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Collaborative Interactions Between MEF-2 and Sp1 in Muscle-Specific Gene Regulation

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Abstract Previous investigations have demonstrated synergistic interactions in vivo between CCAC and A/T-rich nucleotide sequence motifs as functional components of muscle-specific transcriptional enhancers. Using CCAC and A/T-rich elements from the myoglobin and muscle creatine kinase (MCK) gene enhancers, Sp1 and myocyte-specific enhancer factor-2 (MEF-2) were identified as cognate binding proteins that recognize these sites. Physical interactions between Sp1 and MEF-2 were demonstrated by immunological detection of both proteins in DNA binding complexes formed in vitro by nuclear extracts in the presence of only the A/T sequence motif, by coprecipitation of recombinant MEF-2 in the presence of a glutathione-S-transferase–Sp1 fusion protein bound to glutathione beads, and by a two-hybrid assay in *Saccharomyces cerevisiae*. The interaction with Sp1 in vitro and in vivo is specific for MEF-2 and was not observed with serum response factor, a related MADS domain protein. Forced expression of Sp1 and MEF-2 in insect cells otherwise lacking these factors promotes synergistic transcriptional activation of a promoter containing binding sites for both proteins. These data expand the repertoire of functional and physical interactions between lineage-restricted (MEF-2) and ubiquitous (Sp1) transcription factors that may be important for myogenic differentiation. J. Cell. Biochem. 70:366–375, 1998.

Key words: transcription; myogenesis; MADS domain; DNA binding

Promoter regions of more than 20 genes expressed selectively in striated myocytes have been characterized and found to contain binding sites for both muscle-restricted and ubiquitously expressed transcription factors. Previously, we demonstrated the critical importance of a CCAC sequence and an A/T rich element for transcriptional regulation of the human myoglobin gene [Bassel-Duby et al., 1992]. We also demonstrated muscle-specific gene expression from a minimal promoter constructed with only CCAC and A/T sequences linked to a TATA element [Grayson et al., 1995]. Similar CCAC and A/T-rich elements are present within functionally defined transcriptional control regions of several genes that exhibit muscle-specific expression (Fig. 1), suggesting that interactions between factors binding these motifs is conserved as a common mechanism for gene regulation in this cell lineage [Bassel-Duby et al., 1992; Feo et al., 1995].

Myocyte-specific enhancer factor-2 (MEF-2) proteins constitute a small subgroup of the extensive MADS box family of transcription factors and were discovered in a screen for proteins related to serum response factor (SRF) [Pollock and Treisman, 1991]. The four mammalian MEF-2 genes, A–D [Yu et al., 1992; Breitbart et al., 1993; Martin et al., 1993; McDermott et al., 1993], are expressed primarily in skeletal and cardiac muscles, although certain isoforms also are expressed within the central nervous system [Leifer et al., 1993]. The MADS region is an evolutionarily conserved DNA binding motif that is shared with (M)CM1, (A)ganamous, (D)eficiens, and (S)erum response factor. MEF-2 proteins share both the MADS domain and a highly conserved signature motif (MEF-2 box) that defines this subfamily. Null mutations of D-MEF-2, the only MEF-2 gene of *Drosophilia melanogaster*, allow the specification

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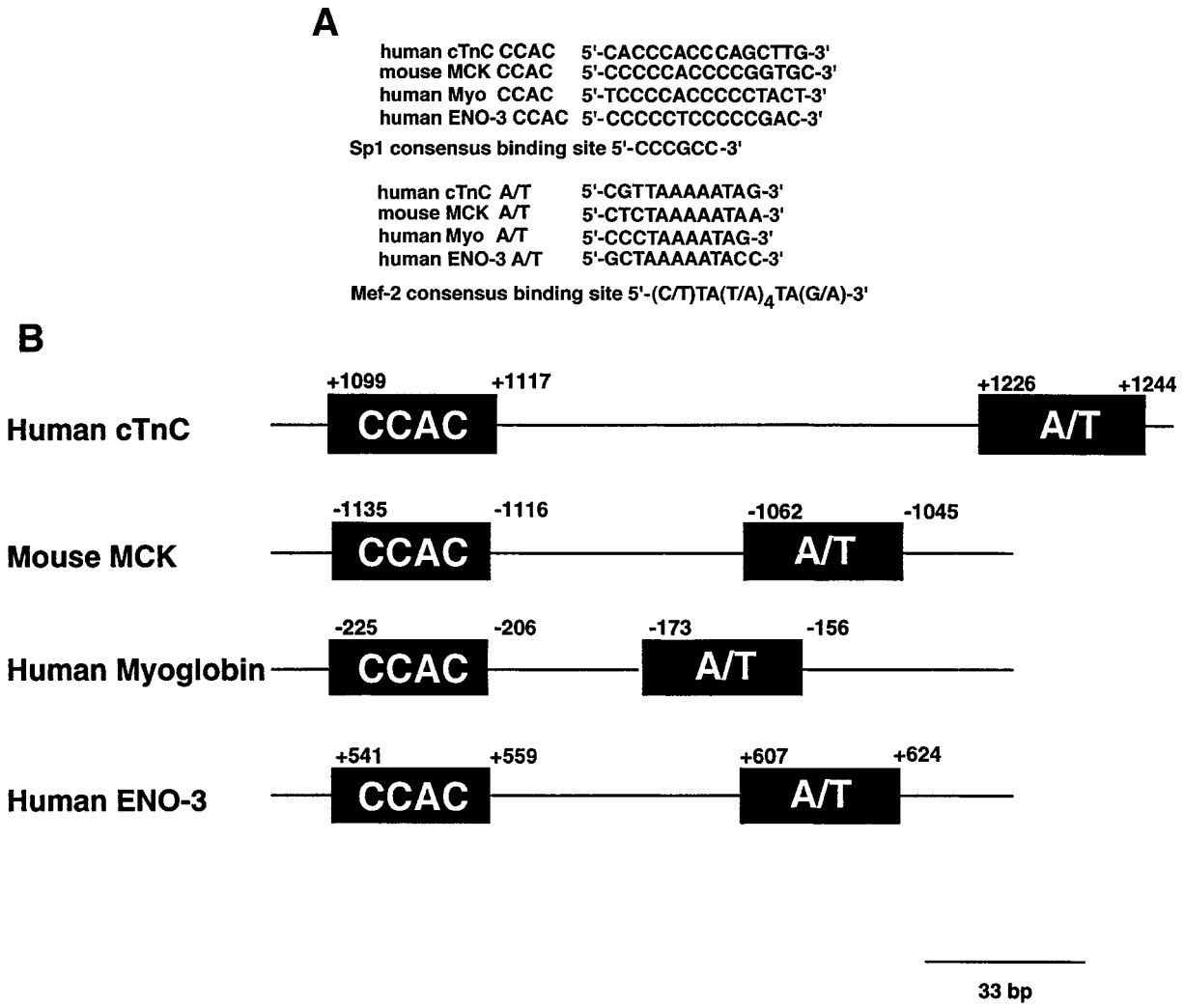

Fig. 1. Alignment of A/T rich and CCAC nucleotide sequences from muscle-specific transcriptional control regions. Sequences (**A**) and physical arrangement (**B**) of CCAC and A/T elements within promoter/enhancer segments from the human slow/cardiac troponin C (cTnC) [Parmacek et al., 1994], mouse muscle creatine kinase (MCK) [Gosset et al., 1989], human myoglobin (Myo) [Bassel-Duby et al., 1992], and human β-enolase (ENO-3) [Giallongo et al., 1993] genes are illustrated and compared with consensus MEF-2 and Sp1 binding sequences.

of myoblasts but prevent differentiation of all types of muscle [Lilly et al., 1995]. In mice, a homozygous null mutation in the MEF-2C gene results in severe abnormalities of cardiac development and embryonic lethality [Lin et al., 1997]. In the skeletal muscle lineage, MEF-2 family members interact physically and functionally with myogenic bHLH proteins of the MyoD family to activate myogenic transcription [Kaushal et al., 1994; Molkentin et al., 1995]. These findings indicate that MEF-2 proteins collaborate with other factors to execute critical decisions within the myogenic program.

Many of the genes that are activated during differentiation of striated muscle contain binding sites for MEF-2, which adhere to a consensus sequence (C/T)TA(A/T)$_4$TA(G/A) [Gosset et al., 1989; Yu et al., 1992]. In some cases, sites binding MEF-2 overlap sequence elements binding other proteins [Grayson et al., 1995]. A common feature of muscle-specific enhancer regions is that A/T-rich MEF-2 binding elements are flanked by a G/C-rich motif termed the CCAC box (Fig. 1) [Feo et al., 1995]. A variety of factors binding to CCAC sequences have been identified and characterized in different degrees of detail. These include the ubiquitously expressed nuclear protein Sp1, the consensus binding motif of which (GGGCGG) is similar to the CCAC box. Sp1 was first identified on the

basis of binding to the SV40 early promoter [Dynan and Tjian, 1983] and contains a zinc-finger DNA binding domain and several dispersed regions comprising transcription activation domains [Kadonaga et al., 1987; Courey and Tjian, 1988]. Four closely related isoforms in mammals are termed Sp1–4 [Hagen et al., 1992; Kingsley and Winoto, 1992]. Recent studies have shown that Sp1 plays a role in cell-cycle-dependent transcription by interacting with E2F1 [Karlseder et al., 1996; Lin et al., 1996], and previous investigations have also demonstrated that Sp1 interacts with MyoD [Sartorelli et al., 1990], p53 [Gualberto and Baldwin, 1995], YY1 [Lee et al., 1993], and SREBP [Sanchez et al., 1995; Yieh et al., 1995]. A homozygous null mutation in the Sp1 gene produces delayed development and early embryonic lethality [Marin et al., 1997]. These findings suggest that Sp1 participates in a diverse spectrum of cellular responses to developmental cues or environmental stimuli.

Our current data demonstrate that MEF-2 and Sp1 collaborate to drive expression of a promoter that includes CCAC and A/T elements. Moreover, MEF-2 and Sp1 interact physically to form protein complexes in vitro and in vivo. The interaction of MEF-2 with Sp1 is a distinctive feature of MEF-2 that is not shared with SRF, another MADS family protein.

MATERIALS AND METHODS

Cell Culture

Monolayers of sol8 myogenic cells were grown as described previously [Grayson et al., 1995]. *Drosophilia melanogaster* SL2 cells were grown in SF-9000 growth medium (Gibco/BRL Research, Gaithersburg, MD) containing penicillin/streptomycin (100 U/ml Gibco/BRL Research) at 25°C.

Plasmid Constructs

Several vectors and constructs used in the present study have been described previously. These include bacterial expression vectors glutathione-S-transferase (GST)–Sp1(1–621) [Karlseder et al., 1996] and pGEX-CS [Parks et al., 1994], *Drosophilia* expression vectors pPAC and pPAC-Sp1 [Courey and Tjian, 1988] and pHT4 [Schneuwly et al., 1987], the in vitro translation plasmid T7 SRF [Norman et al., 1988; Nurrish and Treisman, 1995], and luciferase reporter constructs MCK-TATA-Luc and TATA-Luc (previously termed MCK Guppa.9 and pGuppa.8, respectively) [Grayson et al., 1995]. Other plasmids included pAS1 CYH2, pAS1 CYH2 SNF1, pACT, and pACT-SNF4 (from S. Elledge), and pAS1 CYH2-MEF2C(1–117) (from J. Liu). Plasmid pACT-Sp1 was constructed by inserting a polymerase chain reaction (PCR) fragment encompassing human Sp1 (amino acids 1–621) into the *Xho*I site of pACT. Plasmid pAS1 CYH2-SRF(MADS) was constructed by inserting a PCR fragment that contains the SRF MADS domain into the *Sal*I site of pAS1 CYH2. Plasmid pHT4-MEF-2A was constructed by ligating a 1.95-kb EcoRI MEF-2A cDNA fragment from pMT2-MEF-2A [Yu et al., 1992] into an *Eco*RI site in pHT4.

Transfections and Luciferase Assay

Approximately 10^5 SL2 cells were plated into 35-mm dishes 12 h before transfection. Briefly, 2 μg of DNA were mixed with 8 μl of Cellfectin reagent (Gibco/BRL Research) and placed onto cells in 1 ml of antibiotic free growth medium. After 5 h, the transfection mixture was removed and replaced with 2 ml of growth medium. Expression of MEF-2A was induced 48 h after the start of transfection by placing the cells at 37°C for 30 min. The cells were allowed to recover at 25°C for 3 h before harvesting. Growth medium was aspirated and the cells were washed once with Dulbecco's phosphate buffered saline and overlaid with 400 μl of 1× Lysis buffer (Promega Corporation, Madison, WI). After 15 min, the cells were scraped off the dish with a rubber policeman and quickly frozen in liquid N_2. The samples were thawed at 37°C and centrifuged to clear the lysate of debris. Twenty microliters of sample were assayed in a Berthoid LB9500C Luminometer with 100 μl of Luciferase Assay Buffer (Promega Corporation). All results were derived from four or more independent experiments. Efficiency of transfection was determined by cotransfection of pHT4-Lac-Z, and β-galactosidase activity was monitored as described previously [Grayson et al., 1995].

DNA Probes and Competitors

DNA probes were prepared by using both sense and complementary antisense DNA strands as described previously [Gosset et al., 1989]. Oligonucleotides used in the present study (sense strand) include human myoglo-

bin CCAC (5′-GATCACGCACAACCACCCCAC-CCCC TGTG-3′), HIV-1-LTR-Sp1 (5′-AGG-GAGGCGTGGCCTGGGCGGGACTGGGG-3′), mouse MCK A/T (5′-GATCCTCTAAAAATA-ACCT-3′), and mouse mutant MCK A/T (5′-GATCCTCTAAGGCTAACCT-3′).

Electrophoretic Mobility Shift Analysis (EMSA)

Nuclear proteins were prepared and EMSA were performed as described previously [Grayson et al., 1995]. Antibodies to either human Sp1 or MEF-2A (Santa Cruz Biotechnology, San Diego, CA) were incubated with preformed DNA protein complexes for 20 min at 25°C, as described previously [Grayson et al., 1995].

Purification of GST Fusion Protein

Escherichia coli DH5α cells expressing GST-Sp1 were grown to an optical density at 595 nm of 0.5. Protein expression was induced by addition of isopropyl-B-D-thiogalactopyranoside to a final concentration of 0.1 mM. After 4 h, the cells were pelleted and washed in 10 mM Tris-HCl, pH 8.0, 25% sucrose, 10 mM EDTA, pH 8.0. The cells were then centrifuged at 1,000*g* for 20 min and resuspended in 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethlsulfonyl fluoride, 100 µg/ml leupeptin, and 100 µg/ml pepstatin A. A Branson Sonifier 450 sonicator was used to lyse the cells. The lysate was cleared by centrifugation at 37,000*g* for 1 h. The samples were incubated overnight at 4°C with glutathione-agarose beads (Pharmacia Corp., Piscataway, NJ) in 20 mM HEPES, pH 7.4, 100 mM KCl, 20% glycerol, 1 mM EDTA, pH 8.0, 1% Triton X-100, and 100 µg/ml leupeptin. The beads were then washed five times with the latter buffer, and the fusion protein was stored as a 1:1 slurry (beads:buffer) at 4°C.

In Vitro Translation

Radiolabeled proteins were in vitro translated with the Promega TNT Rabbit Reticulocyte T7 In Vitro Transcription and Translation Coupled system in the presence of ^{35}S-methionine (Amersham, Arlington Heights, IL) by following the manufacturers' instructions.

In Vitro Protein–Protein Interaction Assay

One microgram of GST fusion protein was preincubated with binding buffer (20 mM HEPES, pH 7.9, 40 mM KCl, 1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethlsulfonyl fluoride) for 1 h on a rotating wheel at 4°C. The fusion protein was then incubated with 1/50 of each in vitro translation reaction for 1 h at 4°C on a rotating wheel. The beads were then washed three times with binding buffer, mixed with 2× sodium dodecylsulfate (SDS) loading dye, and loaded onto an 8% SDS–polyacrylamide gel electrophoresis gel. The radiolabeled proteins were viewed using autoradiography.

Yeast Two-Hybrid Assay

Yeast strain SFY526 [Mat a, ade2, lys1, leu2, his3, trp1, Dgal4, Dgal80, (Gal1-LacZ::URA 3)] was used to examine the in vivo interaction between Sp1 and MEF-2C in a two-hybrid assay. Yeast were grown in rich (YEP) or synthetic medium lacking appropriate amino acids to maintain selection for plasmids. Cells were grown until $OD_{600} = 0.5$. Approximately 200 µg of yeast total protein were incubated with 1 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 40 mM β-mercaptoethanol, 1 mM $MgCl_2$, 10 mM KCl, 4 mg/ml O-nitrophenyl-β-D-galactopyranoside) at 37°C. At the appropriate time, 200-µl aliquots were removed, and the reaction was stopped by the addition of 800 µl of 1 M Na_2CO_3. The absorbance at 420 nm was measured. Beta-galactosidase activity was calculated in Miller units $(\Delta Abs\ 420 \times 380)/(\Delta time_{min} \times protein_{mg})$.

RESULTS

DNA:Protein Complexes Formed on the A/T-Rich Motif Contain Both Sp1 and MEF-2A

Previously, we had shown that MEF-2 proteins bind to the A/T region of the myoglobin promoter [Grayson et al., 1995], although MEF-2 bound less avidly to this site than to the A/T-rich element from the MCK enhancer. For the current studies, the higher affinity MEF-2 binding site from the MCK enhancer was employed as the probe for EMSA. Using nuclear extracts from sol8 myotubes, a single major protein:DNA complex was formed (Fig. 2). The mobility of this complex was not altered by the addition of preimmune sera but was supershifted by polyclonal IgG raised to human MEF-2A. The MEF-2:DNA complex also was supershifted and depleted by polyclonal IgG raised against human Sp1. Addition of excess unlabeled MCK A/T oligonucleotide abolished binding, whereas addition of unlabeled myoglobin CCAC oligonucleotide or a mutated MCK A/T-

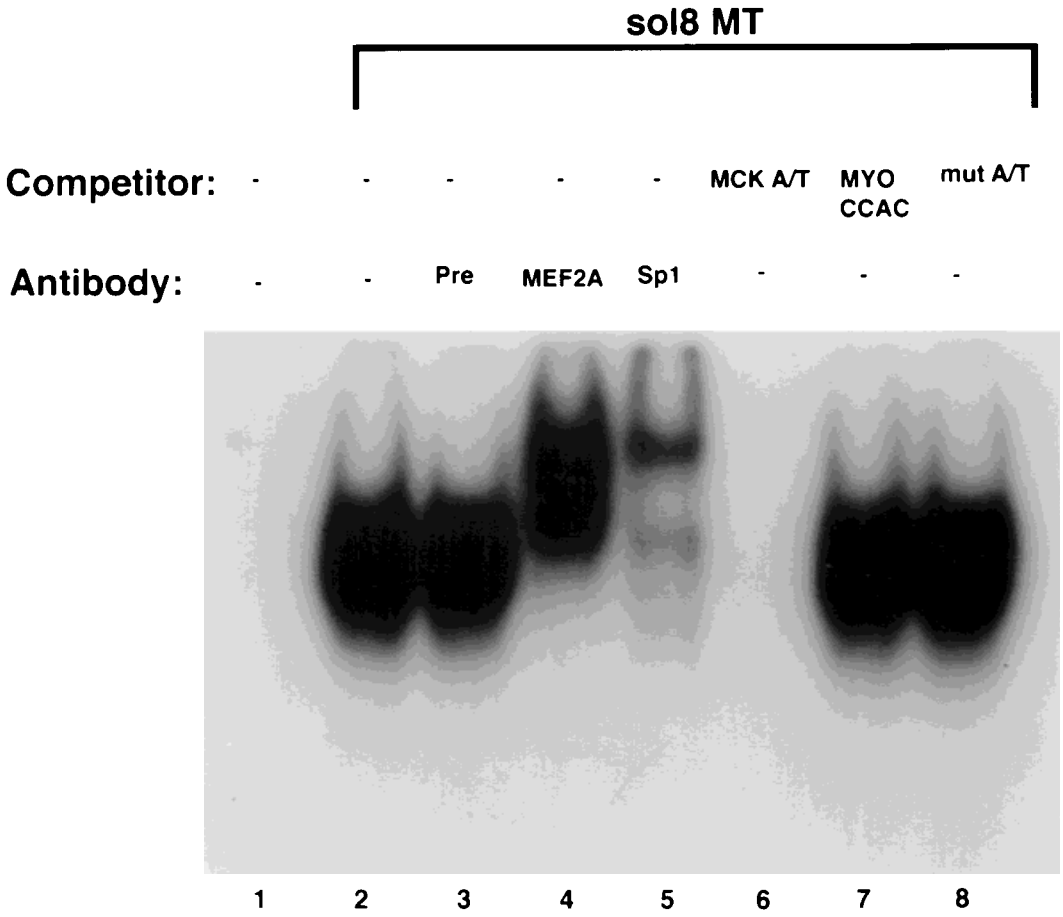

Fig. 2. Sp1 is present in conjunction with MEF-2 in a protein complex binding to an A/T sequence. Protein:DNA complexes were formed by addition of sol8 myotube nuclear extract to a labeled MCK A/T probe and resolved by EMSA, resulting in a slowly migrating complex, the mobility of which is altered by either an MEF-2A or Sp1 antibody. Competing unlabeled oligonucleotides (100 ng) represented the MCK A/T element, the myoglobin (MYO) CCAC motif, or a mutated variant of the MCK A/T element (mut A/T).

rich element (without MEF-2 binding activity) [Yu et al., 1992] failed to disrupt this complex.

DNA:protein complexes formed by nuclear extracts from sol8 myotubes in the presence of the myoglobin CCAC sequence were supershifted by polyclonal IgG antibodies to human Sp1, but immunoreactive MEF-2A could not be detected within this complex (data not shown). This negative result, however, does not contradict the conclusion that the two proteins can associate within a single complex bound to DNA. MEF-2 epitopes recognized by currently available antibodies may be masked within the complex formed on the CCAC motif, or the relative abundance of the two proteins may differ. If Sp1 is more abundant than MEF-2, then only a small proportion of Sp1 present within the sol8 nuclear extract may be complexed with MEF-2, so that the level of MEF-2 present in Sp1:DNA complexes falls below the detection limit of this assay.

Sp1 Binds MEF-2 In Vitro

A fusion protein linking GST to Sp1 (amino acids 1–621) was expressed in bacteria, and the purified recombinant protein was incubated with either MEF-2 or SRF proteins translated in vitro in the presence of ^{35}S-methionine. Labeled proteins bound to GST-Sp1 were separated from unbound proteins on the basis of binding to glutathione–Sepharose beads. The results demonstrated that MEF-2A interacts with GST-Sp1 (Fig. 3A) more avidly than with the related MADS box protein SRF (Fig. 3B). Incubation of GST-Sp1 with in vitro translated MEF-2C produced results similar to those with MEF-2A (not shown). Quantitative results from

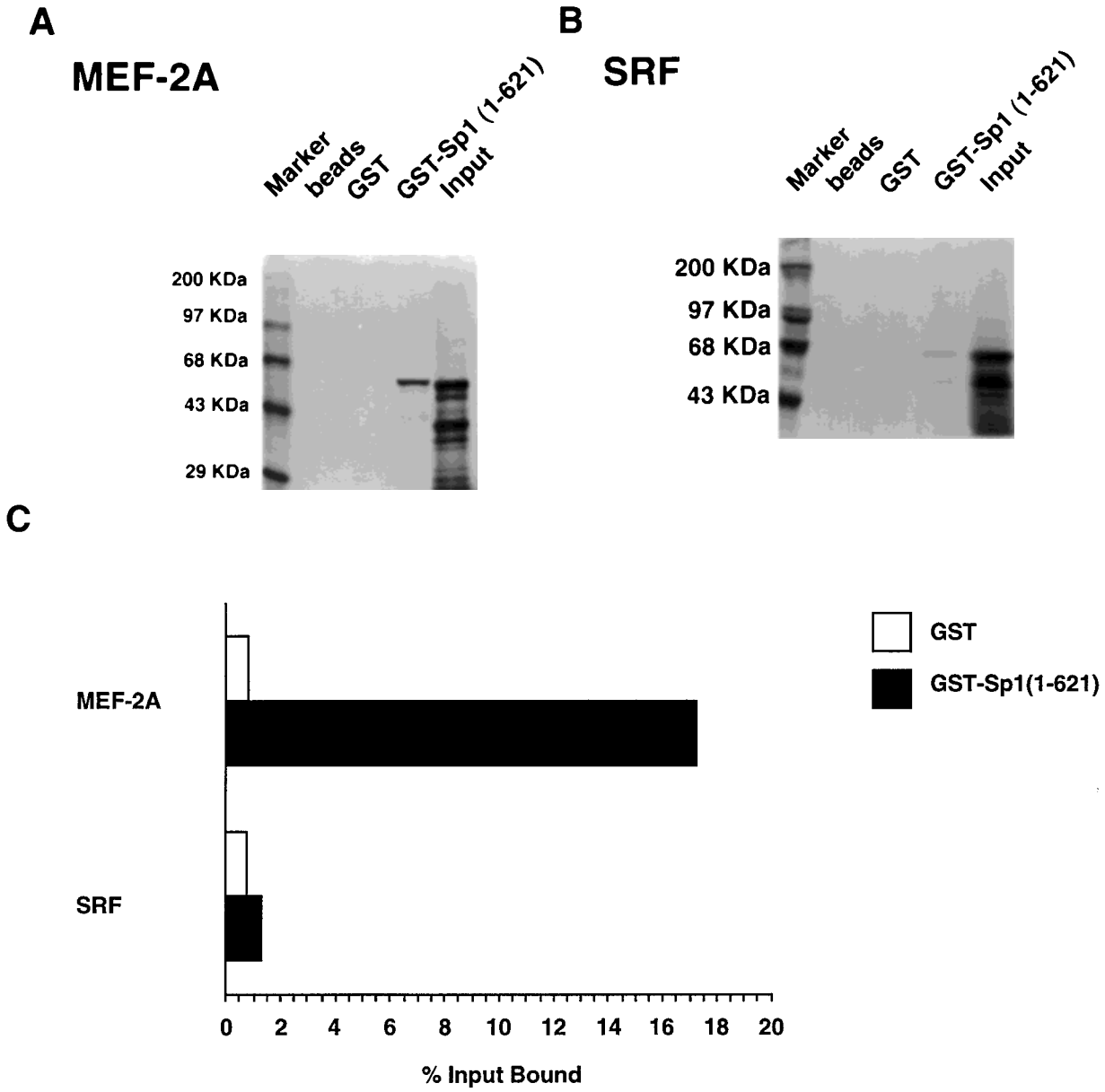

Fig. 3. Sp1 binds MEF-2 in vitro. GST-Sp1 was bound to glutathione–agarose beads and incubated with radiolabeled, in vitro translated MEF-2A (**A**) or SRF (**B**). After incubation, the beads were washed extensively, and bound proteins were resolved by electrophoresis and visualized by autoradiographic film exposure. A quantitative summary of the protein interaction results is illustrated (**C**).

a representative experiment are shown in Figure 3C.

Sp1 Binds MEF-2 In Vivo

MEF-2C and Sp1 were fused to the GAL4 DNA binding domain (GBD; amino acids 1–147) or activation domain (GAD; amino acids 768–881), respectively, and examined in a yeast two-hybrid assay. The interaction between the yeast transcriptional regulators SNF1 and SNF4 (fused to GBD and GAD, respectively) was assessed as a positive control. Each GBD and GAD derivative was transformed into SFY526 cells. This strain of *S. cerevisiae* carries an integrated LacZ gene, controlled by the GAL1 promoter. Protein–protein contacts between heterologous fusion proteins bearing GBD and GAD domains results in β-galactosidase expression, which can be quantitated by enzymatic assay.

SF-Y526 cells were transformed with plasmids expressing each fusion protein, and transformants were identified by growth on selective

media. Five colonies were picked from each set of transformants and β-galactosidase activity was quantified in whole cell extracts (Fig. 4). All the negative controls demonstrated only minimal β-galactosidase activity. These included GBD or GAD domains in the absence of fusion to heterologous protein segments, GDB-MEF2C(1–117) in combination with GAD, GDB-SRF(144–235) in combination with GAD or with GAD-Sp1(1–621), and GAD-Sp1(1–621) alone.

In contrast, combined transformation of GAD-Sp1(1–621) and GBD-MEF-2C(1–117) resulted in a 15-fold increase (mean of five experiments) in β-galactosidase activity in comparison with that observed following expression of GBD-MEF-2C(1–117) plus GAD. The interaction between Sp1 and MEF-2C sequences in this assay was approximately 25% as potent as that of SNF1 and SNF4 tested under similar conditions. These data suggest that MEF-2C and Sp1 can interact in vivo and that this interaction is not observed with the related MADS domain protein SRF.

Sp1 and MEF-2A Act Synergistically as Transcriptional Activators

A/T and CCAC motifs binding MEF-2 and Sp1, respectively, are found in close proximity in many muscle specific enhancers (Fig. 1), and we previously demonstrated a synergistic interaction between these elements in the context of the human myoglobin enhancer [Grayson et al., 1995]. In the present study, we sought to determine whether Sp1 and MEF-2A can function synergistically to activate transcription. We conducted transient transfection experiments in insect cells (SL2 from *D. melanogaster*) to avoid confounding effects from endogenous Sp1 or MEF-2 present in mammalian cell lines. The reporter plasmids included a minimal promoter (TATA element from the human hsp70 gene) controlling the firefly luciferase gene in the presence or absence of the mouse MCK enhancer containing an A/T-rich site (−1,077 to −1,062 bp) adjacent to a CCAC sequence (−1,135 to −1,117 bp) (Fig. 1).

The results of transient transfection assays using these reporter constructions and insect expression vectors driving an Sp1 or MEF-2A transgene, alone or in combination, are shown in Figure 5. In the absence of the MCK enhancer (TATA element only), Sp1 drove a low level of transcription but MEF-2A did not, and no synergistic interaction was observed. When the MCK enhancer was included in the reporter construct, transcription of the luciferase reporter, in the absence of exogenously expressed mammalian proteins, was not increased above the activity of the minimal promoter in the insect cell background. Forced expression of Sp1, however, resulted in an 10-fold increase in transcription from the MCK enhancer-regu-

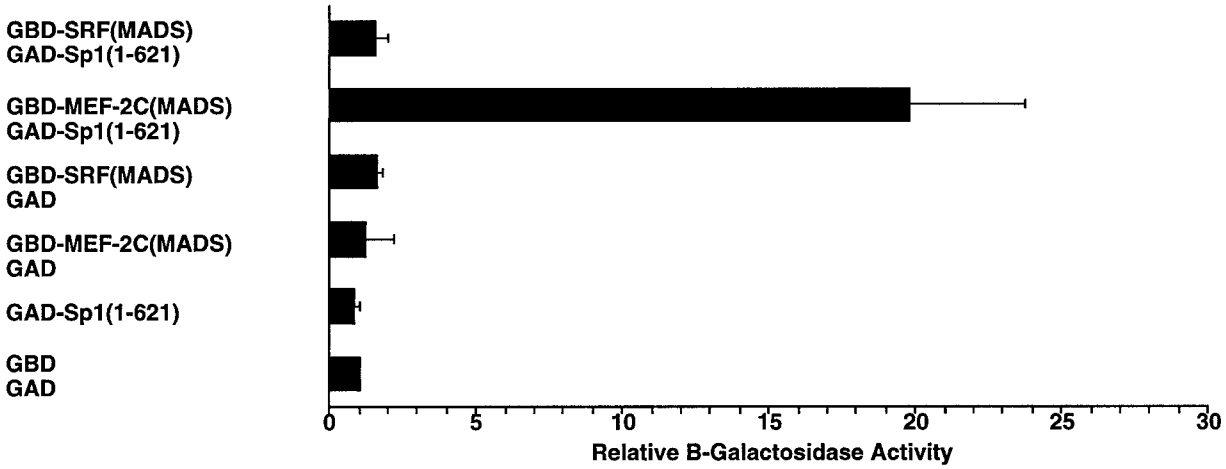

Fig. 4. Sp1 binds MEF-2 in vivo. Fusion proteins representing MEF-2C or SRF fused to the DNA binding domain of Gal4p (amino acids 1–147; GBP) or Sp1 (amino acids 1–621) fused to the transactivation domain of Gal4p (amino acids 768–881; GAD) were expressed from plasmid vectors in a strain of *S. cerevisiae* bearing an integrated lac-Z reporter gene controlled by the GAL1 upstream activation sequence. Beta-galactosidase activity was measured in whole cell extracts and is presented (mean ± S.D. of 5 independent transformants) as multiples of background activity observed in cells expressing GBD + GAD. Only cells simultaneously expressing both GBP-MEF-2C and GAD-Sp1 activated the reporter gene to levels above the background measured in the negative controls.

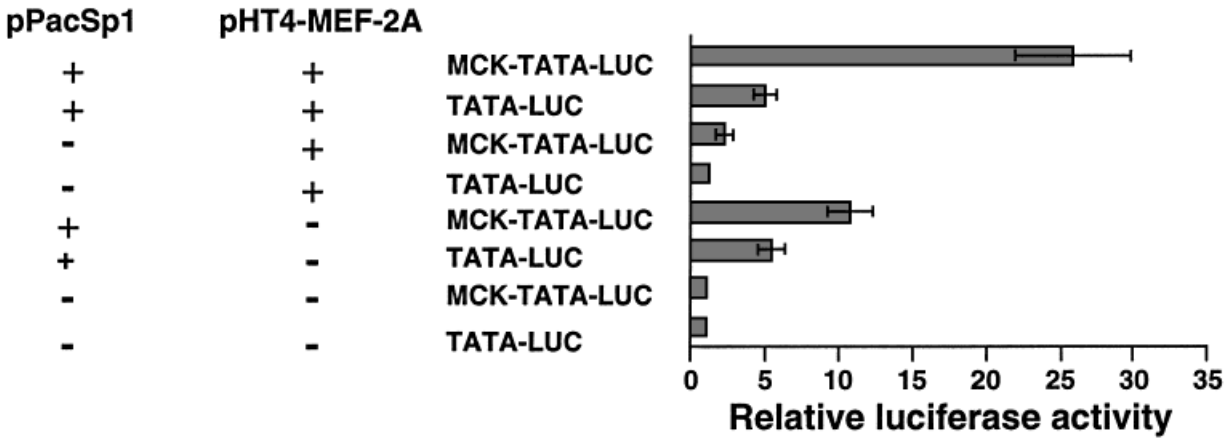

Fig. 5. Sp1 and MEF-2A synergistically activate a promoter that contains CCAC and MEF-2 binding sites. Reporter plasmids TATA-LUC (a minimal hsp 70 promoter linked to the luciferase gene) or MCK-TATA-LUC (MCK enhancer −1,135 to −1,062 bp linked to TATA-LUC; 100 ng) were transfected with 100 ng of pPacSp1 and/or pHT4-MEF-2A. Results were calculated relative to the activity of the minimal promoter alone and are displayed as mean (±S.E.M.) values from four or more independent observations.

lated construct. MEF-2A stimulated transcription only weakly (1.7-fold) in the absence of Sp1, but the combined expression of Sp1 and MEF-2A activated transcription by 27-fold. This synergistic activation was similar to that observed to be mediated via the CCAC and A/T elements in the presence of endogenous transcription factors in mammalian skeletal myotubes [Grayson et al., 1995].

DISCUSSION

Previous studies of transcriptional control elements of the human myoglobin promoter identified two essential upstream activation elements: a GC-rich CCAC box and an A/T box [Bassel-Duby et al., 1992]. These sequences were required for transcription in both skeletal and cardiac myocytes [Bassel-Duby et al., 1993]. We also observed that these two elements, removed from the authentic context of the myoglobin enhancer and linked to a minimal promoter, were sufficient to direct muscle-specific transcription [Grayson et al., 1995]. We identified MEF-2 as one of two proteins binding specifically to the myoglobin A/T sequence [Grayson et al., 1995], and binding of MEF-2 proteins to related A/T-rich elements in other muscle-specific transcriptional control elements has been demonstrated by several other groups [Horlick and Benfield, 1989; Yu et al., 1992; Leibham et al., 1994]. The identity of CCAC-box binding factors has been more controversial. Studies from our group and others have suggested several candidates, including CBF-40 [Bassel-Duby et al., 1992], HT-β [Wang et al., 1993], MNF [Bassel-Duby et al., 1994], and Sp1 [Kadonaga et al., 1987].

Is Sp1 a physiological transactivator that functions in muscle differentiation by binding CCAC elements? Sp1 binds to a CCAC motif with an affinity comparable to its binding to a consensus CCGCCC motif from the HIV-1-LTR, and we demonstrate that Sp1 is capable of transactivation mediated through this element. These data in conjunction with our observation that Sp1 interacts both physically and functionally with the myogenic regulator MEF-2 strongly support the viewpoint that Sp1 is important for muscle-specific gene regulation. The conservation of a nonconsensus Sp1 motif within many muscle-specific enhancers (Fig. 1) suggests, however, that this motif may discriminate among variant isoforms of Sp1 or may bind accessory proteins in a manner different from consensus Sp1 binding sites found in other genes. We speculate that the CBF-40 protein previously identified by our group could represent an alternatively spliced variant of Sp1 because truncated Sp1 isoforms in this size range have been characterized by others [Persengiev et al., 1995].

The major conclusion of our study is that Sp1 and MEF-2 proteins interact both physically and functionally. This interaction plausibly accounts for the transcriptional synergy previously observed to be dependent on adjacent CCAC and A/T-rich elements within muscle-specific enhancer regions [Feo et al., 1995; Gray-

son et al., 1995]. Four lines of evidence are presented in this report to support this conclusion: (1) Sp1 and MEF-2A are present together in DNA:protein complexes detected by EMSAs when using myotube nuclear extracts, (2) recombinant Sp1 and MEF-2 proteins form physical complexes in solution, (3) Sp1 and MEF-2A interact in vivo as assessed in a yeast two-hybrid assay, and (4) the two proteins function synergistically as transcriptional activators when expressed in a null background (insect cells).

The physical basis for transcriptional synergism, in a fundamental sense, remains controversial, although several general mechanisms have experimental support. First, heterologous transcription factors may bind DNA in a cooperative manner [Sanchez et al., 1995]. Preliminary studies with recombinant Sp1 and MEF-2C proteins in our laboratory did not show evidence for cooperative DNA binding of these two factors in vitro (data not shown), but these negative results are not conclusive. A second general mechanism of transcriptional synergy is based on the observation that heterologous transcriptional activators make different contacts with TAFs and other components of the basal transcriptional apparatus. Sp1 has been shown to interact with TAF110 [Gill et al., 1994], but the interactions between MEF-2 proteins and basal transcription factors have not yet been defined. In preliminary experiments using size exclusion chromatography (not shown), we observed that most of the MEF-2A and Sp1 in myotube nuclear extracts is present within complexes much larger than the size of protein monomers or dimers, but our current data are insufficient to support a detailed mechanistic model of how Sp1 and MEF-2 interact. We suspect, however, that these proteins function within large macromolecular complexes that also include p300 [Sartorelli et al., 1997] and bHLH proteins of the MyoD family [Sartorelli et al., 1990; Molkentin et al., 1995].

Protein–protein interactions observed using in vitro systems must be viewed with skepticism with respect to the relevance of such interactions in vivo. Several features of our data, however, suggest that physical interactions between Sp1 and MEF-2 have physiological relevance. The results from the two-hybrid assay indicate that these proteins can form a physical complex within intact cells and under the artificial conditions of the EMSA or glutathione binding assays. The conservation of paired Sp1 and MEF-2 binding motifs within enhancer elements of several muscle-specific genes (Fig. 1) in conjunction with the observation that the interaction surface resides within a segment of MEF-2 that is distinct from ubiquitously expressed MADS domain proteins like SRF suggest a physiological role for this interaction within the myogenic program. Perhaps most importantly, the functional collaboration between MEF-2 and Sp1 demonstrated by cotransfection assays in insect cells supports the contention that physical interactions between these proteins may be pertinent to transcriptional regulation during myogenesis.

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