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Mos Activates Myogenic Differentiation by Promoting Heterodimerization of MyoD and E12 Proteins

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The activities of myogenic basic helix-loop-helix (bHLH) factors are regulated by a number of different positive and negative signals. Extensive information has been published about the molecular mechanisms that interfere with the process of myogenic differentiation, but little is known about the positive signals. We previously showed that overexpression of rat Mos in C2C12 myoblasts increased the expression of myogenic markers whereas repression of Mos products by antisense RNAs inhibited myogenic differentiation. In the present work, our results show that the rat *mos* proto-oncogene activates transcriptional activity of MyoD protein. In transient transfection assays, Mos promotes transcriptional transactivation by MyoD of the muscle creatine kinase enhancer and/or a reporter gene linked to MyoD-DNA binding sites. Physical interaction between Mos and MyoD, but not with E12, is demonstrated in vivo by using the two-hybrid approach with C3H10T1/2 cells and in vitro by using the glutathione *S*-transferase (GST) pull-down assays. Unphosphorylated MyoD from myogenic cell lysates and/or bacterially expressed MyoD physically interacts with Mos. This interaction occurs via the helix 2 region of MyoD and a highly conserved region in Mos proteins with 40% similarity to the helix 2 domain of the E-protein class of bHLH factors. Phosphorylation of MyoD by activated GST-Mos protein inhibits the DNA-binding activity of MyoD homodimers and promotes MyoD-E12 heterodimer formation. These data support a novel function for Mos as a mediator (coregulator) of muscle-specific gene(s) expression.

During myogenesis, transcription of muscle-specific genes is dependent on a family of muscle-specific transcription factors that include MyoD (11), myogenin (13, 63), Myf5 (6), and rat MRF4 (50), also referred to as herculin in the mouse (36) and Myf6 in the human (61). These proteins share a basic-helix-loop-helix (bHLH) domain, which is also common to ubiquitous transcription factors such as the products of the E2A gene, E12 and E47 (40, 53), and the HEB gene product (20), with which myogenic factors mediate dimerization and binding to a DNA consensus sequence known as an E box (CANNTG) (12). An intriguing property of myogenic bHLH proteins is their sensitivity to growth factors and transforming gene products such as v-Src (14), Ras (25, 44), v-Fms (4), v-Fos (26), Jun, (33), Myc (37), and E1A (58) that can silence their activities and inhibit myogenic differentiation (reviewed in reference 46). Some of these oncogene products act in pathways regulated by protein kinase C (PKC), which by itself can inhibit myogenesis. Phosphorylation of myogenin by PKC in vitro prevents DNA binding, and constitutively expressed PKC inhibits transactivation by myogenin (35). Similarly, cyclic AMP-dependent protein kinase (PKA) represses muscle-specific transcription through an indirect mechanism (34, 61). The inactivation of the myogenic regulatory proteins is associated with a loss of their ability to heterodimerize and to bind DNA (7, 8, 35, 39) and/or to activate transcription (45). Thus, the activity of myogenic bHLH factors is extensively regulated by a number of different positive and negative signals. Although much information has been published about the molecular mechanisms that interfere with the process of myogenic differentiation, little is known about the positive signals. We have recently shown that the serine/threonine kinase product of the *mos* proto-oncogene is expressed in adult skeletal muscle tissue (28, 29), whereas its expression was originally described as being restricted to male and female germ cells (16, 49). Mos expression in mammalian gonad tissues appears to play an important role in the maturation of male and female germinal cells (42, 47, 52). However, various other somatic cell lineages and tissues have been found to express significant, if rather low, amounts of Mos RNAs and proteins (19, 32). Mos protein levels are higher in adult skeletal muscle cells than in other somatic cells (29), suggesting a particular(s) function for *mos* in this tissue. Indeed, during postnatal maturation of skeletal muscle, changes in the pattern of $p34^{cdc2}$ expression are correlated with accumulation of Mos protein. A part of the Mos protein is complexed with $p34^{cdc2}$, yielding a 170-kDa complex (30) distinct from that observed in Mos-transformed cells or in oocytes (1, 2, 65). Altogether, our data argued in favor of a muscle-specific function of Mos. We reported previously that in C2C12 myoblasts, overexpression of Mos activated muscle differentiation whereas inhibition of endogenous Mos expression by antisense RNA resulted in reversible blockage of myogenesis, suggesting the likely hypothesis that Mos interacts with the myogenic bHLH proteins (27). Here we undertook a study to determine the molecular mechanisms by which the proto-oncogene product Mos may favor the process of myogenic differentiation. We report that constitutive expression of Mos in C2C12 myoblasts activates MyoD expression as well as myogenesis. In transient transfection assays, we show that Mos enhances the ability of MyoD protein to activate target muscle enhancers. Our results indicate that unphosphorylated MyoD but not its E12 partner physically interacts with Mos. Muta-

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tional analysis of the proteins demonstrates that there is a highly conserved region of Mos proteins that has sequence homologies with the ubiquitous E-protein class of bHLH factors that associate with the helix 2 domain of MyoD. Phosphorylation, at the serine and to a lesser extent at the threonine residues, of recombinant MyoD by the activated glutathione *S*-transferase (GST)–Mos protein inhibits the DNA-binding activity of MyoD homodimers but promotes the formation and DNA-binding activity of MyoD-E12 heterodimers. These results suggest that interaction with and phosphorylation of MyoD by Mos participate in the positive regulation of muscle differentiation.

MATERIALS AND METHODS

Plasmids. pEMSV-E12, pEMSV-MyoD, and pEMSV-MyoD mutants (56) were kind gifts from H. Weintraub. To create MyoD mutant proteins, *Alu*I inserts from MyoD mutants (DM:63-99, DM:102-135, and DM:218-269) were agarose purified and then cloned in frame into the *Pvu*II site of the pRSET C expression vectors (Invitrogen Corporation). Pet-MyoD was constructed by using a PCR-amplified insert as previously described (57). The rat Mos *Xba*I fragment (3.2 kb) was first subcloned in pSp64polyA to yield pSp64A-Mos. A pair of synthetic oligonucleotides (5′GATCCATGCCTTCGCCTCTCATCCTGTGTC GCTACCTCCCTCG3′ and 5′CGAGGGAGGTAGCGACACAGGATGAGA GGCGAAGGCATG3′) and a 2.4-kb *Nru*I-*Bam*HI fragment of the pSp64A-Mos construct, which encoded the first ATG to the 12th amino acid and the 13th to the last amino acids, respectively, of rat Mos protein (28) were inserted in frame into the *Bam*HI site of plasmid pRSET A (pRSET-Mos). GST-Mos was obtained by inserting in frame, into the *Bam*HI site of the pGEX-2T expression plasmid, the *Bam*HI fragment of pRSET-Mos. The full-length wild-type Mos (MosWT) construct pGEX2T-Mos^{WT} was used as the template for mutagenesis. Site-directed mutagenesis resulting in substitution of Lys-88 with Arg to create kinase-inactive Mos (Mos^{KM}) was performed by PCR with an oligonucleotide primer (G-GCC-ATC-AGG-CAA-GTG-AAC-A) to introduce the desired base change. Mutation was confirmed by sequencing. Mos^{WT} used for in vitro translation experiments was constructed as follows. DM:1-12 is a 2.4-kb *Nru*I-*Eco*RI insert from pSp64A-Mos cloned in frame into pRSET A. DM:1-30 is a 2.3-kb *Xho*I-*Eco*RI fragment from pSp64A-Mos cloned in frame into pRSET B. DM: 1-139 is a *Kpn*I-*Kpn*I fragment from pSp64A-Mos cloned in frame into the *Kpn*I site of pRSET B. pRSET-Mos was cut by *Hin*dIII to remove the 385-bp *Hin*dIII fragment, and the plasmid was self-annealed to give the TM:221 mutant plasmid. pRSET-Mos was cut by *Hpa*I and *Eco*RI to remove the 1.9-kb *Hpa*I-*Eco*RI fragment. The resulting plasmid was filled in with Klenow polymerase before self-annealing to give TM:181. TM:137 was constructed by subcloning a *Bam*HI-*Kpn*I blunt-ended fragment (by T4 polymerase) into the *Bam*HI-*Pvu*II site of the pRSET A. DM:1-139;TM:221 contained a 0.25-kb *Kpn*I-*Hin*dIII fragment resulting from a *Hin*dIII-*Hin*dIII deletion of mutant DM:1-139. Finally, DM:137-220 was constructed from pRSET-Mos. The plasmid was first cut by *Kpn*I to remove the 1,271-bp *Kpn*I-*Kpn*I insert and was blunt ended by T4 polymerase. The 385-bp *Hin*dIII-*Hin*dIII fragment of the *mos* insert was first filled in by the Klenow polymerase and ligated in frame to the blunt-end *Kpn*I site of pRSET-Mos. These constructs were controlled by partial sequencing, and results obtained with these constructs were confirmed by using two independent clones.

The resulting restriction fragments of pSp64A-Mos, *Sal*I-*Eco*RI (MosΔ1) and *Nru*I-*Eco*RI (MosΔ4), were subcloned into pcDNA3 under the control of the cytomegalovirus (CMV) promoter. pM and pVP16AD vectors were purchased from Clontech Laboratories, Inc. pVP16-Mos was constructed by inserting an *Nru*I-*Bam*HI fragment, corresponding to amino acids 13 to 339 of Mos protein, into the *Sma*I-*Bam*HI sites of the pVP16AD vector. Deletion mutant pVP16-MosΔ137-220 was constructed by inserting *Bam*HI fragment from mutant DM: 137-220 into the *Bam*HI site of pHK3NVP16 vector (provided by D. Trouche). Plasmids pVP16-MyoD, pVP16-E12, pM-MyoD, pM-MyoDΔ128-172, and pM-E12 were constructed by inserting the corresponding complete coding sequence, obtained by the PCR as previously described (17), into the *Eco*RI sites of pVP16AD and pM vectors. These constructs were controlled by sequencing across the junctions of the fusion genes. The reporter plasmid MCK-CAT (generously provided by S. Hauschka) contains the promoter-enhancer region from the mouse muscle creatine kinase (p1256 MCK expression vector) (8). Plasmid 4R-tk-CAT (generously provided by A. Lassar) contains four tandem copies of the high-affinity right-hand E box from the MCK enhancer linked to the thymidine kinase gene basal promoter (60).

Cell cultures and DNA transfections. C3H10T1/2 cells and the mouse skeletal muscle cell line C2C12 were maintained in growth medium (GM; 20% fetal calf serum in Dulbecco's modified Eagle's medium. To initiate myogenic differentiation, GM was replaced with Dulbecco's modified Eagle's medium containing 2% fetal calf serum (DM). For transfection, about 5×10^6 cells were plated in 75-cm^2 flask. Twenty-four hours later, cultures were refed with fresh GM for 2 h before transfection. The cells were exposed to calcium phosphate precipitates for 4 h (31). Cells were washed and refed with growth medium. Forty-eight hours after transfection, cells were harvested and chloramphenicol acetyltransferase (CAT) activity was determined in aliquots of cell extracts containing equivalent quantities of proteins. One microgram of plasmid pCH110 (Pharmacia) was included in transfections as an internal control for transfection efficiency. CAT assays were performed as previously described (31). CAT activities were determined by separating substrate and products by thin-layer chromatography and counting the radioactivity in the different products with a phosphorimager (Fuji, Tokyo, Japan). All reactions were normalized to an equal amount of total protein, and the results were expressed after normalization of the β-galactosidase activity of each transfectant. Experiments were done in triplicate and repeated at least twice.

Mammalian two-hybrid assay. C3H10T1/2 cells were maintained and transfected as described previously (31). Cells were transfected with 2 μg of the Gal-dependent reporter plasmid pG5E1bCAT and 10 μg of each of the indicated plasmids and brought to 25 μg of total DNA with salmon sperm DNA. Following transfection, cells were maintained in GM for 24 h and/or were then transferred to DM for 48 h before harvesting. CAT assays were performed as described above. Experiments were done in triplicate and repeated at least three times.

SDS-PAGE and immunoblotting. Cells were solubilized in radioimmunoprecipitation assay buffer containing 10 mM EGTA and processed essentially as described previously (29). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analyses were performed on 10% polyacrylamide gels with a 5% polyacrylamide stacking gel. Electrophoretic transfer of proteins from SDS-polyacrylamide gels to nitrocellulose membranes and Western blotting were performed with an enhanced chemiluminescence system (Amersham, Little Chalfont, United Kingdom). Exposure was done with Agfa Curix RP2 films and intensifying screens.

Purification of MyoD proteins. MyoD wild-type (WT) protein was purified essentially as described previously (57). The MyoD mutant proteins produced by the pRSET constructs in *Escherichia coli* BL21/DE3 were purified by nickel affinity column as described by the supplier (Qiagen).

Preparation of active GST-Mos protein. GST and recombinant GST-Mos fusion proteins was purified from *E. coli* (55) and washed three times with NTEN buffer (20 mM Tris [pH 8], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40 [NP-40]) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml, and 10 μg of aprotinin per ml), 0.5 mM Na_3VO_4, 80 μM β-glycerophosphate, and 10 mM NaF. Purity of the GST and GST-Mos proteins, by SDS-PAGE analysis, was estimated to be 70 to 80% by Coomassie brillant blue staining of the gels. GST-Mos^{WT} and GST-Mos^{KM} were activated by incubation in rabbit reticulocyte lysate as described previously (47). Typically 1.5 μg of purified GST-Mos was incubated in 200 μl of reticulocyte lysate for 60 min at 30°C. The extract was diluted with 5 ml of ice-cold NTEN buffer containing protease and phosphatase inhibitors. Activated GST-Mos was affinity purified by incubation with glutathione-agarose beads and then washed three times with a buffer containing 10 mM TPO4 (pH 7), 150 mM NaCl, 1 mM EDTA, 1 mM sodium pyrophosphate, and 1% NP-40 and then stored at −80°C.

In vitro phosphorylation of purified MyoD proteins by the Mos kinase. In vitro phosphorylation of MyoD proteins by activated GST-Mos immobilized on glutathione-agarose was done as described previously (29). Protein kinase reaction mixtures (100 μl, final volume) included 1 mM sodium pyrophosphate, 10 mM sodium phosphate (TPO_4) (pH 7), 150 mM NaCl, 10 mM $MnCl_2$, 2 mM dithiothreitol, 20 μM ATP, 50 μCi of [γ-^{32}P]ATP (3,000 Ci mmol^{-1}), and 0.5 to 2 μg of purified MyoD proteins. The reaction was started by adding 400 ng of activated GST-Mos beads and incubated for 15 min at 25°C. The reaction was terminated by adding 50 μl of 2× SDS sample buffer. The mixture was boiled for 10 min and analyzed by SDS-PAGE. For the electrophoretic mobility shift assays (EMSAs), phosphorylation of MyoD was performed as described above except that [γ-^{32}P]ATP was omitted in in vitro phosphorylation and replaced by 1 mM cold ATP.

In vitro binding (GST pull-down) assay. GST, GST-MyoD, and/or GST-Mos beads were prepared by the methods of Lassar et al. (24) except that the fusions proteins were not eluted. After four washes at 4°C in NTEN buffer, aliquots of beads were frozen at −80°C. pEMSV-E12, MyoD, Mos, and various mutants were in vitro translated and/or cotranslated by using a TNT translation kit as instructed by the manufacturer (Promega). The programmed lysates (1 to 10 μl) were incubated with the GST alone or GST-MyoD and/or GST-Mos overnight at 4°C. Beads were washed four times in NTEN buffer at room temperature and then mixed with 1 volume of 2× SDS loading buffer, and bound proteins were analyzed by SDS-PAGE by using standard procedures.

EMSA. For a typical EMSA, the dimerization and DNA binding reactions were carried out for 20 min at 30°C in a final volume of 20 μl containing 80 mM NaCl, 10 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM dithiothreitol, 5% glycerol, 0.05% NP-40, 50 ng of poly(dI-dC) per ml, 50 ng of bovine serum albumin (Boehringer) per ml, 2×10^4 cpm of DNA probe, 10 to 100 ng of MyoD and/or Mos proteins, and up to 10 μl of reticulocyte lysate, where indicated, containing the E12 protein synthesized in vitro. The E-box DNA sequence, 5′-AGCTTCC AACACCTGCTGCAAGCT-3′, was derived from the creatine kinase gene (24) and was labeled with [α-^{32}P]dCTP by fill-in with Klenow polymerase. The binding reactions were analyzed on 4% (wt/vol) native polyacrylamide gels at 120 V for 2.5 h at room temperature.

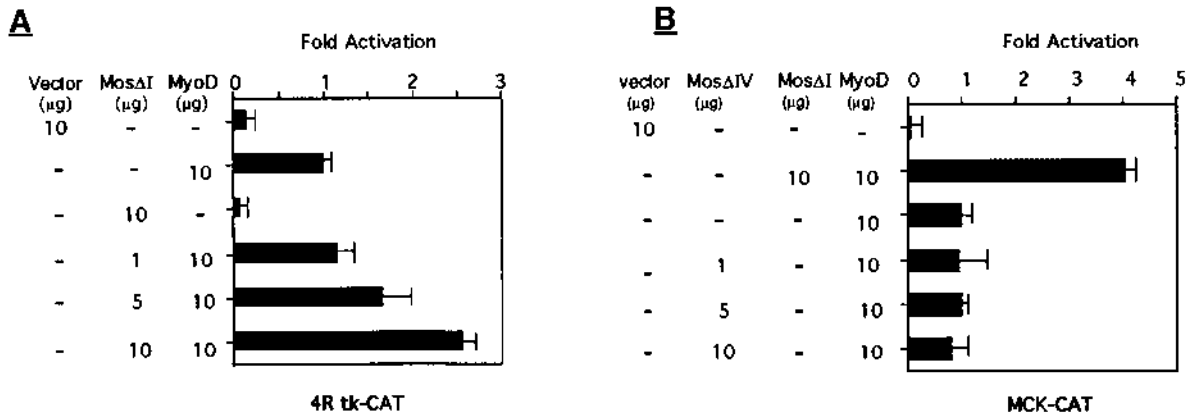

FIG. 1. Mos enhances MyoD-dependent transactivation of the MCK enhancer. (A) C3H10T1/2 cells were cotransfected with 5 μg of 4R-tk-CAT, 10 μg of pEMSV-MyoD, and increasing amounts of CMV-MosΔ1. Forty-eight hours following transfection, CAT expressions were assayed in aliquots of cell extracts normalized to β-galactosidase activity. (B) C3H10T1/2 cells were transfected as for panel A with 5 μg of the muscle-specific p1256 MCK-CAT reporter gene, 10 μg of pEMSV-MyoD, and increasing amounts of the ATP-binding deleted Mos mutant CMV-MosΔ4. Error bars represent the standard error of the mean.

Phosphoamino acid analysis. Phosphoamino acid analysis was done by two-dimensional separation on thin-layer plates as described previously (5).

RESULTS

Ectopic expression of Mos enhances transcriptional transactivation by MyoD. We previously showed that overexpression of rat Mos induced increasing expression of muscle-specific genes expression in C2C12 transfectants whereas inhibition of Mos expression by antisense RNA resulted in blockage of myogenesis (27).

To determine whether the in vivo effects of Mos could be reproduced with a cloned target reporter gene, C3H10T1/2 cells were transfected with MyoD and Mos expression plasmids together with a CAT reporter gene driven by either the MCK enhancer-promoter or four copies of MyoD DNA-binding sites. Figure 1 shows that MCK-CAT and 4R-tk-CAT were not expressed in C3H10T1/2 cells when transfected alone but could be activated efficiently by cotransfection with a MyoD expression vector. When transfection assays were performed in the presence of pCMVMosΔ1 expression vector, transactivation of both 4R-tk-CAT and MCK-CAT by MyoD was enhanced in a dose-dependent manner by Mos (Fig. 1). At the highest level of pCMV-MosΔ1 tested, we observed 2.5- and 4-fold increases in the level of CAT expression driven by the 4R-tk-CAT and MCK-CAT constructs, respectively. The transcriptional stimulation was not observed with the control plasmid pCMV alone or with the truncated MosΔ4 expression plasmid that is biologically inactive (Fig. 1). These results indicate that transactivation of 4R-tk-CAT and MCK-CAT expression by MyoD is enhanced by the addition of a Mos expression vector that synthesized the complete Mos protein. Taken together, the results demonstrate a functional interaction between Mos and MyoD.

MyoD but not E12 binds to Mos in vitro and in vivo. To test the hypothesis of a physical interaction between Mos and MyoD proteins, a biochemical approach was used (21). GST- or GST-Mos^{WT}-covered beads were incubated with ^{35}S-labeled in vitro-translated MyoD and E12, separately or together (Fig. 2). Results demonstrated that neither E12 alone (Fig. 2, lane 5) nor the MyoD-E12 heterodimer (lane 4) showed significant binding to Mos, while a significant amount of binding (about 20% of the input) could be detected when MyoD was added individually (lane 6). The converse experiments, in which beads were coated with MyoD and or E12, confirmed these results (see Fig. 5 and 6 and discussion below). To determine whether Mos interacted with MyoD in vivo, we used the two-hybrid approach with C3H10T1/2 cells. We used the GAL4 DNA-binding domain (DBD)-dependent reporter system, in which MyoD or E12 was fused to the DBD of yeast GAL4 (pM vector) and MyoD, E12, or Mos was fused to the VP16 activation domain (pVP16AD vector). The expression vectors were transfected singly or pairwise into C3H10T1/2 cells along with the pG5E1bCAT reporter vector. As illustrated in Fig. 3, expression of either pM-MyoD, pM-E12, or the VP16-MyoD, VP16-E12, or VP16-Mos polypeptide failed to induce significant CAT activity in transfected C3H10T1/2 cells. Coexpression of MyoD (in the pM-MyoD moiety) and E12 (in the pVP16-E12 moiety) generated a large increase in CAT activity to levels 200-fold (in GM [Fig. 3A]) or 800-fold (in DM [Fig. 3B]) higher than those found with the empty pM and pVP16AD vectors. These data show that heterodimers are formed in vivo through a stable interaction between the bHLH domains of MyoD and E12. Experiments with pM-MyoD, pM-E12, and VP16-Mos revealed in vivo association between Mos and MyoD but not with E12 and confirmed the results observed with the GST pull-down experiments (Fig. 2). Coexpression of MyoD and Mos induced 25-fold (in GM)- and 250-fold (in DM)-higher CAT activity than the control vectors. In contrast, pairwise expression of pM-E12 and VP16-Mos did

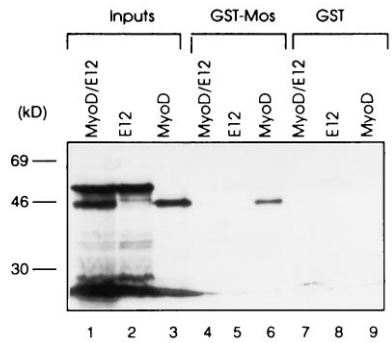

FIG. 2. Specific binding of MyoD but not E12 in vitro. E12 (lane 2) and MyoD (lane 3) were in vitro translated alone or in combination (lane 1). Translation products were incubated with beads covered with GST-Mos^{WT} (lanes 4 to 6) or GST alone (lane 7 to 9). Bound products were analyzed by SDS-PAGE and autoradiography.

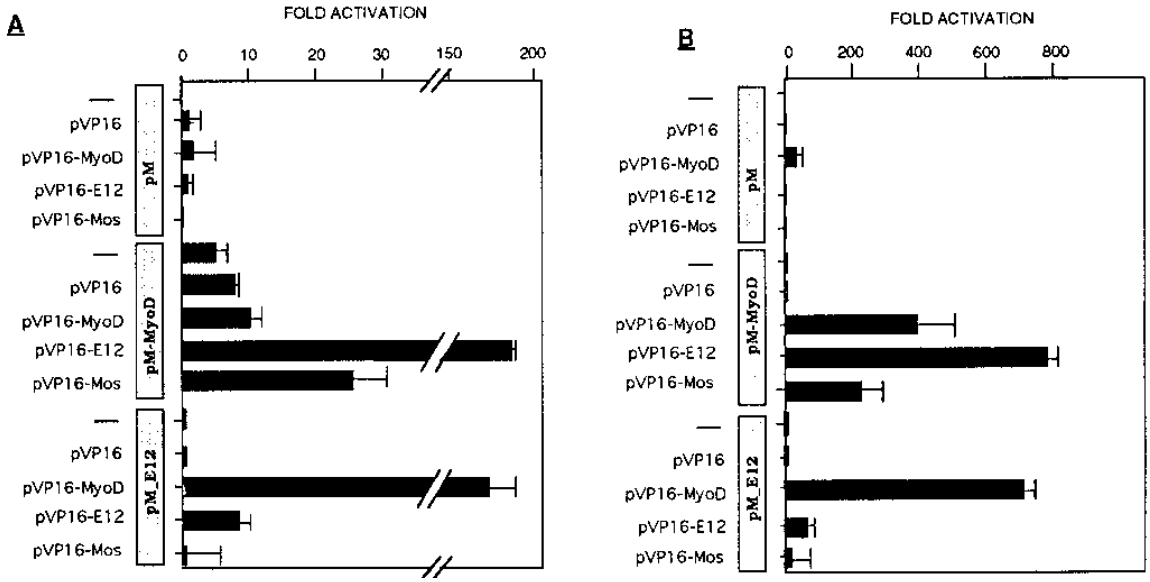

FIG. 3. In vivo interactions between Mos and MyoD evaluated by the two-hybrid assay. C3H10T1/2 cells were transiently transfected with 2 μg of the pG5E1bCAT reporter plasmid and 10 μg of each expression plasmid. Cells were harvested from GM 48 h after transfection (A) or shifted from GM to DM 24 h after transfection and harvested 48 h later (B). CAT activities were determined in aliquots of cell extract containing equivalent amounts of total cellular protein. Values are expressed as the fold activation of the reporter gene relative to the level of expression observed with the pM and pVP16AD vectors. Error bars represent the standard error of the mean.

not produce a detectable increase in CAT activity (Fig. 3). These data demonstrated the ability of Mos to interact with MyoD although the magnitude of activation was less than observed between E12 and MyoD proteins. This difference probably reflects a weaker affinity between Mos and MyoD than between E12 and MyoD. The bHLH domain of MyoD protein could mediate homodimer formation in vivo as measured in this assays.

A complex between MyoD and Mos was also detected when total cellular extracts from proliferating and differentiated myogenic cells were used (Fig. 4). Cellular proteins were extracted at various times of cell culture and incubated with GST- or GST-Mos^{WT}-covered agarose beads. Bound proteins were analyzed by SDS-PAGE followed by Western blotting, using a specific antibody to the COOH-terminal MyoD. Interestingly, two different migrating species of MyoD were observed in total cell lysates (Fig. 4, lanes 1 to 3). The slowly migrating species is probably hyperphosphorylated relative to the more rapidly migrating species (56). In vitro phosphorylation by the Mos kinase of purified MyoD induced changes in the apparent molecular mass (data not shown). As shown in Fig. 4, only the faster-migrating species of MyoD bound to GST-Mos^{WT} (Fig. 4; compare lane 1 to 3 with lanes 5, 7, and 9). Thus, it appears that Mos can bind selectively in vitro to the unphosphorylated form of MyoD.

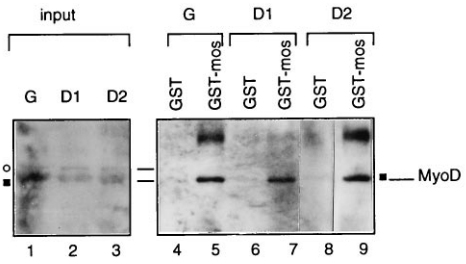

FIG. 4. Ex vivo, GST-Mos^{WT} binds the faster-migrating form of MyoD. Myoblasts were kept for 48 h in GM (G) or for 24 h (D1) and 48 h (D2) in DM. Total C2C12 protein extracts were incubated with GST or GST-Mos^{WT} protein beads. Total cellular proteins (lanes 1 to 3) and bound cellular proteins (lanes 4 to 9) were analyzed by Western blotting using anti-MyoD antibody either directly (lanes 1 to 3 contain 1/10 of the input used in lanes 4 to 9) or after absorption onto GST-covered beads (lanes 4, 6, and 8) or GST-Mos^{WT}-covered beads (lanes 5, 7, and 9). The circle indicates the slower-migrating form of MyoD, and the squares show the faster-migrating form of MyoD which binds the GST-Mos^{WT}-covered beads.

A highly conserved sequence in Mos proteins, related to the helix 2 of E-protein class of bHLH factors, mediates interaction with the helix 2 domain of MyoD. The domains of each polypeptide required for this interaction were mapped by in vitro protein binding experiments (Fig. 5 and 6). Full-length in vitro-translated MyoD efficiently bound to GST-Mos^{WT} but not to GST alone (Fig. 5B). Removing amino acids 63 to 99 from the N terminus or amino acids 218 to 269 from the C terminus of MyoD did not affect the interaction with GST-Mos^{WT}. In a MyoD protein in which the basic region and helix 1 were deleted (DM:102-135), association with the GST-Mos^{WT} protein was not affected. Likewise, the mutants MME12 basic and/or MMT4 basic (mutants of MyoD in which the basic domain has been replaced by the E12 basic and/or the T4 basic domain, respectively) showed no modification in the binding to GST-Mos^{WT} beads. On the other hand, a mutation that deleted the loop and helix 2 of MyoD (DM:128-172) suppressed association with Mos. These data indicate that binding of MyoD to Mos is mediated by the loop and helix 2 but not by the basic and helix 1 domains. All of the MyoD mutants excepted DM:218-269 and at a lesser extend DM: 63-99 bind to Mos less well than WT MyoD. This should be related to the modification of the bHLH region of MyoD that could modify the α-helical secondary structure and probably the affinity for the Mos protein (12).

To confirm these results, we performed reciprocal binding experiments with in vitro-translated Mos^{WT} and GST-MyoD. Figure 6B shows that full-length Mos bound to GST-MyoD. Mutations that deleted amino acids 1 to 12 and 1 to 30 in the

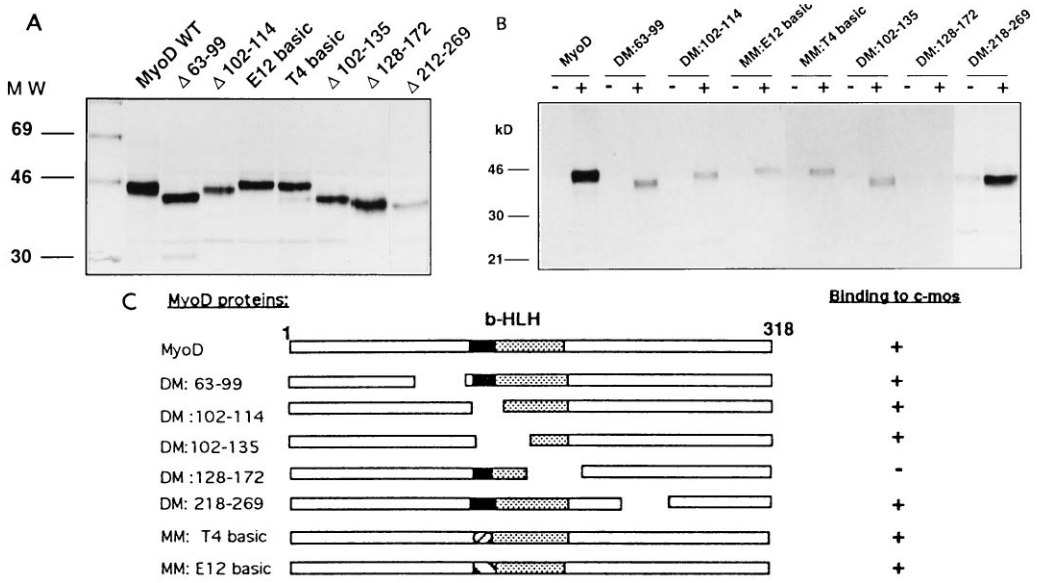

FIG. 5. The helix 2 domain of MyoD mediates interaction with Mos. (A) Various MyoD mutants were translated by programmed reticulocyte lysate, and 2 μl of the products were analyzed by SDS-PAGE. Molecular weights (MW) are indicated in thousands. (B) Binding of various MyoD mutants to GST-Mos^{WT}. Similar amounts of various [^{35}S]methionine-labeled MyoD proteins were incubated with GST (−) or GST-Mos^{WT} (+). Bound proteins were eluted and analyzed by SDS-PAGE and autoradiography. (C) Summary of the results in panel B.

N terminus (DM:1-12 and DM:1-30) or amino acids 222 to 339 of the C terminus (TM:221) did not affect the interaction with GST-MyoD. The deletion of the N-terminal part of Mos (DM: 1-139) and mutations that deleted amino acids 138 to 339 (TM:137) and 182 to 339 (TM:181) showed reduced association. Both the N- and C-terminal regions of the molecule could be deleted, and the resulting minimal central domain containing amino acid residues 140 to 221 was sufficient for a high

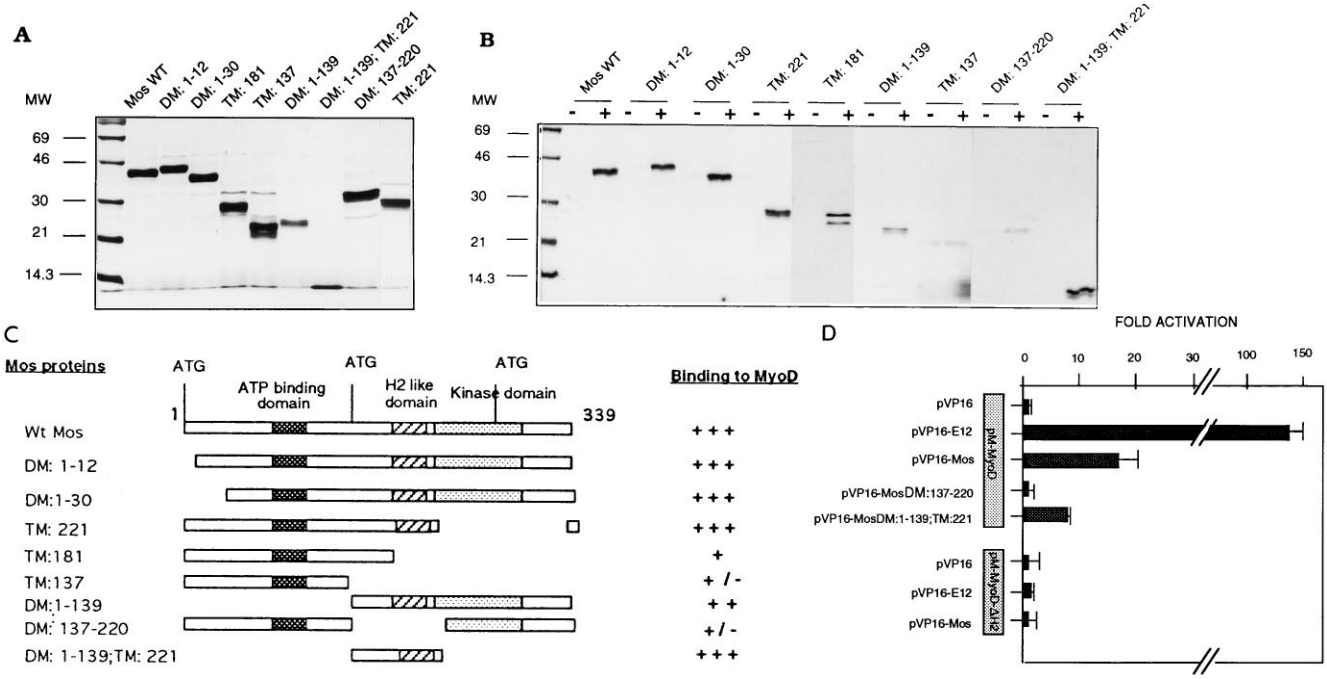

FIG. 6. An internal domain of Mos is sufficient for binding to MyoD. (A) Various Mos deletion mutants were translated by programmed reticulocyte lysate, and 2 μl of the products were analyzed by SDS-PAGE. The double bands observed in some deleted Mos mutant translations are due to the alternative use of either the translational start site of the pRSET vector or the first and/or the conserved internal ATG in the Mos inserted sequence. Molecular weights (MW) are indicated in thousands. (B) Binding of an internal domain of Mos protein to GST-MyoD. Similar amounts of various [^{35}S]methionine-labeled Mos proteins were incubated with GST (−) or GST-MyoD (+), and the bound proteins were analyzed by SDS-PAGE and autoradiography. (C) Summary the results in panel B. (D) In vivo interactions between deletion mutants Mos and MyoD evaluated by the two-hybrid assay. C3H10T1/2 cells were transiently transfected with 2 μg of the pG5E1bCAT reporter plasmid and 10 μg of expression vectors encoding GAL4 DBD-MyoD (pM-MyoD), GAL4 DBD-MyoDΔ128-172 (pM-MyoD-ΔH2), pVP16-E12, pVP16-Mos, pVP16-MosDM:137-220, and pVP16-MosDM:1-139;TM:221. Cells were harvested from GM 48 h after transfection, and CAT activities were determined as described for Fig. 3.

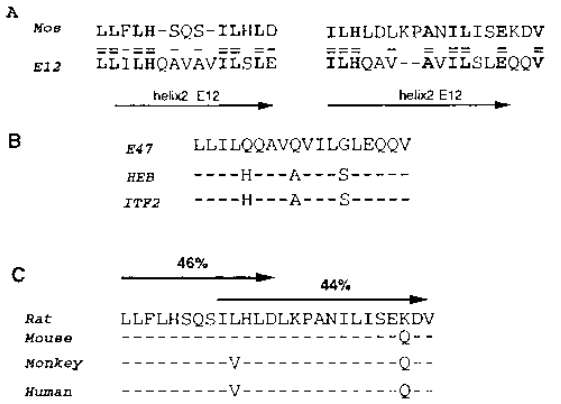

FIG. 7. Sequence comparison of Mos proteins and the helix 2 of E-protein class of b-HLH factors. (A) Protein alignments were made to maximize homology. Amino acid identitities in Mos, E12, E47, ITF2, and HEB are in boldface. The double lines indicate absolute identity; the single lines indicate conservative substitutions of the amino acids. (B) Alignment of primary sequences of Mos mammalian family proteins containing the region similar to helix 2 of E proteins.

specific binding to GST-MyoD. Deletion of these amino acids (mutant protein DM:137-220) resulted in little association to the same level as mutant TM:137. Finally, the association between MyoD and Mos proteins was not observed in vivo by using the deletion mutants pM-MyoDΔ128-172 and pVP16-MosΔ137-220 in the two-hybrid approach in C3H10T1/2 cells (Fig. 6D).

The protein sequence of this minimal domain covering amino acids 140 to 221 was used to search available databases. Amino acids 184 to 209 of the Mos protein contains two overlapping sequences having, respectively, 46 and 41% similarity to the helix 2 domain of the ubiquitous transcription factors E12, E47, HEB, and ITF2 (Fig. 7A). Furthermore, this amino acid domain (residues 184 to 209 of the rat Mos protein) is highly conserved in the four mammalian Mos proteins (Fig. 7B). Thus, Mos contains an amino acid sequence, related to helix 2 of the E-protein class of bHLH factors, that correlates with its ability to physically associate with MyoD. In addition, a domain in the NH_2 region of Mos protein (between amino acids 31 and 136) seems to contribute to the association with MyoD but with a lower affinity than the sequence related to the helix 2 of E12 and ITF2. This domain is currently under investigation.

Phosphorylation of MyoD by Mos in vitro. Because MyoD is known to be phosphorylated, we wanted to determine whether MyoD was a direct substrate for Mos kinase. The purified GST-Mos^{WT} showed no detectable autophosphorylation activity in vitro (data not shown). Purified maltose-binding protein–*Xenopus* Mos was also not autophosphorylatable in vitro and could be activated (by an unknown mechanism) by incubation in cell extracts prepared from rabbit reticulocyte lysate (47). The synthesis and/or incubation of Mos protein in rabbit reticulocyte lysates induced the activation of endogenous MKK and mitogen-activated protein kinase (MAPK) (48). In particular, p44^{ERK1} and p42^{ERK2}, which are present in such lysates, can be activated by MKK. The phosphorylation of a threonine residue and a tyrosine residue reduces the gel mobility of ERKs when tested by SDS-PAGE (47, 48). We tested the ability of Mos to phosphorylate MyoD in such lysates. Increasing amounts of GST, GST-Mos^{WT}, and GST-Mos^{KM} were treated by incubation in the lysates containing purified MyoD protein. An anti-ERK2 immunoblot showed that Mos^{WT}, but not Mos^{KM} or GST alone, caused the band shift of ERKs,

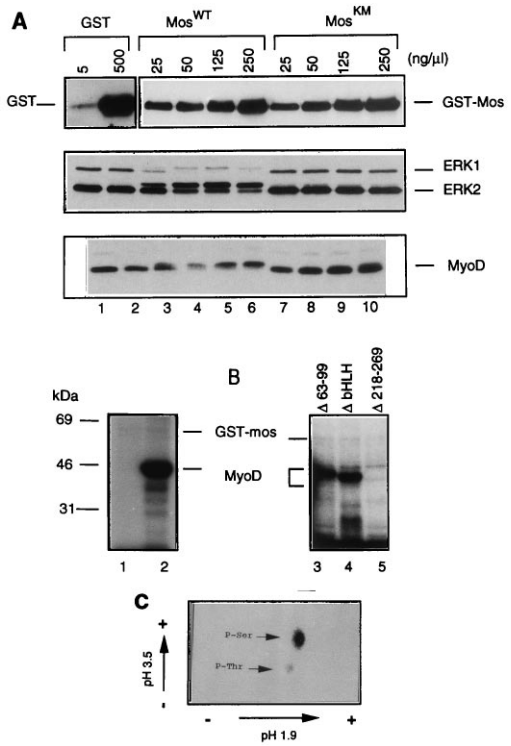

FIG. 8. MyoD is a substrate for phosphorylation by the Mos^{WT} protein kinase. (A) Mos kinase activity is required for MyoD phosphorylation in rabbit reticulocyte lysates. Increasing amounts of GST, GST-Mos^{WT}, or kinase inactive GST-Mos^{KM} were incubated in the presence of 10 ng of MyoD protein in rabbit reticulocyte lysates (50 μl) at 30°C for 1 h. Samples (2 μl) were analyzed by blotting with antibodies against GST, GST-Mos, MyoD, and ERK2 MAPK. The anti-ERK2 antibody (Santa Cruz Biotechnology, Inc.) also recognized p44^{ERK1}. Phosphorylation decreases the electrophoretic mobility of MAPKs (47, 48) and MyoD (56). (B) Phosphorylation of bacterially expressed MyoD proteins by activated GST-Mos^{WT}. Activated GST-Mos^{WT} beads were incubated in the absence (lane 1) or in the presence (lane 2) of 1 μg of MyoD and then analyzed by SDS-PAGE and autoradiography. Purified His-tagged MyoD mutants (1 μg of each) were incubated in the presence of activated GST-Mos^{WT}. Lane 3, deletion of amino acids 63 to 99 (D:63-99); lane 4, deletion of amino acids 102 to 135 (D:102-135); lane 5, deletion of amino acids 218 to 269 (D:218-269). Exposure time was 2 h. (C) Gel-purified MyoD phosphorylated in vitro by Mos was subjected to phosphoamino acid analysis followed by autoradiography. The positions of unlabeled phosphoamino acid standards detected by ninhydrin are shown.

corresponding to phosphorylated forms (Fig. 8A) (47, 48). Interestingly, an anti-MyoD Western blot showed that only the Mos^{WT} was able to phosphorylate MyoD, as evidenced by the reduced gel mobility of MyoD (56). The ability of Mos^{WT} to phosphorylate MyoD was correlated with the amount of GST-Mos^{WT} protein being added. The amount of GST and/or GST-Mos^{KM} was at least in 10-fold excess in this assay, relative to the minimal quantity of Mos^{WT} used in lane 3, indicating that Mos kinase activity is necessary for MyoD phosphorylation.

Active GST-Mos^{WT} recovered from rabbit reticulocyte lysates by binding to glutathione-agarose beads showed autophosphorylation activity (Fig. 8B, lane 1). The activated GST-Mos^{WT} was able to phosphorylate bacterially produced MyoD in an in vitro kinase reaction (Fig. 8B, lane 2), whereas as expected GST alone or GST-Mos^{KM} could not phosphorylate MyoD (Fig. 8A). Purified MyoD previously incubated alone in rabbit reticulocyte lysates, immunoprecipitated with anti-MyoD, showed no detectable phosphorylation when added to the kinase buffer, indicating that Mos kinase activity is neces-

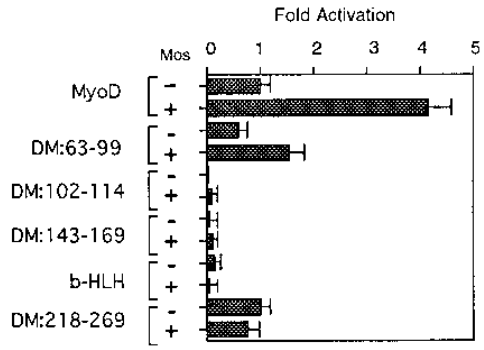

FIG. 9. Activation of transcriptional transactivation of MyoD proteins by Mos. The MyoD expression vector (5 μg) and each of the mutant MyoD expression vectors (5 μg) were cotransfected with the p1256 MCK-CAT reporter plasmid (5 μg) in C3H10T1/2 cells. As indicated, 10 μg of Mos expression plasmids (+) or empty vector (−) was added to the cotransfected MyoD expression vectors and MCK-CAT reporter plasmids. Twenty-four hours after transfection, cultures were transferred from GM to DM, and CAT activity was assayed in cells extracts 48 h later. The fold activation was calculated by quantification with a phosphorimager (Fuji). The transfections experiments were done twice in duplicate. Error bars represent the standard error of the mean.

sary for MyoD phosphorylation in this in vitro kinase assay (data not shown).

Previous reports have shown that bacterially expressed MyoD and/or myogenin were phosphorylated by PKC and PKA on the serine and threonine residues located in the basic domains of their bHLH domains (34, 35). Different MyoD mutant proteins were tested for their abilities to be phosphorylated by Mos. Mutants in which the basic-helix 1 region was deleted (D:102-135) and which lacked amino acids 63 to 99 in the N terminus (D:63-99) continued to be phosphorylated by Mos (Fig. 8B, lanes 3 and 4). Surprisingly, a mutation that deleted amino acids 218 to 269 in the COOH-terminal domain (D:218-269) showed a dramatically reduced level of phosphorylation (~10% of the WT MyoD level). These data indicate that in vitro phosphorylation sites map mainly in the COOH domain of MyoD. The sites for phosphorylation of MyoD by Mos were analyzed by phosphoamino acid analysis and revealed that MyoD was phosphorylated predominantly on serine residues (Fig. 8C).

We also tested whether Mos could activate transcriptional transactivation of MyoD mutants by using the MCK-CAT expression vector. MyoD mutant DM:63-99, which lacks the Cys/His-rich region, could be positively regulated by Mos. Mutants DM:102-114 (Δb) and DM:143-169 (ΔH2) were unable to activate expression of MCK-CAT because they could not bind to DNA and/or could not heterodimerize with E12 protein. The mutant containing only the bHLH domain was also unable to activate expression of MCK-CAT because this mutant lacks the NH_2- and COOH-terminal transactivation domains. In contrast, mutant DM:218-269, which lacks the COOH region, was able to activate the expression of the reporter gene, but it was not transactivated by Mos (Fig. 9). This region, which contains nine serines and two threonines, is mainly phosphorylated by the Mos kinase (Fig. 8B). These data strongly suggest that transcriptional transactivation of MyoD by Mos is dependent on the phosphorylation of the COOH region of MyoD.

Phosphorylation of MyoD by Mos inhibits the DNA-binding activity of homodimers but not MyoD-E12 heterodimers. Transactivation of the MCK enhancer by MyoD is dependent on a high affinity E box known as the MEF-1 site (8). MyoD alone can bind the E box as homodimers when expressed at high concentrations as bacterial fusion proteins and/or in the

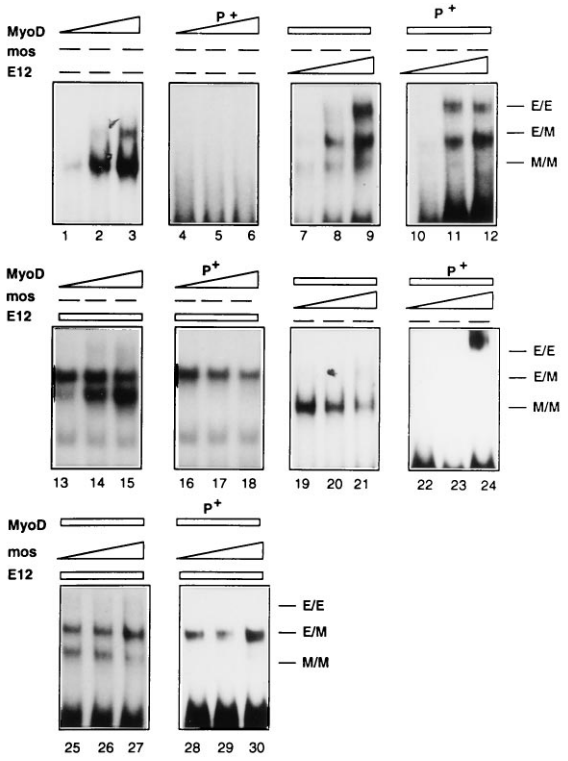

FIG. 10. EMSA with unphosphorylated and phosphorylated MyoD proteins, E12 protein synthesized in reticulocyte lysate, and His-tagged Mos protein produced in bacteria. DNA-binding activity was monitored by EMSA using the MCK E box as a probe. The proteins are indicated above each lane. P+, binding reaction using 25, 75, and 225 ng of phosphorylated MyoD protein by activated GST-Mos (lanes 4 to 6 and 16 to 18) or 50 ng of phosphorylated MyoD protein (lanes 10 to 12, 22 to 24, and 28 to 30). Identical quantities of unphosphorylated MyoD proteins were used in gel shift assay experiments (lanes 1 to 3 and 13 to 15 for increasing amounts and lanes 7 to 9, 19 to 21, and 25 to 27 for constant amounts of unphosphorylated MyoD protein). E12 was synthesized in reticulocyte lysate, and 1, 3, and 9 μl of extracts were added to the binding reactions lanes 7 to 9 and 10 to 12 or 2 μl was added in lanes 13 to 18 and 25 to 30; 50, 100, and 200 ng of purified His-tagged Mos protein were added in lanes 19 to 30. Note that MyoD-Mos complexes do not exhibit DNA-binding activity (lanes 19 to 24). The minor slower retarded bands observed in lanes 2 and 3 are not MyoD specific. M/M, MyoD-MyoD homodimers; E/M, E12-MyoD heterodimers; E/E, E12-E12 homodimers.

presence of $MgCl_2$ (38), but it binds with significantly higher affinity as a heterodimer with E12 or E47 protein (9, 41). To examine the effect of phosphorylation by Mos on the DNA-binding activity of MyoD, we compared the DNA-binding properties of bacterially expressed MyoD before and after phosphorylation in vitro by Mos. Unphosphorylated MyoD bound in a concentration-dependent manner to the E-box element as homodimer (complex M/M in Fig. 10, lanes 1 to 3), whereas phosphorylation greatly reduced the DNA-binding activity of MyoD homodimers (lanes 4 to 6). We next investigated whether phosphorylation of MyoD by Mos inhibits DNA binding in the presence of increasing amounts of E12. For these experiments, E12 was translated in vitro and the translation product was incubated with unphosphorylated and/or phosphorylated MyoD. Binding between MyoD and E12 (complex E/M) was more efficient when phosphorylated MyoD was used in the reaction, as judged by the comparative band intensities of the DNA-protein complexes (Fig. 10; compare lanes 7 to 9 and 10 to 12). At saturating levels of E12 protein, E12 homodimer formation is observed (complex E/E). The dimerization and the DNA binding of the same unphosphorylated

and phosphorylated MyoD proteins were then analyzed in the presence of a constant restricted amounts of E12 protein. Unphosphorylated MyoD dimerized with E12 and bound to DNA (complex E/M), but increasing amounts of MyoD induced formation of homodimers which bound to the E box (complex M/M). In the same conditions, phosphorylated MyoD did not form homodimers even at high amounts of proteins (Fig. 10, lanes 13 to 15 and 16 to 18). Since we have shown a direct physical association between MyoD and Mos proteins (Fig. 4 to 6), we next analyzed if the MyoD-Mos complex exhibited DNA-binding activity. In the absence (Fig. 10, lanes 19 to 21) or/and in the presence (lanes 25 to 27) of E12 protein, increasing amounts of Mos protein reduced the formation of unphosphorylated MyoD homodimers but did not interfere with the formation of MyoD-E12 heterodimers. Addition of Mos protein to the binding reactions with phosphorylated MyoD proteins did not modify the band shifts (Fig. 10, lanes 22 to 24 and 28 to 30). These data indicate that the physical association of Mos with MyoD competes for the formation of MyoD homodimers. Phosphorylation of MyoD by the Mos kinase inhibits its homodimer formation. The MyoD-Mos complex does not exhibit DNA-binding activity. Interaction between MyoD and E12 proteins is stronger than that observed between MyoD and Mos because MyoD homodimers but not MyoD-E12 heterodimers were reduced in the presence of increasing quantities of Mos protein (Fig. 9, lanes 25 to 27), and phosphorylated MyoD did not bind to Mos protein.

DISCUSSION

MyoD has been implicated as a master regulatory gene in the process of muscle differentiation, but its activity is highly controlled in particular by growth factors, oncogenes, and negative HLH proteins such as Id (3). The mechanisms that negatively modulate MyoD activity are beginning to be well characterized (for a review, see reference 60), but little is known about the positive regulation of MyoD. We have shown that the Mos protein, in contrast to other oncogenic proteins, when overexpressed in myoblasts facilitated their differentiation in myotubes (27). The absence of transformation in the sublines of Mos transfectants was surprising with regard to previous observations showing that transfection of fibroblasts with Mos sequences placed under the control of a potent promoter, in particular the murine sarcoma virus long terminal repeat, resulted in their neoplastic transformation (15, 43, 54). According to a previous report, *mos* genes from different vertebrate species transform NIH 3T3 cells with different efficiencies, and these efficiencies correlate with the Mos protein kinase activities (64). In contrast, overexpression of the v-*mos* oncogene in human monocytes induces their differentiation in macrophages (22, 23). These data suggest that the Mos product functions differently in cell differentiation and transformation.

Mos up-regulates the expression of both MyoD and MCK genes. Ectopic expression of Mos results in dose-dependent transcriptional transactivation of MyoD in C3H10T1/2 cells. We recently showed that the expression of antisense Mos sequences, linked to the metallothionein promoter, resulted in a reversible dose-dependent inhibition of MyoD and myogenesis in C2C12-derived transfectants (27). The apparent positive regulation of MyoD by Mos suggests several possibilities: Mos induces the expression of cellular factors that act positively on MyoD expression, Mos competes for the expression of a repressor(s) of myogenic regulators, and/or Mos directly associates with and activates MyoD by specific phosphorylation. Here we demonstrate a physical interaction between the two proteins through the helix 2 domain of MyoD and a highly conserved region of Mos which displays a great similitude with the helix 2 of E proteins. Phosphorylation of the COOH domain of MyoD by the Mos kinase enhances the transcriptional transactivation of MyoD by promoting its heterodimerization. One important feature of this conserved domain of Mos protein is its capacity to associate with this myogenic bHLH factor and not with nonmyogenic bHLH proteins such as E12, E47, and USF, the HLH protein Id, and/or the serum response factor (unpublished data). It would thus appear that there are specific structural constraints and affinities that govern interactions between Mos and the bHLH myogenic factors. Despite the fact that MyoD and E12 form excellent heterodimers, there is no association between E12 and Mos. Mos physically interacts with MyoD homodimers, but not with the MyoD-E12 heterodimer, and competes for its formation, as observed in the DNA binding experiments. The oligomeric composition of these interacting species has not been addressed. MyoD monomers possibly interact with Mos monomers, but these MyoD-Mos complexes have no DNA-binding activity. The interaction between MyoD and Mos differs from the homotypic association between E12 and MyoD. Nevertheless, the competition for binding to MyoD between E12 and Mos demonstrates that it is the same binding site. Our DNA binding data show that the MyoD-E12 complexes bound to the E box are stable in the presence of excess Mos. However, when Mos activity was removed from muscle cells by modulable expression of Mos antisense RNAs, reversible repression of MyoD and myogenic differentiation was observed (27). These data suggest that Mos is involved in the formation and/or stabilization of active MyoD-E12 protein complexes via specific interactions through helix 2 of MyoD and by a specific phosphorylation of MyoD. This particular interaction between Mos and MyoD is under investigation.

Although it is known that MyoD is a phosphorylated nuclear protein (56), the kinase or kinases involved in the transcription-activating potential of MyoD by phosphorylation are not known at this time. We show here that MyoD protein phosphorylated by Mos behaves differently from the unphosphorylated protein or the MyoD protein phosphorylated by the serine/threonine kinases such PKC and PKA (34, 35). Substantial indirect evidence suggested that active kinase Mos was implicated in the transcriptional activity of MyoD (27). As shown in Fig. 1, the product of a Mos deletion mutant (p25Δmos) which lacks the NH_2-terminal and ATP-binding domains and has no biological activity (30a) is inactive in the CAT assays, while the complete Mos protein stimulates MyoD activity in a dose-dependent manner. It has been previously shown that fibroblast growth factor inactivates myogenic bHLH proteins through phosphorylation of a conserved PKC site in their DNA-binding domains. This site is phosphorylated by PKC and mediates repression of the myogenic program through a loss in DNA-binding activity (35). PKA inhibits the activity of myogenic bHLH proteins to activate endogenous and exogenous muscle-specific genes, and transcriptional repression by PKA is targeted to the E-box motif. However, although myogenic bHLH proteins contain PKA phosphorylation sites in their basic regions, these sites are not required for repression by PKA, indicating that this kinase represses muscle-specific transcription through an indirect mechanism (34). By contrast, we show that Mos is able to phosphorylate MyoD and that a great proportion of the phosphorylation sites are located in the COOH-terminal portion of the protein (Fig. 8B). The role of this phosphorylation is demonstrated in Fig. 9: phosphorylation by the Mos kinase inhibits the DNA binding of MyoD homodimers and favors MyoD-E12 heterodimers. Our data are consistent with the hypotheses that Mos protein

in myogenic cell serves at least two functions. Phosphorylation of MyoD by Mos greatly reduces or eliminates the DNA-binding activity of MyoD homodimers to the same target sequence as the MyoD-E12 heterodimer. This modification would favor a change in the homodimer-heterodimer equilibrium toward the formation of either the transcriptionally active DNA-binding complex with E-protein family or the nonbinding complex with inhibitory HLH proteins such as Id (3). Interestingly, neither the E2A gene products E12 and E47 nor Id binds to the Mos protein, while the MRF4 protein that accumulates during postnatal development of skeletal muscle (as does the Mos protein) is able to associate with and is phosphorylated by Mos (unpublished data). Modulation of the MyoD homodimer-heterodimer equilibrium through specific binding and phosphorylation could serve to regulate the amount of active heterodimer.

Our data strongly suggest that Mos protein is involved in myogenic differentiation. However, this hypothesis is challenged by the recent creation of Mos nullizygous mice (10, 18). Although we have confirmed the absence of Mos protein in adult skeletal muscles of homozygous Mos−/− mice (data not shown), these mice are viable and have in particular no obvious muscular defect. Mutant males are fertile, but the fertility of the females is very low. Oocytes from these females matured to the second meiotic metaphase both in vivo and in vitro but were activated without fertilization. These results indicate that in mice, Mos does not seem to be essential for the initiation of oocyte maturation, spermatogegesis, or somatic cell cycle. Perhaps Mos behaves in a species-specific manner, as suggested by the fact that, unlike *Xenopus* Mos, in homozygous Mos−/− mice, Mos protein is not essential for normal oocyte matutation. Alternatively, the small number of normal offspring that arise from double Mos−/− mothers and the normal fertility of males suggest that they could derive by a selective pressure to substitute a different molecule to perform the essential cellular functions of Mos in particular for maturation initiation (58). Such MyoD−/− mice are also fairly "normal" (51). We suspect that the same sort of compensation may occur for the absence of gross muscular abnormalities in Mos−/− mice. Identification and analysis of a functional domain(s) such as the helix 2-related sequence that could determine the substrate specificity of Mos kinase may shed light on the molecular mechanisms of Mos expression in the different phenotypes in various cell types.

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