Overexpression of Mos^{rat} proto-oncogene product enhances the positive autoregulatory loop of MyoD

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Abstract The myogenic b-HLH transcription factor MyoD activates expression of muscle-specific genes and autoregulates positively its own expression. Various factors such as growth factors and oncogene products repress transcriptional activity of MyoD. The c-*mos* proto-oncogene product, Mos, is a serine/threonine kinase that can activate myogenic differentiation by specific phosphorylation of MyoD which favors heterodimerization of MyoD and E12 proteins. Here we show that overexpression of Mos enhances the expression level of MyoD protein in myoblasts although phosphorylation of MyoD by Mos does not modify its stability but promotes transcriptional transactivation of the MyoD promoter linked to the luciferase reporter gene. Moreover, co-expression of MyoD with Mos^{wt} but not with the kinase-inactive Mos^{KM} greatly enhances expression of endogenous MyoD protein and the DNA binding activity of MyoD/E12 heterodimers in 10T1/2 cells. Our data suggest that Mos increases the ability of MyoD to transactivate both muscle-specific genes and its own promoter and could therefore participate in the positive autoregulation loop of MyoD and muscle differentiation.

Key words: Mos; Positive autoregulation; MyoD

1. Introduction

During myogenesis the transcription of muscle-specific genes is dependent on a family of muscle-specific factors which includes MyoD [1,2], myogenin, [3,4], Myf5 [5], MRF4 [6], known as herculin in mouse [7] and Myf6 in human [8]. These proteins share a basic helix-loop-helix domain (b-HLH), which is also common to ubiquitous transcription factors such as E12 or E47 [9,10] and the HEB gene products [11] with which myogenic factors mediate dimerization and binding to a DNA consensus sequence known as a E-box (CANNTG) [12]. In addition to activation of muscle-specific genes, some members of the MyoD family also activate their own transcription and are able to transactivate expression of other family members [13]. Two proximal E-boxes in the MyoD promoter were identified as targets of MyoD binding as well as autoregulation of MyoD gene expression [14]. It was postulated that the autoregulatory interactions amplify the expression of these factors above a threshold for activation of the muscle differentiation program and stabilization of the myogenic phenotype. Myogenesis is inhibited by some mitogens [15] and overexpression of oncogenes and/or proto-oncogenes [16,17]. Myogenic b-HLH proteins are sensitive to growth factors and oncogene products which can silence their activities and inhibit myogenic differentiation [18]. Inactivation of the myogenic b-HLH proteins is associated with a loss of their ability to heterodimerize and to bind DNA [19–22] and/or of their transcriptional activity [23]. We previously showed that the c-*mos* proto-oncogene product, serine/threonine kinase Mos, is up-regulated during skeletal muscle development [24,25]. Its expression was originally described as being restricted to male and female germ cells [26]. In mammalian gonad tissues Mos appears to play an important role in maturation of male and female germinal cells [27–29]. However, various other somatic cell lineages and tissues express significant amounts of Mos products [30–32]. Accumulation of Mos during skeletal muscle development suggested a particular function(s) of Mos in this tissue [33]. Indeed, ectopic expression of Mos activated muscle differentiation while the inhibition of endogenous Mos expression by antisense RNA resulted in reversible blockage of myogenesis [33], suggesting that Mos may interact with myogenic b-HLH proteins. Thus, unphosphorylated MyoD but not its E12 partner physically interacts with Mos [34]. Mutational analysis of the protein reveals a highly conserved region in Mos proteins which shares sequence homologies with the ubiquitous E12/E47 proteins and associates with the helix 2 domain of MyoD. Phosphorylation of the COOH domain of MyoD by Mos inhibits the DNA binding activity of MyoD homodimers but promotes the formation and DNA binding activity of MyoD-E12 heterodimers [35]. As the MyoD promoter contains two E-boxes identified as targets of MyoD binding and autoregulation of MyoD gene expression, we investigated if Mos could enhance the transcriptional activity of MyoD on its own promoter as it does on muscle gene promoters. We found that ectopic expression of Mos causes a increase in the accumulation of MyoD gene products in proliferating C2C12 myoblasts and MyoD-expressing 10T1/2 cells. Neither Mos^{wt} nor the kinase-inactive Mos^{KM} modifies the stability of MyoD protein. On the other hand, Mos^{wt} but not the kinase-inactive Mos^{KM} increases the levels of DNA binding and transcriptional activities of MyoD on its own promoter. Thus these data indicate that Mos favors the capacity of MyoD to up-regulate its own expression and promotes the levels of MyoD products. These results suggest that Mos participates in the positive autoregulation of MyoD and thus in activation of muscle differentiation.

2. Materials and methods

2.1. Cell cultures, DNA transfections and luciferase assays

The mouse skeletal muscle cell line C2C12 and the fibroblast cell line 10T1/2 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% and 15% fetal calf serum respectively. To obtain G418-resistant colonies, C2C12 cells were stably transfected by the calcium phosphate procedure with 1 μg of super-

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coiled pSV_2-neo plasmid and 10–15 μg of the expression vectors pCMV, pCMV-Mos^{wt} or pCMV-mosΔNH2 as described previously [36]. 10T1/2 cells were transfected using polyethyleneimine (PEI, Sigma) essentially as described [37]. The total amount of DNA used for each plate was normalized with the respective empty expression vehicle. Forty-eight hours after transfection, cells were harvested and luciferase activity determined on aliquots of cell extracts containing equivalent amounts of proteins with a luminometer (Lumat LB 9507-DLA, Berthold). Five hundred nanograms of the plasmid pCH110 (Pharmacia) was included in the assays as an internal control for transfection efficiency. Experiments were performed in triplicate and repeated at least twice.

2.2. Plasmid constructions

To create pCMV-Mos, the *Bam*HI restriction fragment of pRSET-Mos was subcloned into pcDNA3 (Invitrogen) under the control of the cytomegalovirus (CMV) promoter [34]. Expression vectors pEMSV-E12 and pEMSV-MyoD have been previously described [1,12]. Expression vectors pCMVHA-MyoD, pCMVHA-Mos^{WT} and pCMVHA-Mos^{KM} were generated by cloning the hemagglutinin (HA) epitope tag in triplicate at the amino-terminus of cDNA inserts in pcDNA3. Expression vectors pEMSV-Mos^{wt} and pEMSV-Mos^{KM} were generated after excision of the *Bam*HI inserts from pGEX2T-Mos^{wt} and pGEX2T-Mos^{KM} constructs previously described [34]. Inserts were filled in with Klenow DNA polymerase and inserted in pEMSV at the filled in *Eco*RI site. Expression vector pCMV-MosΔNH2 was created by inserting a *Nru*I-*Xba*I fragment encoding aa 141–339 of the Mos^{wt} protein into the *Sma*I-*Xba*I sites of pcDNA3 vector. The MyoD promoter fragment *Hin*dIII-*Bsm*I [14] was filled in with Klenow DNA polymerase and cloned into the *Sma*I site of pGL3-Luc plasmid harboring the firefly luciferase reporter gene (Luc) (Promega).

2.3. RNA analysis

For Northern blot analyses, total RNA was isolated by the guanidinium isothiocyanate procedure. Hybridization conditions were as previously described [33]. Randomly primed DNA insert probes used in hybridizations were for MyoD a 1.8 kb *Eco*RI fragment of the mouse MyoD cDNA clone pEM-MyoD [1] and for Gapdh a 1300 bp *Pst*I fragment of the rat glyceraldehyde 3-phosphate dehydrogenase cDNA clone pRGAPDH 13 [38].

2.4. SDS-PAGE immunoblotting

Cells were lysed and total proteins solubilized in RIPA buffer containing 10 mM EGTA and processed as previously described [24]. After electrophoretic transfer of proteins onto nitrocellulose membranes, immunodetection was performed with the 12CA5 monoclonal antibody (dilution: 1/1000, Boehringer) or the MyoD antibody (dilution: 1/1000, Santa Cruz). After exposure to the secondary antibody, horseradish peroxidase-linked sheep anti-rabbit or anti-mouse immunoglobulin G (Sigma), antigen-antibody complexes were revealed by the chemoluminescence system (ECL, Amersham). Exposure was performed with Agfa Curix RP2 films and intensifying screens.

2.5. Electrophoretic mobility shift assay (EMSA)

MyoD DNA binding activity in presence or in absence of co-transfected Mos^{wt} and Mos^{KM} was determined as described [39]. Twenty micrograms of whole-cell extract were diluted to a final volume of 20 μl in a reaction mixture containing 80 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM dithiothreitol, 5% glycerol, 0.05% NP40, 1 μg/ml poly(dI-dC), 500 ng of bovine serum albumin and incubated with 0.1–0.3 ng of ^{32}P-labeled E-box probe (5′-AGCTTCCAA-CACCTGCTGCAAGCT-3′) derived from the creatine kinase gene promoter [39]. After a 15 min incubation at 25°C, antibodies were added for an additional 15 min incubation, at that time 5 μl of 0.005% bromophenol blue/xylene cyanol was added and the binding reactions analyzed on 4% (w/v) native polyacrylamide gels at 120 V for 2.5 h at room temperature.

3. Results and discussion

3.1. Stable overexpression of Mos induces increasing expression of MyoD protein in C2C12 transfectants

Proliferating C2C12 myoblasts were stably transfected with the neomycin resistance and CMV-Mos constructs. G418-resistant colonies were selected and serially passaged. Transfectants displayed both the normal phenotype and a capacity to carry out the myogenic program of the parental C2C12 line. When analyzed by Western blot during exponential growth and compared to C2C12 control cells (which were transfected with the empty vehicle), transfectants carrying the mos^{wt} gene displayed variable amounts of MyoD and Mos^{rat} proteins (Fig. 1A). However, at the same stages of growth, the levels of MyoD and Mos proteins were found to be higher in C2-Mos^{wt} transfectants than in control cells (Fig. 1B). These results reveal an increase in the level of MyoD protein expression when Mos is stably transfected in C2C12 myoblasts. In

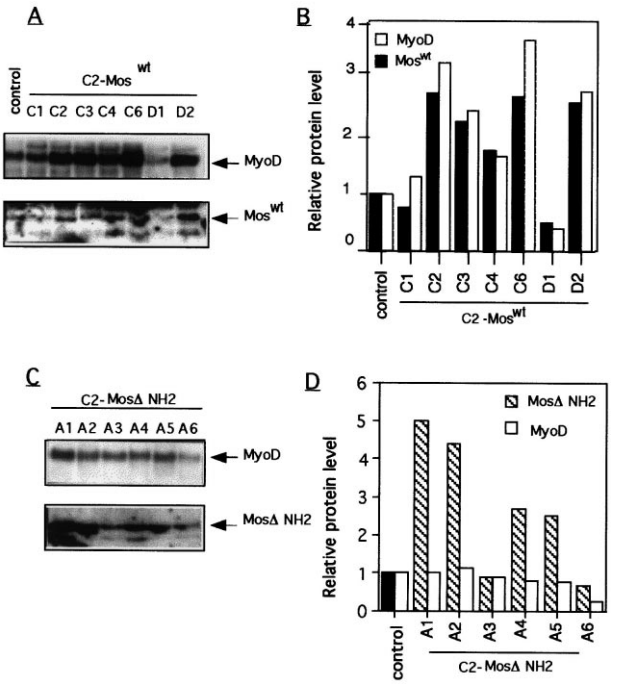

Fig. 1. Protein expression in Mos-transfected myoblasts C2C12. A: C2C12 cells were stably transfected with the empty vector (control), the complete coding c-mos^{rat} cDNA (C2-Mos^{wt}: clones C1–D2) or (C) with MosΔNH2 (C2-MosΔNH2, clones A1–A6), a Mos mutant with the NH2 domain deleted and maintained in DMEM supplemented with 20% FCS for 48 h. 50 μg of total cell extract was used for quantitative immunoblots probed with either the affinity-purified polyclonal anti-MyoD or the affinity-purified anti-Emos2 antibody [24] and developed using enhanced chemiluminescence detection. B, D: The signals were quantitated with a gel scan (Pharmacia).

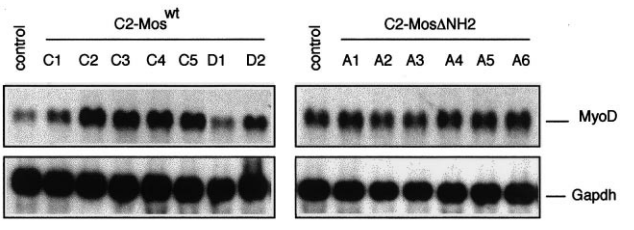

Fig. 2. MyoD mRNA expression in Mos-transfected myoblasts C2C12. Total RNA was prepared from stable transfectants as described in Fig. 1. 15 μg of each RNA sample was run on a 1% agarose-formaldehyde-MOPS gel, transferred to nitrocellulose filter and hybrized with a mouse MyoD probe. For MyoD, exposure time was 6 h. The blots were stripped and reprobed with a rat Gapdh probe and it was confirmed that approximately equal amounts of RNA were loaded. Exposure time was 18 h.

contrast, the level of MyoD protein expression was not affected when an inactive Mos protein with the NH2 domain deleted is stably expressed at high levels in the same cells (C2-MosΔNH2 transfectants) (Fig. 1C,D). Several hypotheses could explain these results: (I) Mos could enhance the stability of MyoD protein; (II) Mos acts at the transcriptional level to increase expression of MyoD gene; (III) Mos could act positively on the stability of MyoD mRNA.

3.2. Mos does not modify the stability of MyoD

To elucidate the mechanism by which the level of co-expressed MyoD is augmented by Mos, we tested whether Mos affected transcription or translation of the co-transfected MyoD. We first examined the effect of Mos expression on the stability of co-expressed MyoD in 10T1/2 cells. The metabolic stability of MyoD was investigated by a pulse-chase experiment. Immunoprecipitation analyses with a MyoD-specific polyclonal antiserum revealed that MyoD was unstable with a half-life of 50–60 min, in good agreement with its previously reported half-life of 30–60 min [13]. Surprisingly, MyoD when co-expressed with Mos^{wt} or kinase-inactive Mos^{KM} showed a half-life very similar to that observed for MyoD alone (data not shown). This result suggests that the much higher level of co-expressed MyoD compared to that of the singly expressed MyoD was not due to its metabolic stabilization by Mos expression. To determine whether Mos^{wt} protein can activate expression of the MyoD gene, we analyzed the expression of endogenous MyoD mRNA in C2C12 myoblasts that were either transfected by the empty vehicle (control), the Mos^{wt}

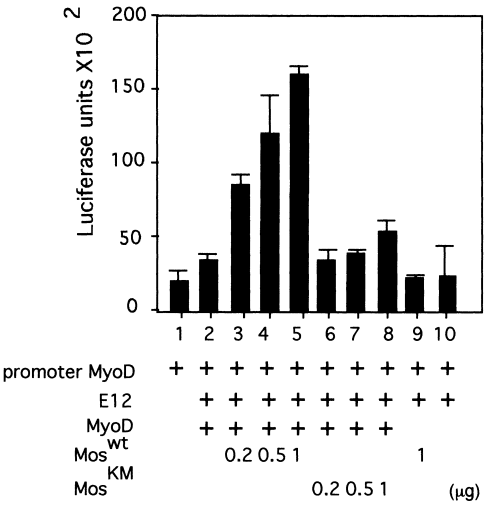

Fig. 3. Mos enhances MyoD transcriptional transactivation of its own promoter. 10T1/2 cells were co-transfected with 0.5 μg of a plasmid containing the *Hin*dIII/*Bsp*MI fragment of the mouse MyoD promoter cloned upstream of the luciferase reporter gene, 0.5 μg of pEMSV-E12, 0.5 μg of pCMVHa-MyoD (lane 2) together with pCMVHA-Mos^{WT} (lanes 3–5) or pCMVHA-Mos^{KM} (lanes 6–8). Forty-eight hours following transfection, cells maintained in DMEM supplemented with 15% FCS were harvested and luciferase activity was assayed in aliquots equivalent to 10 μg of whole cell extracts. Differences in transfection efficiencies were corrected based upon the level of β-galactosidase activity from the co-transfected pCH110 plasmid. Each bar represents the average of at least three independent tranfections.

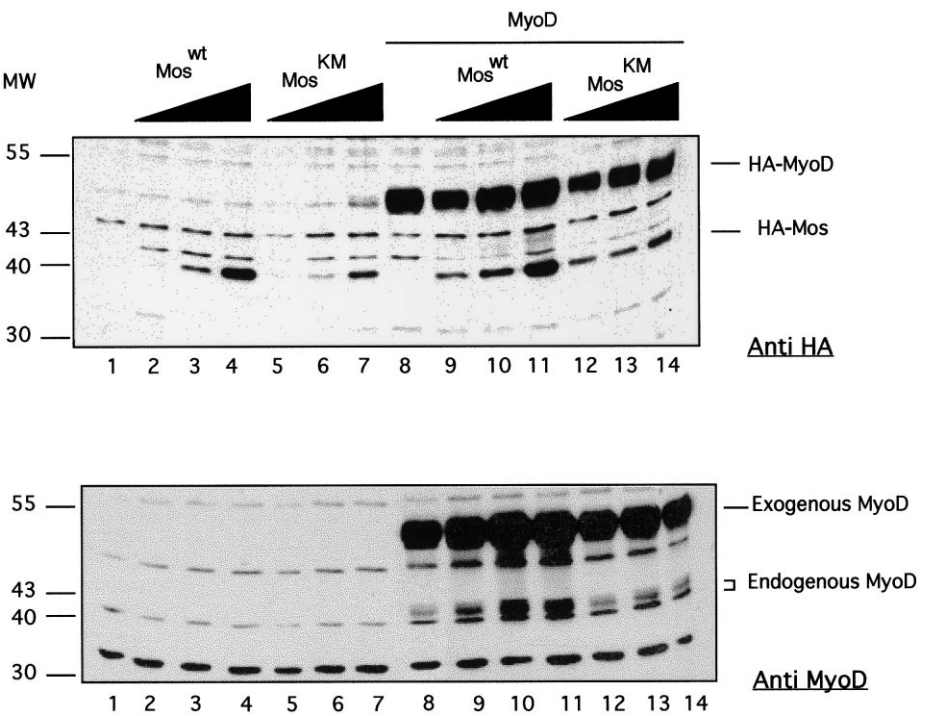

Fig. 4. Mos co-expressed with MyoD activates expression of the endogenous MyoD protein. 10T1/2 cells were co-transfected with 0.5 μg of pEMSV-E12 (lanes 1–14), 0.2, 0.5 and 1 μg of pCMVHA-Mos^{WT} (lanes 2–4 and 9–11) or pCMVHA-Mos^{KM} (lanes 5–7 and 12–14) alone or in combination with 0.5 μg of pCMVHa-MyoD (lanes 8–14). Forty-eight hours following transfection, cells maintained in DMEM supplemented with 15% FCS were harvested and Western blot analysis was performed. A: Ten micrograms of whole cell extracts was solubilized in SDS loading buffer. After SDS-PAGE, HA-tagged proteins were detected by immunoblotting with the 12CA5 monoclonal antibody. B: Fifty micrograms of the same whole extracts was used for SDS-PAGE. Proteins were detected by immunoblotting with the affinity-purified polyclonal anti-MyoD antibody.

expression vector (C2-Mos^{wt}) and/or the truncated Mos expression plasmid (C2-MosΔNH2). Fig. 2 shows that expression of MyoD mRNA in stably transfected cells differed significantly between co-expressed Mos^{wt} and the truncated MosΔNH2 mutant. Densitometric analysis of MyoD hybridization bands versus those of Gapdh, a housekeeping gene, showed that the C2-Mos^{wt} transfectants expressed higher quantities of MyoD mRNA than control C2C12 or C2-MosΔNH2 transfectants. These results suggest that Mos^{wt} affects an event at the transcriptional and/or post-transcriptional level of MyoD.

3.3. Mos enhances MyoD expression at the transcriptional level

Phosphorylation of MyoD by Mos^{wt} inhibits the DNA binding activity of MyoD homodimers, favors the binding of MyoD-E12 heterodimers and promotes MyoD transcriptional transactivation of the muscle creatine kinase promoter-enhancer [34]. We wondered if Mos could also enhance MyoD transcriptional transactivation of its own promoter which contains two proximal E-boxes known to be the targets of its autoregulation loop [14]. 10T1/2 cells were transfected with HA epitope-tagged MyoD and/or together with Mos^{wt} and/or the kinase-inactive Mos^{KM} expression vector and a luciferase reporter gene driven by the *Hin*dIII/*Bsp*MI fragment of the mouse MyoD promoter. As previously shown [14], in 10T1/2 cells, MyoD promoter is poorly activated by MyoD alone (Fig. 3, lane 2). On the other hand, when MyoD is co-expressed with Mos^{wt}, transactivation of the MyoD promoter was enhanced in a dose-dependent manner by Mos^{wt} (Fig. 3, lanes 3–5). At the highest level of Mos^{wt} tested, we observed a 6–8-fold increase in the expression of luciferase activity. In contrast, Mos^{KM} expression stimulated the MyoD promoter very poorly (Fig. 3, lanes 6–8). Finally, transcriptional stimulation was not observed in the presence of Mos^{wt} or E12 alone (Fig. 3, lanes 9 and 10). These results show that Mos^{wt} strongly enhances MyoD expression at the transcriptional level suggesting that Mos is not only able to increase the transcriptional activity of MyoD on muscular gene expression but is also able to enhance the capacity of MyoD to activate its own expression.

3.4. Co-expression of Mos and MyoD in 10T1/2 cells activates expression of endogenous MyoD protein

Stable ectopic expression of MyoD cDNA in 10T1/2 cells led to the endogenous expression of MyoD [13]. As Mos has the capacity to enhance transcriptional activity of MyoD on its own promoter, we decided to verify the effects of co-expressed Mos and MyoD on the expression of endogenous MyoD in 10T1/2 cells. Protein lysates identical to those used in the luciferase assays were analyzed by two Western blot experiments (Fig. 4A,B). Total cellular proteins were probed with the monoclonal antibody 12CA5 directed against HA-tagged MyoD and Mos proteins. The results showed that HA-MyoD is strongly expressed (Fig. 4, lanes 8–14) and that increasing amounts of HA-Mos^{wt} (lanes 2–4 and 9–11) and HA-Mos^{KM} (Fig. 4A, lanes 5–7 and 12–14) expression vectors are well correlated with the increasing amounts of corresponding tagged proteins. Moreover, as the exogenous MyoD is linked to a triplicate HA epitope, its molecular weight on SDS polyacrylamide gel is increased to 48 kDa instead of 43 kDa, the molecular weight of endogenous MyoD. This allowed us to distinguish between the exogenous and the endogenous forms of MyoD proteins as observed on a second Western blot probed with a MyoD polyclonal antibody. Fig. 4B shows that when Mos^{wt} or Mos^{KM} are expressed alone in 10T1/2 cells, no expression of endogenous MyoD is observed (Fig. 4A, lanes 2–7). This result confirms that Mos alone does not activate MyoD expression. When HA-MyoD is singly expressed in these cells, we detect a small expression of endogenous MyoD (Fig. 4B, lane 8). On the other hand, when HA-MyoD is co-expressed with increasing amounts of HA-Mos^{wt} expression vector, expression of endogenous MyoD protein is enhanced in a dose-dependent manner by Mos^{wt} (Fig. 4B, lanes 9–11). In contrast, the kinase-inactive HA-Mos^{KM} had no effect on the expression of endogenous MyoD protein. Expression of endogenous MyoD is unaffected and is identical to that observed with HA-MyoD transfected alone (Fig. 4B, lanes 12–14). Taken together, these results demonstrate that Mos increases the transcriptional activity of MyoD on its own promoter and increases the potentiality of MyoD to activate the expression of endogenous MyoD protein.

3.5. Mos activates MyoD DNA binding activity in 10T1/2 cells

To elucidate the mechanism by which the transcriptional activity of co-expressed MyoD is augmented by Mos, we examined the DNA binding activity of MyoD when expressed alone or in combination with Mos in 10T1/2 cells. Total protein extracts from transfected 10T1/2 cells were analyzed by Western blots with results similar to those shown in Fig. 4. Aliquots of total proteins were used in EMSA experiments

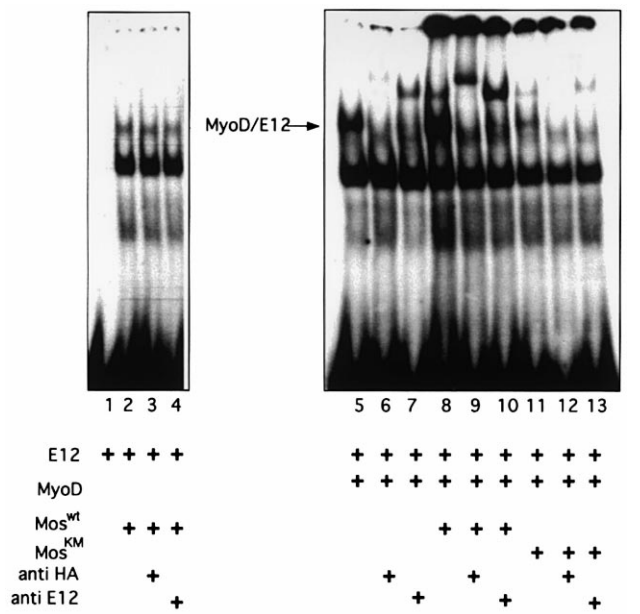

Fig. 5. The DNA binding activity of MyoD is increased in 10T1/2 cells when co-transfected with Mos. 10T1/2 cells were transiently transfected with 0.5 μg of pEMSV-E12 (lanes 1–13), 0.5 μg of pCMV-HA-MyoD (lanes 5–13), 1 μg of pCMV-HA-Mos^{WT} (lanes 2–4 and 8–10) and 1 μg of pCMV-HA-Mos^{KM} (lanes 11–13). Twenty micrograms of whole extracts was incubated with a ^{32}P-labeled E-box probe. HA antibody (anti HA) or E12 antibody (anti E12) was added as indicated, and the binding mixtures were separated by non-denaturing PAGE. MyoD/E12 complexes are indicated by an arrow. The extra band in lane 8 which migrates at the same level as the band supershifted by anti-E12 antibody (lane 7) is probably an artifact. In long running gels, its migration is slightly faster than the supershifted band observed with anti-E12 antibody and especially this retarded band is not currently observed.

with a ^{32}P-labeled MEF1 oligonucleotide. As expected, Mos does not bind to the E-box DNA probe (Fig. 5, lanes 2–5). Addition of anti-HA antibody in the binding mixture did not modify the non-specific protein-DNA complexes observed in all lanes. When MyoD is expressed ectopically in 10T1/2 cells, we detected a specific DNA binding complex constituted of MyoD/E12 heterodimers. When supershift experiments were performed with antibodies directed against HA epitope or E12 protein, both of these antibodies specifically supershifted this complex (lanes 5–7). Co-expression of Mos^{wt} with MyoD induced an important increase in the retarded bands corresponding to the DNA binding activity of MyoD/E12 heterodimers (compare lanes 5 and 8). Quantitation of the specific retarded bands showed a 3–5-fold increase in their intensity. In contrast, the kinase-inactive Mos^{KM} did not significantly affect the DNA binding activity of MyoD/E12 complexes (compare lanes 5 and 11). These data show that Mos kinase increases DNA binding activity of MyoD/E12 heterodimers in 10T1/2 cells.

In conclusion, together these data demonstrate that Mos can promote the formation of active MyoD/E12 heterodimers and increase the ability of MyoD to transactivate both muscle-specific genes [34] and its own promoter and could therefore participate in the positive autoregulation loop of MyoD and muscle differentiation. Given that the four b-HLH muscle factors identified thus far form a family of nuclear phosphoproteins themselves related to a larger family of regulatory factors, it is tempting to assume that Mos could play a part in their activation (Benayoun et al., submitted).

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