same cells (lanes 4 and 5, same extract as in Fig. 2), another MM14 myoblast nuclear extract (lanes 13 and 14) from a culture with fewer than 0.5% of nuclei in cells staining positive for myosin, and C2C12 myoblast nuclear extract (lanes 28 and 29) from a culture with ~6% of nuclei in myosin-positive cells. Myocyte extracts: MM14 myocyte cytoplasmic extract (lanes 6 and 7) and nuclear extract from the same cells (lanes 8 and 9, same extract as used in Fig. 5 and 7) harvested 20 h after a switch to FGF-deficient medium, another MM14 myocyte extract (lanes 15, 16, 34, and 35) with ~80% of nuclei in cells positive for myosin at harvest 38 h after a switch to FGF-deficient medium (a third MM14 myocyte extract was used for Fig. 2 and Table 1, and a fourth MM14 myocyte extract was used for Fig. 4), C2C12 myocyte cytoplasmic extract (lanes 30 and 31) and nuclear extract from the same cells (lanes 32 and 33, same extract as in Fig. 2 and 5) harvested 48 h after a switch to low-mitogen medium with ~50% of nuclei in cells positive for myosin. Nonmyogenic cell extracts: Nuclear extract from hybridoma 2 cells (lanes 10 and 11), nuclear extract from hybridoma 1 cells (lanes 23 and 24, same extract as Fig. 2 and 5), nuclear extract from MM14-derived differentiation-defective DD1 cells (lanes 17 and 18) (these cells were quiescent but not differentiated in the manner of parental MM14 cells under similar culture conditions [~1% of nuclei pulse-labeled with [^{3}H]thymidine, <0.2% of nuclei in myosin-positive cells]), nuclear extract from 10T1/2 mesodermal stem cells (lanes 19 and 20), nuclear extract from L cells (lanes 21 and 22), nuclear extract from HeLa cells (lanes 25 and 26), another HeLa cell nuclear extract (lanes 38 and 39; same extract used in Fig. 2), and cytoplasmic extract from the same cells (lanes 36 and 37). Positions of faint, competable bands seen in some muscle and nonmuscle cells are indicated (⇦) and are most clearly seen in lanes 17 and 25. Positions of MEF 1 bands, seen most clearly in myocyte lanes 8, 15, and 32, are also indicated (⇦); as noted above, these bands were overexposed to allow detection of minor bands in other lanes and are seen more clearly in the lighter exposures (bottom right panel).

densities and at low concentrations of serum, DD1 cells become quiescent, but even after a week without proliferation no more than a few percent have differentiated. Therefore, quiescent DD1 cells were used as an example of a cell type closely related to myocytes by descent and by their proliferation status but dissimilar in their differentiation state. Since no band corresponding to MEF 1 was seen (Fig. 6, lanes 17 and 18), it appears that MEF 1 is specific to myocyte nuclei and does not occur in myoblasts or nonmuscle cells, including cells with the potential to become myogenic or in differentiation-defective cells derived from myogenic cells.

In addition to the sequence-specific factor MEF 1, a less abundant binding activity was observed in some myocyte and nonmuscle extracts. The stippled arrows in Fig. 6 indicate this complex, seen most clearly in lanes 17 and 25. In addition, nonspecific DNA-binding proteins (not subject to competition by fragment E) are present in the binding mixtures. Most produce bands with mobilities different from that of MEF 1. The HeLa cytoplasmic and nuclear extracts (lanes 25, 26, and 36 to 39) produce bands with similar mobility to the MEF 1 complex, but the factors are distinguished from MEF 1 on the basis of lack of competition by fragment E.

Subcellular localization of MEF 1. To address the question of subcellular localization of MEF 1, several cytoplasmic extracts were examined. Faint bands corresponding to MEF 1 were seen in the cytoplasmic extracts of MM14 myocytes (Fig. 6, lane 6 versus 7) or C2C12 myocytes (lane 30 versus 31), while nuclear extracts from the same cells produced dominant bands (lane 8 versus 9, MM14; lane 32 versus 33, C2C12). Since the cell fractionation was not absolute, it is likely that all or part of the signal from cytoplasmic extracts was in fact derived from nuclear factors. Thus, the subcellular location of MEF 1 in myocytes appears to be nuclear. Since nuclear extracts from myoblasts and nonmyogenic cells are lacking in significant MEF 1, it might be hypothesized that these cells produce MEF 1 but that it is not transported to the nuclei. Contrary to this hypothesis, neither MM14 myoblast (Fig. 6, lane 2 versus 3) nor HeLa cell (lane 36 versus 37) cytoplasmic extracts contained MEF 1-binding activity. Therefore, it appears that MEF 1 is specific to differentiated myocytes and that it has a nuclear location.

Sequence specificity of MEF 1. From comparisons of competitors such as pUC, which contains ~3,300 nt of bacterial sequences, and pUC-E, with the additional 206-nt MCK 5′-enhancer region, it seemed apparent that MEF 1 binding was fairly specific for the MCK upstream enhancer sequences. To determine whether MEF 1 might recognize DNA sequences besides that of the MCK 5′ enhancer, we tested other sequences for their ability to compete for MEF 1 binding. Since nonspecific heterogeneous DNA can compete for the binding of MEF 1 (e.g., Fig. 5, lane 3 versus 5; a much stronger signal is seen with poly[d(I-C) · d(I-C)] than with a similar amount of plasmid DNA), the amounts of both heterogeneous DNA and total DNA were kept constant. To allow comparisons of equal molar amounts of variously sized fragments, the B nonenhancer fragment, which competed poorly for MEF 1 binding (Fig. 5, lane 3 versus 2), was used to bring all mixtures to a constant level of heterogeneous DNA. The MCK 5′-enhancer fragment at a 30-fold molar excess over oligomer probe (Fig. 7, lane 3) competed for MEF 1 binding much better than did several other regulatory regions, including the SV40 enhancer (Fig. 7, lane 9), the Rous sarcoma virus long terminal repeat promoter-enhancer (lane 10), and the mouse immunoglobulin κ and μ chain enhancers (lanes 11 and 12). The E fragment was an efficient competitor, nearly eliminating the signal at a 30-fold excess over probe and significantly decreasing the signal at lower amounts (Fig. 7, lanes 4 and 5). The bacterial vector pUC and total mouse genomic DNA were also tested as competitors at the same concentrations as the total heterogeneous DNA used in Fig. 7, lanes 2 to 12. Neither pUC (Fig. 7, lane 13) nor genomic DNA (lane 14) competed significantly for MEF 1 binding. This indicated that MEF 1 does not recognize a sequence which is highly repeated in the mouse genome.

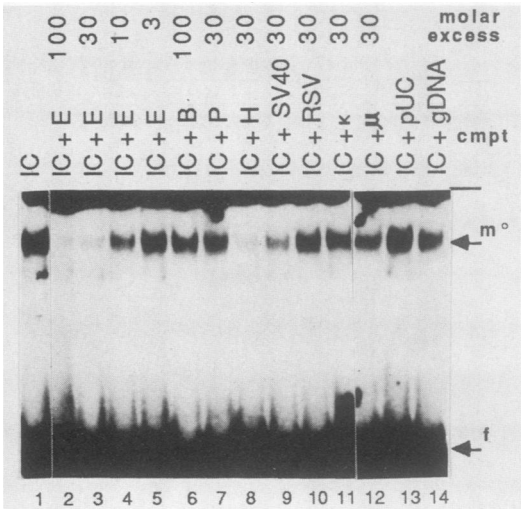

FIG. 7. Specificity of MEF 1 binding as revealed by competition with various DNA fragments in a gel mobility shift assay. Complementary DNA oligomers containing the region of the MCK sequence centered on the footprint produced by binding of a myocyte factor (Fig. 3) were end labeled and hybridized. MM14 myocyte nuclear extract was incubated with this probe and a variety of unlabeled competitor DNAs as indicated above the lanes. Lane 1 contains no heterogeneous DNA; E, B, P, and H are fragments of the MCK gene as indicated in Fig. 1 (of these, E is the enhancer-containing fragment including the MCK sequences in the oligomer probe); SV40, fragment containing the enhancer and part of the early promoter from SV40; RSV, fragment containing the Rous sarcoma virus long terminal repeat enhancer-promoter; κ, fragment containing the murine immunoglobulin κ chain enhancer; μ, fragment containing the murine immunoglobulin μ chain enhancer; pUC, linearized bacterial vector; gDNA, *Bam*HI-cut mouse genomic DNA. The molar excess of fragments over probe is indicated at the top. Arrows indicate positions of free probe (f) and the MEF 1 band (m°).

MEF 1 binds both MCK enhancers but not the MCK proximal promoter. Since a fragment from within the first MCK intron (Fig. 1, H) has enhancer activity (54; Jaynes, Ph.D. thesis), we were particularly curious to see whether this fragment would compete for MEF 1 binding. Interestingly, the 900-nt intron fragment competed about as well as did the 206-nt 5′-enhancer fragment (Fig. 7; compare lanes 3 and 8). Therefore, it seems likely that MEF 1 is a transcriptional factor which interacts with both the upstream and intron 1 regulatory regions. In addition, the ability of the myosin light-chain-1/3 enhancer region to compete for MEF 1 binding (see above) (Fig. 4) suggests that MEF 1 may be involved in regulating many muscle-specific genes.

The more proximal region of the MCK promoter (nt −800 to the transcription start site [P in Fig. 1]), which also contains muscle-specific positive regulatory elements (29), did not compete for MEF 1 binding (Fig. 7, lane 7). This suggests that distinct muscle-specific *trans*-acting factors interact with the two MCK enhancers and the proximal MCK promoter region.

DISCUSSION

We have characterized DNA-binding factors which interact with the upstream region of the MCK gene containing a muscle-specific enhancer. In initial gel mobility shift experiments using a 206-nt probe (E, Fig. 1), MM14 myocyte

nuclear extract produced two sequence-specific bands (m and n), while a hybridoma extract produced a band in only one of these positions (Fig. 2b). This was consistent with the possibility that a myocyte-specific factor (MEF 1) recognizes the MCK upstream enhancer. Further observations supported this possibility. A C2C12 myocyte nuclear extract produced a band in the position of the putative myocyte-specific band, while neither MM14 myoblast nor HeLa cell nuclear extract produced such a band (Fig. 2c). The HeLa extract did produce a dominant sequence-specific band of lesser mobility than the myocyte-specific band; however, subsequent experiments (see below) showed that the HeLa factor recognizes a different region of the probe; thus, it is not merely a variant of MEF 1 with altered mobility.

To determine the sites of binding more precisely, mobility shift assays with smaller probes (Table 1) and then DNase I footprinting techniques (Fig. 3) were employed. These experiments showed that the band common to myocytes and hybridoma cells (Fig. 2, band n) correlated with a footprint within the f1 fragment (Fig. 1) near the 3′ end of the 206-nt probe (data not shown). This region is not required for enhancer function (J. E. Johnson, J. N. Buskin, and S. D. Hauschka, unpublished), suggesting that the f1-binding factor is not critical for MCK gene activation during early MM14 terminal differentiation. Since the footprint overlapped the sequence TAAAAATAA, located at nt −1076 to −1068 (29), it is possible that the f1-binding factor is the α protein which binds to adenine-thymine-rich DNA (53) or a TATA box-binding factor such as TFIID (49). Another possibility is that the adenine-thymine-rich region represents a topoisomerase-binding site; this is intriguing in light of reports that nuclear matrix attachment regions containing topoisomerase binding sites have been found near other cellular enhancers (10, 18). In contrast to our results, a myocyte-specific factor has been reported as binding to this site (D. J. Kelvin, L. A. Gossett, E. A. Sternberg, and E. N. Olson, submitted for publication). Since different cell types and binding and electrophoretic conditions were used, it is difficult to directly compare these results.

The MEF 1-binding site was found to occur in the 110-nt internal fragment f4 (Fig. 1 and 3; Table 1), the smallest fragment tested that retained transcriptional activity in myocytes similar to that of the 206-nt fragment E. We have tested the function of a mutant fragment f4, which does not bind MEF 1 in vitro (Fig. 4), for its ability to stimulate transcription in myocytes. The mutant f4 fragment had a significant positive effect on expression in MM14 myocytes (four- to eightfold, depending on orientation), but it was about 25 times less active than the wild-type fragment (Table 2).

The residual activity of the mutant, non-MEF 1-binding f4 fragment taken together with several pieces of preliminary evidence showed that the MEF 1-binding site is insufficient for full enhancer activity. (i) Neither the 30- or 80-nt *Bst*XI fragment within f4 (Fig. 1) conferred full enhancer activity, although both fragments have a positive effect on expression (J. E. Johnson, J. N. Buskin, and S. D. Hauschka, unpublished results); since the MEF 1-binding site is within the 80-nt fragment, we inferred that an element overlapping the *Bst*XI site and/or within the 30-nt fragment is important for enhancer function. (ii) The possibility of an element overlapping the *Bst*XI site is supported by our observation that small deletions at this site (4 to 8 nt) greatly reduced enhancer function; however, since these deletions also affected the relative positions of elements within the 30- and 80-nt parts of f4, this does not prove that a positive element overlaps the *Bst*XI site. (iii) Insertion of the footprinted sequences as monomer or dimer oligomer inserts (see below) in several test plasmids increased expression but did not produce full enhancer activity. Consistent with the idea of multiple binding sites within the 110-nt f4 enhancer region, we have observed additional shifted bands with some myocyte extracts (Fig. 2c and 4b), and studies by other investigators have revealed several factors which bind within the 5′-enhancer region (25, 26; Kelvin et al., submitted).

Synthetic oligonucleotides based on the footprint in fragment f4 (Fig. 3) were used to further characterize MEF 1. The oligomers were sufficient to bind MEF 1 from either MM14 or C2C12 myocytes, while no corresponding binding factor was observed in extracts from six nonmyogenic cell lines (Fig. 5 and 6). Of particular interest were 10T1/2 cells, which are thought to represent a pluripotent mesodermal stem cell analogous to the developmental precursors of myoblasts (56), and DD1 cells, a differentiation-defective variant derived from MM14 myoblasts (34). Among the nonmyogenic lines tested, only HeLa cells gave a prominent band with mobility similar to the myocyte-specific band. However, this oligomer-binding band does not result from the same binding factor, as it was not reduced by inclusion of unlabeled MCK 5′-enhancer fragment compared with the nonspecific B fragment. The absence of a dominant, slowly migrating, competable band (Fig. 6, lane 25 versus 26) demonstrated that the HeLa factor producing the major competable band in Fig. 2c, lane 8 binds elsewhere. The evidence presented in this study that MEF 1 is a myocyte-specific binding activity is complemented by preliminary in vivo footprinting studies which demonstrate differences in the MEF 1-binding site between myocytes and fibroblasts (P. R. Mueller, J. E. Johnson, and B. J. Wold, unpublished results).

To examine the developmental-stage specificity of MEF 1, extracts were prepared from myoblasts. The three myoblast extracts shown in Fig. 6 produced weak bands in the positions of the competable bands in the corresponding myocyte lanes. Although this could indicate low-level MEF 1 occurrence in myoblasts, we believe that myoblast MEF 1 is actually contributed by the small proportion of myocytes present in myoblast cultures. It is possible, however, that an inactive form of MEF 1 exists in myoblasts or in nonmyogenic cells. If so, its activation would be analogous to the mechanisms proposed for factors E2F (48) and NF-κB (51). Preliminary results suggest that MEF 1 is identical to the myogenic determination factor MyoD1 (see below); if so, it would indicate that an inactive form of MEF 1 does exist, since MyoD1 can be antigenically detected in myoblasts. The existence of several phosphorylated forms of MyoD1 (55) may be related to this hypothesis.

Comparison of myocyte nuclear and cytoplasmic extracts indicated that most or all of MEF 1 is localized in the nucleus (Fig. 6). It was also possible that cells lacking nuclear MEF 1 might contain cytoplasmic MEF 1, analogous to the situation of steroid receptors which bind more tightly to nuclear components in the presence of steroids (62). However, the lack of MEF 1 in cytoplasmic extracts of myoblasts or HeLa cells (Fig. 6) indicated that the binding activity of MEF 1 is induced, rather than repositioned, upon myogenic differentiation.

We also looked for binding factors in mouse tissues. Although no MEF 1 activity was detected in brain, liver, or muscle nuclear extracts, the absence of MEF 1 could be due to inappropriate extraction protocols. If MEF 1 is actually lacking in adult muscle, this suggests that MEF 1 may be

required only during early stages of myogenesis, as represented by the MM14 and C2C12 myocyte extracts used in this study. This situation would be similar to those of the immunoglobulin heavy-chain and SV40 enhancers, which have been reported to be necessary to establish high level transcription but not to maintain it (31, 60).

Muscle tissue extracts did, however, contain sequence-specific binding factors; in particular, binding was observed within fragment f2. This fragment contains the sequence TCCCCAGGCC (in reverse orientation), which is a good match to the consensus-binding sequence for the transcriptional activator AP-2 (27, 40); also within the fragment is the sequence CCATGTAAGG, which is a good match to the CArG or CBAR consensus which has been proposed to play a role in muscle-specific gene regulation (4, 21, 39, 41). While CArG/CBAR sequences are contained in nonmuscle-specific genes as well as in a variety of muscle genes, differences between myogenic and nonmyogenic cell factors which bind to CArG/CBAR-containing fragments have been reported (58, 59). Although the 110-nt f4 fragment, which does not overlap the f2 region, functions as a muscle-specific enhancer (Table 2 and unpublished data), our preliminary data suggest that the 206-nt E fragment is somewhat more active than the 110-nt fragment. Thus, it is possible that the f2 region does play a role in muscle-specific gene expression.

The binding specificity of MEF 1 was investigated by using other sequences as test competitor DNAs. An enhancer-containing region of the MCK gene located in the first intron competed well, as did a fragment from the rat myosin light-chain-1/3 enhancer region (13) which contains a 10-of-10-nt match with the MEF 1 footprint region (Fig. 4 and 7). Thus, MEF 1 binds at least three myocyte-specific enhancers. In contrast, neither the muscle-specific regulatory elements of the MCK proximal region (29) nor several general enhancers competed for MEF 1 binding (Fig. 7).

We believe that MEF 1 is a novel factor, in part because of its cell-type occurrence; only one other factor has been reported to be specific to differentiated myocytes (Kelvin et al., submitted), and this binds to a different site of the MCK gene. Another factor, MAPF 2, has been reported to occur in myoblasts and myocytes, while an analogous binding activity, MAPF 1, occurs in other cell types (58, 59). MEF 1 appears to be distinct from MAPF 2 because their binding sequences are dissimilar and because MEF 1 is lacking in MM14 and C2C12 myoblasts, whereas MAPF 2 is present in L6 myoblasts. On the basis of the effects of mutating the MEF 1 site in the upstream MCK enhancer on myocyte expression and its recognition specificity, we believe that MEF 1 is involved in the muscle-specific regulation of the MCK gene and possibly of other muscle genes.

Recently, four different genes or cDNAs whose products cause mesodermal stem cell-like 10T1/2 cells to become myogenic have been reported (7a, 11, 47, 60b). Since entry into the myogenic lineage involves acquisition of the ability to induce muscle-specific genes upon specific external stimuli (e.g., absence of mitogens), it seemed reasonable to speculate that the myogenic-transforming agents might be directly involved in gene activation. In collaboration with A. B. Lassar, R. L. Davis, D. Lockshon, and H. Weintraub, we have investigated a link between the myogenic transforming protein MyoD1 (11) and MEF 1. Antibodies produced against MyoD1 recognize MEF 1; furthermore, bacterially produced MyoD1-fusion proteins bind to the MCK 5′ enhancer in the same region as MEF 1. We conclude that MEF 1 includes MyoD1 or a closely related protein. Since MyoD1 is present in myoblasts, the possible identity of MEF 1 with

SITE	SEQUENCE
MEF 1	C A G G C A G C A G G T G T T G G G G G
	* * * * * * * *
κE2	T C C C A G G C A G G T G G C C C A G A
	* * * * * * * * * * * * * *
Bst X I	A C C C A G A C A T G T G G C T G C C C

FIG. 8. Comparison of MEF 1-binding site with κE2 region of Igκ enhancer and *Bst*XI region of MCK enhancer. MEF 1 sequence was from the mouse MCK 5′-enhancer region (29) noncoding strand shown in this paper to bind a muscle-specific nuclear factor, MEF 1, and to be an essential part of the enhancer. κE2 sequence was from the mouse immunoglobulin light-chain-κ enhancer, showing similarity to other immunoglobulin-binding sites (8, 52). *Bst*XI sequence was from the mouse MCK 5′-enhancer region (29) surrounding the *Bst*XI restriction site (Fig. 1). Four- to eight-nucleotide deletions within this sequence diminish enhancer activity (J. E. Johnson, J. N. Buskin, and S. D. Hauschka, unpublished data). Bases which are identical between two sequences are indicated (*).

MyoD1 suggests that the MEF 1 protein is present in myoblasts in a form incapable of binding the MCK enhancer regions. Appropriate environmental stimuli, such as the absence of FGF in the case of MM14 cultures, would produce the enhancer-binding form of MEF 1. Since MyoD1 is not present in all cells producing MCK, it is possible that a family of related proteins with similar or identical binding specificity can induce MCK binding. It is interesting in this light that two other myogenic-transforming proteins, myogenin and myf5, have extensive sequence similarity to MyoD1 (7a, 60b).

The myogenic lineage-specific MEF 1-MyoD1-myogenin proteins may be part of a larger class of related DNA-binding proteins. This is suggested by examination of the factors binding to the immunoglobulin κ enhancer E2 site (recognized by sequence similarity to other immunoglobulin binding sites [8] and subsequently referred to as κE2 [52]). Recently, two cDNAs cloned via their binding to the κE2 site have been reported to bear sequence similarity to MyoD1 over a limited portion of their protein-coding regions (42a). Although the immunoglobulin κ site does not compete for MEF 1 binding, the sequences of the binding sites are similar (7-of-7-nt identities). In addition, the region of the MCK 5′ enhancer containing the *Bst*XI site that also appears to contribute to enhancer activity contains a similar sequence. The three sequences shown in Fig. 8 may represent binding sites for three members of a family of transcription factors which have evolved to recognize distinct but related sequences. On the basis of comparisons between the MEF 1-binding site in the MCK enhancer with regulatory regions of other muscle-specific genes (3a, 13, 22a, 26, 29, 46a, 56a, 60a, 62a), we propose the following consensus sequence for muscle-specific binding:

$$\begin{matrix} C & N & G & G & & C & C & & T & G & C & C & N & C \\ G & & A & A & C & A & G & G & & & T & T & & G \end{matrix}$$

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