The human M creatine kinase gene enhancer contains multiple functional interacting domains

Robert V.Trask, Joseph C.Koster, Michael E.Ritchie and Joseph J.Billadello

Department of Medicine, Washington University School of Medicine, St Louis, MO 63110, USA

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ABSTRACT

Cis-elements (– 933 to – 641) upstream of the human M creatine kinase gene cap site contain an enhancer that confers developmental and tissue-specific expression to the chloramphenicol acetyltransferase gene in C₂C₁₂ myogenic cells transfected in culture. Division of the enhancer at –770 into a 5' fragment that includes the MyoD binding sites (– 933 to – 770) and a 3' fragment that includes the MEF-2 binding site (– 770 to – 641) resulted in two subfragments that showed minimal activity but in combination interacted in a position- and orientation-independent fashion to enhance activity of the SV40 promoter in transient transfection experiments. A 5' enhancer construct (– 877 to – 832) including only one (the low affinity) MyoD binding site was active when present in multiple copies. In contrast, a 3' enhancer construct (– 749 to – 732) including the MEF-2 binding site was inactive even when present in multiple copies. However, if the 5' construct was extended to include the high-affinity MyoD binding site (– 877 to – 803) the 5' and 3' constructs interacted in a position- and orientation-independent fashion to activate the SV40 promoter. Thus, the human M creatine kinase enhancer comprises multiple functional interacting domains.

INTRODUCTION

During skeletal muscle development proliferating mononucleated myoblasts withdraw from the cell cycle, fuse to form multinucleated myotubes and transcriptional activation of many contractile proteins and metabolic enzymes necessary for the development of a mature muscle cell phenotype occurs (1–8).

An important question has been whether coordinate expression of the many unlinked muscle-specific genes is governed by common or diverse trans-acting factors. Recent studies have shown the existence of a family of regulatory factors including MyoD (9), myogenin (10,11), Myf-5 (12) and MRF-4 (13) that share extensive amino acid homology within the DNA binding domain and the domain that mediates heterodimerization with other trans-acting factors. These myogenic factors are able to convert the multipotential mesodermal stem cell line 10T1/2 to the myogenic lineage. Interestingly, all of these myogenic factors are expressed exclusively in skeletal muscle with no detectable expression in cardiac muscle, a tissue in which many of the

sarcomeric protein genes, including M creatine kinase (14), are also expressed. These same myogenic factors form heterodimers with E12, the ubiquitous immunoglobulin enhancer-binding factor (15,16,17), and bind to a consensus sequence CANNTG (E-box or MEF-1 motif) that is present in two copies (a high affinity [MyoDR] site and a low affinity [MyoDL] site) in the M creatine kinase enhancer. The MyoD binding sites represent one essential component of this enhancer (18,19) and indicate the potential for myogenic factors to activate muscle genes directly as well as by triggering the myogenic developmental program. However, not all muscle genes have MEF-1 binding motifs in regions known to be critical for their expression (20–22). Similarly, although the MyoD binding site is important for trans-activation of the mouse M creatine kinase enhancer by myogenin in 10T1/2 cells (16) and is required for muscle-specific activity of the enhancer in C₂ myotubes (18,23) it is not the only site within this enhancer that interacts with sequence-specific DNA-binding factors (18,24,25) nor is this site alone sufficient to generate high-level muscle-specific enhancer activity. The mouse (24) and rat (25) M creatine kinase enhancers contain an AT-rich sequence in the 3' end of the enhancer that is conserved among multiple muscle-specific genes. This motif, which is necessary for full function of the enhancer, interacts with a factor termed myocyte-specific enhancer-binding factor –2 (MEF-2) (24) or TA-rich recognition protein (TARP) (26). Hence, evidence has been presented for the presence of multiple functional domains within the M creatine kinase enhancer. However, the ability of these domains to cooperate or synergize to enhance transcription has not been determined.

We describe two enhancers with different functional properties within the M creatine kinase enhancer.

EXPERIMENTAL PROCEDURES

Cells and cell culture

Cell culture conditions for C₂C₁₂ and NIH/3T3 cells have been described previously (2).

Construction of Human M Creatine Kinase-Chloramphenicol Acetyltransferase (CAT) Gene Chimeras

Human M creatine kinase promoter deletion constructs were prepared with the plasmid MCKCAT2620 previously described (2).

The plasmids MCKCATE933/641(+) (Figure 2B) and MCKCATE933/641(−) (Figure 2C) were constructed by inserting the the SmaI-BstEII fragment (position −933 to −641 base pairs upstream of the cap site) into the unique BamHI site of MCKCAT641 (Figure 2A) 3' to the CAT gene in both orientations.

SV40 Early Promoter-M Creatine Kinase Enhancer Constructs

The plasmid pUC19pCAT was generously provided by Nadia Rosenthal (Boston University). This vector contains the SV40 early promoter (without the SV40 enhancer) upstream of the CAT gene. All constructs in the plasmid pUC19pCAT were prepared with the use of the BamHI or PstI sites 3' to the CAT gene or the KpnI site 5' to the CAT gene (Figure 3A). The orientation of the inserts was determined by DNA sequence analysis. The construct pCATE933/641 contains the SmaI-BstEII fragment in the 3' BamHI site in the sense orientation (Figure 3A). The plasmid pCAT2E933/641 contains two copies of the SmaI-BstEII fragment in the sense orientation in the BamHI site (Figure 3B). Deletion of base pairs −933 to −863 was discovered in one recombinant during the construction of pCATE933/641. Sequence analysis of the plasmid designated pCATE862/641 demonstrated no other deletions or point mutations (Figure 3C). In pCAT1.75E862/641 an additional copy of the SmaI-BstEII fragment was inserted into the plasmid pCATE862/641 in the sense orientation (Figure 3D). A convenient BglI site at −770 (Figure 3A) was used to prepare additional constructs comprising the 5' (−933 to −770) and 3' (−770 to −641) portions of the M creatine kinase enhancer. The plasmid pCATE933/770 was constructed by placing the SmaI-BglI fragment (position −933 to −770) into the intact BamHI site of pUC19pCAT in the sense orientation (Figure 3E). The plasmid pCATE770/641 was prepared by ligating the BglI-BstEII fragment (position −770 to −641) into the BamHI site of pUC19pCAT in the sense orientation (Figure 3F). Plasmids pCATE933/770Kpn(+) and pCATE933/770Kpn(−) were prepared by ligating the BglI-BstEII fragment (position −770 to −641) into the Kpn site of pCATE933/770 5' to the CAT gene in the sense and antisense orientations, respectively (Figures 3G and 3H).

Synthetic oligonucleotides were synthesized on an Applied Biosystems 380B automated DNA synthesizer. Constructs 01, 06, 07, 08, 09 were prepared by ligating one or more copies of annealed synthetic oligonucleotides in the BamHI site of pUC19pCAT in the sense orientation (Figure 5). Construct 03 was prepared by ligating one or more copies of the annealed synthetic oligonucleotide 03 in the PstI site of pUC19pCAT in the sense orientation as shown in Figure 5. Constructs 08+03 and 09+03 were prepared by ligating oligonucleotide 03 into the PstI site of constructs 08 and 09 in the sense orientation and copy number designated in Figure 6 or in the text.

Cell transfection and chloramphenicol acetyltransferase assays

Transfection and CAT assays were performed as described previously (2,28). C₂C₁₂ cells were plated at a density of 3.0×10^5 cells per 60 mm dish 24 h before transfection. Precipitates contained a total of 20 μ g of plasmid DNA comprised of 15 μ g of test plasmid and 5 μ g of the reference plasmid pMSV β gal. Myoblasts were harvested 96 h after transfection and myotubes were harvested 96 to 120 h after transfection. An assay for β -galactosidase was performed to normalize for transfection efficiency and yield of extract (29).

Gel mobility shift assays. Nuclear protein extracts were prepared from C₂C₁₂ myoblasts, C₂C₁₂ myotubes, Hep G2 and HeLa cells with a modification of the procedure described by Heberlein et al. (30), quantified (31) and stored frozen at −80°C.

For gel mobility shift assays 1.0×10^4 dpm of double-stranded [³²P]-labeled probe was incubated with 5 μ g of nuclear protein extract, 3.0 μ g poly [d(I-C)] or 1.0 μ g sonicated herring sperm DNA in a solution containing 50 mM KCl, 5% glycerol, 12.5 mM Hepes (pH 7.6), 6.25 mM MgCl₂, 0.05 mM EDTA, in a volume of 20 μ l at room temperature for 20 min. The reaction was then subjected to electrophoresis at 160 volts in a 5% polyacrylamide gel with $0.5 \times$ TBE (45 mM Tris-Borate, pH 8.3, 1.25 mM EDTA) as the buffer.

Figure 1. Promoter deletion analysis. A, The restriction sites StuI, SmaI, and BstEII were used to create deletions within the human M creatine kinase 5'-flanking DNA. The fragments were inserted into the HindIII site of pSVOCAT in the 5' to 3' orientation upstream of the CAT structural gene. The resulting plasmids are designated MCKCAT2620, MCKCAT1562, MCKCAT933 and MCKCAT641. The CAT activity present in extracts prepared from myotubes, transfected with the constructs described in panel A, is expressed relative to that of MCKCAT2620.

RESULTS

M creatine kinase promoter deletion analysis

To locate the cis-acting sequence elements responsible for developmentally regulated expression of the human M creatine kinase gene, promoter deletion mapping was used. In Figure 1, panel B, CAT activity in extracts prepared from myotubes is expressed relative to that of MCKCAT2620. Results represent the average of six or more transfection experiments with two different plasmid preparations for each promoter deletion construct. Deletion of sequences between -933 and -641 base pairs upstream of the cap site reduced expression of CAT to the basal level seen with the promoterless plasmid pSVOCAT. Thus, sequences between -933 and -641 base pairs are vital to expression of the human M creatine kinase gene in transfected C₂C₁₂ myotubes.

M creatine kinase enhancer studies

To determine whether sequence elements within -933 to -641 base pairs had the functional properties of an enhancer. The plasmids MCKCATE933/641(+), MCKCATE933/641(-), MCKCAT641 and MCKCAT933 were transfected into C₂C₁₂ myoblasts. In Figure 2 each construct is shown schematically with autoradiograms from a representative transfection experiment. Transfections were repeated four to 12 times with two different plasmid preparations. MCKCATE933/641(+) and MCKCATE933/641(-) were expressed 13.7- and 13.9-fold higher than MCKCAT641, respectively, representing 94% and 97% of the activity of MCKCAT933, which contains the SmaI-BstEII fragment in its native position and orientation. The constructs MCKCATE933/641(+) and MCKCATE933/641(-) were expressed in a developmentally regulated manner with 5.2- and 3.8-fold induction during differentiation, respectively. At the time of harvest the myoblasts had become confluent and biochemical differentiation had begun, as confirmed by determination of creatine kinase activity in extracts prepared from the myoblasts

Figure 2. M creatine kinase enhancer studies. The plasmids MCKCAT641 (A), MCKCATE933/641(+) (B) and MCKCATE933/641(-) (C) were transfected into C₂C₁₂ myoblasts as described. Myoblasts and myotubes were harvested 72 h after media change. The products of a representative CAT assay; 3-acetyl[¹⁴C]-chloramphenicol (3AC), 1-acetyl[¹⁴C]-chloramphenicol (1AC) and [¹⁴C]chloramphenicol (Ch) are shown. CAT activity relative to MCKCAT641 (normalized to 1) in myotubes (T) and myoblasts (B) and fold induction during differentiation are shown below the thin-layer chromatography panels.

(data not shown). Hence, CAT activity was observed in transfected myoblasts. Thus, sequences between -933 to -641 base pairs upstream of the human M creatine kinase cap site display the characteristics of a developmentally active enhancer, activating transcription in an orientation- and position-independent manner.

Heterologous promoter studies

The SmaI-BstEII fragment (-933 to -641) was inserted in both orientations in the unique BamHI site in pUC19pCAT. The constructs containing the M creatine kinase enhancer in the sense and antisense orientations were expressed 9.3- and 10.8-fold higher than pUC19pCAT, respectively, in transfected myotubes and demonstrated 3.6- and 3.8-fold induction of CAT activity during differentiation from myoblasts to myotubes (n = 12 experiments). Thus, the human M creatine kinase enhancer confers developmentally regulated expression to the heterologous SV40 early promoter.

To determine whether the M creatine kinase enhancer conferred tissue-specific expression to the SV40 promoter we transfected the plasmids pCATE933/641 and pCAT2E933/641 (Figures 3A

Figure 3. Heterologous promoter studies. The complete SmaI/BstEII enhancer fragment and subfragments derived from the enhancer were subcloned into the vector pUC19pCAT as described in 'Experimental Procedures.' The orientation and position of each fragment relative to the CAT gene are shown schematically. Plasmid constructs were transfected and CAT activity in extracts from myotubes were determined as described in Figures 1 and 2. CAT activity relative to expression of pCATE933/641 (normalized to 1) is shown. N indicates the number of plates of cells transfected with each construct. At least two preparations of each plasmid were used for all transfection studies.

and 3B) into mouse fibroblast NIH/3T3 cells. Neither plasmid was expressed in transfected NIH/3T3 cells (data not shown). Thus, the human M creatine kinase enhancer contains the elements necessary to confer muscle-specific expression to a heterologous promoter.

Results of transfection experiments with constructs containing the SV40 promoter and fragments derived from the M creatine kinase enhancer in C₂C₁₂ myotubes are shown relative to pCATE933/641 (normalized to one) in Figure 3. Results of initial transfection experiments presented in Figure 3 (B–D) led us to suspect that the M creatine kinase enhancer showed a modular organization of multiple functional interacting sequence elements typical for viral and certain cellular enhancers (27,32,33,34). As shown in Figure 3C, the plasmid pCATE862/641 demonstrated only minimal activity (less than two-fold above background).

Results of transfection experiments with the construct pCAT1.75E862/641 (Figure 3D) showed that sequence elements present within –862 to –641 were capable of interacting with the complete enhancer, with resultant activity intermediate between a single copy and two copies of the intact enhancer (see Figure 3B). These results show that deletion of sequences from –933 to –862, which are outside the MyoD binding sites and the MEF-2 binding site, results in marked loss of expression of the M creatine kinase enhancer and that the remaining elements from –862 to –641 were capable of interacting with the intact enhancer to increase expression of the SV40 promoter.

Additional constructs were prepared with the use of a convenient BglI site at –770 (Figure 3A) to create two subfragments of the enhancer without interrupting the essential sequence motif present at –862 (Figure 3C). The constructs

Figure 4. Comparison of the nucleotide sequences of the human and mouse M creatine kinase enhancers. The mouse M creatine kinase enhancer (35,36) and the human M creatine kinase enhancer (2) are shown. The sequences of the sense strand of synthetic double-stranded oligonucleotides 01, 02, 03, 03-S, 04, 06, 07, 08 and 09 derived from the human enhancer are represented by solid lines. The numbering shown above the sequences is based on the human sequence. The coordinates of the mouse enhancer are from –1256 to –1050.

Figure 5. Determination of functional domains within the M creatine kinase enhancer. Oligonucleotides 01, 08, 09, 06 and 07 were prepared with BamHI ends and ligated in the sense orientation in one or more copies into the BamHI site of pUC19pCAT as shown. Oligonucleotide 03 was placed in the sense orientation in one or more copies in the PstI site of pUC19pCAT. The relationship of the sequences within the synthetic oligonucleotides to the functional elements and restriction sites within the complete enhancer is shown schematically. CAT activity in extracts prepared from C₂C₁₂ myotubes after transfection is expressed relative to pCATE933/641 (normalized to 1). At least two preparations of each plasmid were evaluated in transfection experiments with multiple batches of C₂C₁₂ cells. N represents the number of plates of C₂C₁₂ myoblasts transfected with each plasmid construct. A diagram of the vector pUC19pCAT is shown in the lower panel.

pCATE933/770 and pCATE770/641 containing the 5' and 3' portions of the enhancer, respectively, showed less than 50% of the activity of the intact enhancer (Figure 3E and 3F) and like pCATE862/641 their expression was less than two-fold above background.

Because enhancers may comprise distinct functional domains that exhibit little activity on their own but can cooperate in a position- and orientation-independent fashion (34), we prepared constructs in which the native position and orientation of the 3' element relative to the 5' element were disrupted by ligating the BglI-BstEII fragment into the KpnI site of pCAT933/770 in both orientations with the use of KpnI linkers. The expression of these constructs was compared with that of the intact enhancer and to the plasmids pCATE933/770 and pCATE770/641. The expression of each composite construct (Figure 3, G and H) was consistently greater than the expression of either deletion construct and was always greater than two-fold above background, although it was never equal to the level of expression of the complete enhancer. Thus, two separate interacting elements are present within the M creatine kinase enhancer.

To delineate the functional sequences within each enhancer element we prepared double-stranded synthetic oligonucleotides based on the results of our transfection experiments and on regions of sequence identity between the human and the mouse M creatine kinase enhancers (Figure 4). All constructs were sequenced in their entirety to determine insert copy number and orientation and to exclude the presence of inadvertent mutations. Important sequence motifs present within the oligonucleotide constructs shown in Figure 5 include a C[A/T-rich]G or CArG box (CCTTGTAAGG) essential for regulation of human skeletal and cardiac α -actin genes (3), the high-affinity (right) MyoD binding site (MyoDR) and low-affinity (left) MyoD binding site (MyoDL) (19,23) and the binding site for MEF-2 (24). The expression of each oligonucleotide construct in transfected C₂C₁₂ myotubes. The constructs 01, 08 and 09 were designed to determine the importance of the CArG box motif and the MyoD low- and high-affinity binding sites to the function of the 5'-enhancer element. Constructs containing a single copy of 01, 08 or 09 were all expressed inefficiently (less than two-fold above background), in agreement with the results shown in Figure 3E.

Because certain enhancer fragments, which have only a negligible effect by themselves, become potent enhancers when present in multiple copies (37), we determined the activity of plasmids containing multiple copies of 01, 08 and 09. Interestingly, plasmids containing two copies of either of the three 5' oligonucleotides were expressed with activity approaching that of the complete enhancer (Figure 5). Comparison of the activity of plasmids containing two copies of 01 or 09 showed that the CArG box motif could be deleted without loss of function. Similarly, deletion of the high-affinity MyoD binding site resulted in an insignificant decrease in activity in the context of two copies of the 5' element (compare 09 with 08). In contrast, deletion of both MyoD binding sites resulted in an inactive construct 06. Construct 06, which contains sequence elements common to 08 and 09 but lacks both the low- and high-affinity MyoD binding sites, was not expressed over background. Similarly, the low-affinity MyoD binding site, even when present in multiple copies, was inactive when isolated from the other 5' sequence elements (see 07). Thus, sequences within the MyoD low-affinity site and additional sequences within the region spanned by 06 are both important for functional expression of the 5'-element. Additional evidence for the presence of functionally important sequence elements within 06 was obtained from transfection experiments with a construct containing one intact copy of 09 in tandem with a mutant 09 in which the four base pairs AATT (-850 to -847) were deleted. The expression of this construct was identical to that of constructs containing a single copy of 09 ($n = 16$, data not shown), indicating that sequence elements within the region spanned by 06 are important for the function of the 5' enhancer motif.

Construct 03 was designed to test the functional importance of the MEF-2 domain (24). The oligonucleotide 03 represents the region within the BglI-BstEII fragment that shows a high degree of sequence identity with the mouse enhancer and contains the MEF-2 binding site. As shown in Figure 5, constructs containing the MEF-2 binding site are not expressed above background and their expression does not increase with copy number, in contrast to the 5' enhancer constructs.

Because cooperativity of enhancer motifs may result from the tandem repetition of a given motif or from the association of two

Figure 6. Interacting functional domains within the M creatine kinase enhancer. Plasmids were prepared with synthetic oligonucleotides 03 and 08 or 09 alone or in combination with oligonucleotide 03 in pUC19pCAT as described in the text. The orientation and copy number of each synthetic oligonucleotide is shown. Plasmids were transfected into C₂C₁₂ myoblasts and CAT activity in extracts from C₂C₁₂ myotubes relative to cells transfected concurrently with pCATE933/641 (normalized to 1) is shown.

different motifs (41), we prepared constructs containing 5' sequence elements in combination with 03. The orientation and copy number of each oligonucleotide is shown in Figure 6. When constructs containing 08 in combination with 03 were transfected into C₂C₁₂ cells marked augmentation of activity was seen relative to constructs containing 08 only. The same magnitude of augmentation of activity by three copies of 03 was noted when 08 was present in two copies in the antisense orientation ($n = 12$, data not shown). Interestingly, augmentation by one copy of 03 was approximately one-half that seen with three copies of 03 ($n = 8$, data not shown). Thus, the sequence motif present in 03 interacts with those present in 08 in a position-independent and copy number-dependent fashion. In striking contrast, only insignificant augmentation of activity of the 09 construct occurred when 03 was combined with 09. This effect plateaued with one copy of 03 ($n = 8$, data not shown) and did not change when the copy number of 03 was increased from one to three. Similarly, only slight augmentation of activity was seen when three copies of 03 were placed in the sense orientation in combination with three copies of 07 (data not shown). These results show that both the MyoD high-affinity binding site and the MyoD low-affinity binding site must be present for optimal interaction of the MEF-2 domain with the 5' enhancer elements.

DNA-Protein Interaction Studies

To characterize the interaction of sequence elements representing functional domains of the human M creatine kinase enhancer with trans-acting factors we used gel mobility shift assays. Figure 7A illustrates results of experiments in which [³²P]-labeled SmaI-BglI fragment (−933 to −770) was incubated with nuclear protein extracts and subjected to polyacrylamide gel

Figure 7. Gel mobility shift assays with genomic enhancer fragments. [³²P]-labeled SmaI/BglI fragment (panel A) and BglI/BstEII fragment (panel B) were incubated with nuclear protein extracts prepared from C₂C₁₂ myoblasts (Blasts or B), C₂C₁₂ myotubes (Tubes or T) and HeLa cells and DNA-protein complexes were resolved by polyacrylamide gel electrophoresis and detected by autoradiography. Uncomplexed probe is designated F. DNA protein complexes that were consistently observed are designated 1 and 2 (see arrows). **A**, lanes 1 and 6, Reactions without additional specific DNA competitor. In lanes 2, 4 and 7, a DNA fragment of the same length as the SmaI/BglI fragment representing an unrelated sequence was added in molar excess to the [³²P]-labeled fragment as a competitor. A molar excess of unlabeled SmaI/BglI fragment was added to the reactions as a specific competitor in lanes 3, 5 and 8. **B**, Lanes 9 and 10 represent gel mobility shift assays with the BglI/BstEII fragment without additional specific DNA competitor.

electrophoresis and autoradiography. Two DNA protein complexes designated 1 and 2 were consistently present in myoblast, myotube and HeLa cell extracts. When unlabeled SmaI-BglI fragment was added to the binding reaction in molar excess to [³²P]-labeled fragment, complexes 1 and 2 were not seen. However, addition to the binding reaction of a molar excess of an unlabeled fragment of the same length as the SmaI-BglI fragment but representing an unrelated sequence did not result in competition for binding of trans-acting protein factors. Therefore, complexes 1 and 2 represent the specific interaction of ubiquitous trans-acting protein factors with the 5'-element of the enhancer. In Figure 7B gel mobility shift assays with the BglI-BstEII fragment (−770 to −641) that contains the functional MEF-2 domain did not reveal DNA-protein interaction. Thus, the 5'-enhancer element interacts with trans-acting factors that are not muscle cell or differentiation specific, while the 3' element did not interact with nuclear proteins in any of the extracts tested.

To localize the sites of DNA protein interaction within the 5'-element, we performed gel mobility shift assays with double-stranded synthetic oligonucleotides 08 and 09, which were shown to have functional activity in transfection experiments. Figure 8, panel A, illustrates results when synthetic oligonucleotide 08 was labeled and used as a probe. DNA protein complexes with electrophoretic mobility identical to that of complexes 1 and 2 (Figure 7) were formed with protein extracts from myotubes and myoblasts as shown in lanes 1 through 6. Panel B illustrates results when oligonucleotide 09 was labeled and used as a probe. Complexes 1 and 2 were again present in nuclear extracts from myoblasts, myotubes and HeLa cells (lanes 7 through 9). For each extract tested, complexes 1 and 2 represented specific DNA-protein interaction. A molar excess of an unlabeled double-stranded oligonucleotide representing an unrelated sequence did not compete with [³²P]-labeled 09 for binding of trans-acting

Figure 8. Gel mobility shift assays with the 5'-enhancer element. [³²P]-labeled synthetic oligonucleotides 08 (**A**) and 09 (**B**) were incubated with C₂C₁₂ myoblast (Blasts or B), C₂C₁₂ myotube (tubes) and HeLa cell nuclear extracts as described in the 'Experimental Procedures.' DNA protein complexes 1 and 2 are depicted with arrows. Unbound probe is designated as F. Lanes 1, 4, 7, 8, 9 and 14 represent reactions without additional specific competitor DNA. In lanes 2, 5 and 10 a synthetic double-stranded oligonucleotide representing an unrelated sequence was used in molar excess as a competitor. In lanes 3 and 6 unlabeled 08 was added to the reaction as a specific competitor. Unlabeled oligonucleotides 07 (lanes 11 and 16), 06 (lane 12), 04 (lane 13) and 02 (lane 15) were also used as specific competitors.

factors (lane 10, data shown for myotube extract only), but an excess of unlabeled 09 did compete (data not shown). To further delineate the cis-acting sequences that interact with nuclear proteins to form complex 1 and 2, unlabeled oligonucleotide 07 was used as a competitor. As shown in lanes 11 and 16, when a molar excess of 07 was added to the binding reaction complex 2 was not detected. Thus, the DNA sequence elements that form complex 2 correspond to 07 (the MyoD low affinity binding site). Of interest is the fact that 02, which represents the MyoD high-affinity binding site, did not compete with [32 P]-labeled 09 for formation of complex 2 (lane 15). Because sequence elements present in 06 are also important for functional expression of the M creatine kinase enhancer, we used 06 as a specific competitor in gel shift assays. As shown in lane 12, a molar excess of oligonucleotide 06 competes with [32 P]-labeled 09 for formation of complex 1. Sequences present within 06 can be divided into a GC-rich domain designated 04 (–877 to –859) and an AT-rich region (–859 to –843). To determine the sequence elements within 06 that give rise to complex 1, unlabeled 04 was used as a specific competitor in gel mobility shift assays with [32 P]-labeled 09. As shown in lane 13, 04 competes with 09 for formation of complex 1. A double-stranded oligonucleotide comprising –859 to –843 did not compete with 09 for formation of complex 1 (data not shown). Therefore, DNA-protein interactions with the functional 5'-enhancer element have been localized to the sequences corresponding to the MyoD low-affinity binding site and to a separate GC-rich sequence domain spanned by 04.

To characterize the interaction of the MEF-2 sequence element with trans-acting factors, gel mobility shift assays were performed with synthetic oligonucleotide 03, which was functionally

important in transfection experiments. Incubation of 03 with myotube, myoblast, Hep G2 and HeLa cell extract was shown to result in a complex pattern with numerous DNA-protein complexes present after autoradiography (Figure 9, panel A). However, competition experiments showed that a single DNA-protein complex represented specific interaction with trans-acting factors. This complex was present when 03 was incubated with myotube but not myoblast extracts, indicating that the DNA-protein interaction was differentiation specific. Although the complex was not found when Hep G2 extracts were analyzed, it was detected in HeLa cell extracts. Thus, the trans-acting factors that complex with 03 are not muscle cell specific. A 12 base pair oligonucleotide (03-S) that contained only the highly conserved (AT-rich) sequences within 03 was also used as a probe (Figure 9, panel B). When herring sperm DNA was included in the binding reaction in place of poly[d(I-C)], nonspecific interaction of the DNA probe with nuclear proteins was eliminated and only a single band was seen after autoradiography.

DISCUSSION

Our results show that the human M creatine kinase enhancer is complex and comprises several distinct domains. At least the low-affinity (left) MyoD binding site must be present in addition to sequence elements contained in 06 (–877 to –843) for expression of the 5' enhancer element. The importance of the high-affinity (right) MyoD binding site was noted in experiments designed to determine whether sequence elements contained within 03 (the MEF-2 domain) were capable of interacting with the 5' enhancer element to activate the SV40 promoter (Figure 6). Although inactive by itself, even when present in multiple copies, the MEF-2 domain shows a cooperative effect in a position- and orientation-independent manner with 5' constructs that contain the high-affinity MyoD binding site, indicating the importance of protein-protein interactions in bringing together the two enhancer domains and the SV40 promoter. Sequence elements contained in oligonucleotide 06 (–877 to –843) include a GC-rich region and an AT-rich region. The importance of each of these sequence elements was shown by experiments with constructs in which the GC-rich domain was interrupted at –862 and with constructs in which a portion of the AT-rich domain was deleted. Interestingly, sequences corresponding to the 5' border of 06 are the site of a myotube-specific *in vivo* footprint (38) of the mouse enhancer.

Both the 5' and 3' enhancer domains interact with sequence-specific DNA binding proteins. The results of gel mobility shift assays show that ubiquitous trans-acting protein factors bind specifically to the low-affinity MyoD binding site and the GC-rich domain of 06. In contrast to the 5' enhancer element the MEF-2 domain interacts specifically with trans-acting factors that are present in myotubes but not myoblasts and are hence differentiation specific. However, these factors are not muscle cell specific because they are found in HeLa cells.

The 5' and 3' borders of the minimal enhancer element from the rat M creatine kinase gene (25,26) correspond almost exactly to those of the human M creatine kinase enhancer. The results of gel mobility shift experiments and DNase I footprinting showed that trans-acting factors binding to the 5' enhancer element were not differentiation specific. Factors interacting with the 3' enhancer element were not muscle specific. Oligonucleotides corresponding to the rat MyoD high-affinity binding site (E4) did not compete with the rat gene enhancer for

Figure 9. Gel mobility shift studies with the MEF-2 sequence element. [32 P]-labeled double-stranded synthetic oligonucleotides 03 (A) and 03-S (B) were incubated with C₂C₁₂ myotubes (Tubes), C₂C₁₂ myoblast (Blasts), Hep G2 and HeLa cell nuclear extracts. The arrow to the left of A and to the right of B designates the MEF-2 DNA protein complex. Unbound [32 P]-labeled DNA is designated F. In lanes 1–9, poly[d(I-C)] was used as a nonspecific competitor. In lanes 10–12, herring sperm DNA (1 μ g) was used instead of poly[d(I-C)]. No additional specific competitor was present in lanes 1, 4, 5, 6, 9 and 10. In lanes 2, 7 and 11 an unlabeled synthetic oligonucleotide representing an unrelated sequence was added in molar excess. In lanes 3 and 8 unlabeled 03 was added to the reaction. In lane 12, unlabeled 03-S was added to the reaction.

binding of trans-acting factors. Our results are in agreement with these findings. In addition, mutation or deletion of the 3' portion of the rat gene enhancer (E3 corresponding to the human construct 03) that eliminated the MEF-2 domain resulted in marked loss of enhancer activity to 20% or less than that of the wild-type enhancer in the context of a minimal rat M creatine kinase promoter. Thus, results of experiments with both the rat and human enhancers point to the importance of multiple functional domains and trans-acting factors for regulated expression of the M creatine kinase gene.

Similarly, characterization of the mouse M creatine kinase gene enhancer (18) showed that, although constructs containing the low- and high-affinity MyoD binding site (the 110 base pair MEF-1 binding fragment [F4]) activated expression of the minimal M creatine kinase promoter, a 206 base pair enhancer fragment that included sequences 5' of the F4 fragment as well as the MEF-2 domain was more active than the 110 base pair F4 fragment, in agreement with our findings. Further work with the mouse gene enhancer has also showed that deletion of sequences 5' of the *Ava*I site at -1204 (corresponding to -862 in the human gene sequence) or deletion of the MEF-2 domain results in loss of function when compared with that of the complete enhancer (24). Although inactive by itself, when present in multiple copies the MEF-2 domain functioned as an enhancer, activating the mouse M creatine kinase promoter in transfected myotubes and myoblasts. Our results show that even when present in multiple copies the MEF-2 domain is not able to activate the SV40 promoter, indicating that interactions between the MEF-2 enhancer and specific promoter sequences may be important determinants of activity (39).

Cellular and viral enhancers have a complex modular organization (33). Enhancer function depends on the combination of various sequence motifs or modules, each contributing to the overall activity of the enhancer (32,33). For example, the SV40 enhancer can be divided into two domains, each of which shows little enhancing activity on its own. Increasing the copy number of either domain results in a linear increase in transcription, while combining both domains in a relatively position-, orientation- and distance-independent fashion results in marked synergism of activity (27). Each domain is composed of a complex array of regulatory elements (enhancers) that appear to be the basic units of enhancer structure (33,40). Some enhancers are functional when present in multiple copies. Others form a functional module when combined with a nonidentical enhancer (33). Other domains that are functionally important within the complete enhancer and can cooperate with different motifs to generate an enhancer element show no activity by themselves, even when present in multiple copies (40,41). Short oligonucleotides that encompass these sequences exhibit enhancer activity when present in multiple copies. Thus individual segments of the SV40 enhancer, when present in multiple copies, function as well as the complete enhancer (32). The modular structure of the M creatine kinase enhancer that we present is strikingly similar to that of the SV40 enhancer.

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