Biochemical and Biophysical Research Communications **265,** 222–232 (1999)
Article ID bbrc.1999.1390, available online at http://www.idealibrary.com on IDEAL®

Transcriptional Activation of the Myogenin Gene by MEF2-Mediated Recruitment of Myf5 Is Inhibited by Adenovirus E1A Protein

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Received August 16, 1999

The basic helix-loop-helix (bHLH) transcription factor myogenin plays a crucial role in terminal differentiation of committed myoblasts into mature myocytes. Transcriptional activation of the myogenin gene requires coordinate action of myocyte enhancer factor 2 (MEF2) proteins and the myogenic bHLH regulators, MyoD or Myf5. Here we show that transcription of the myogenin gene in differentiated cells correlates with MEF2 and NF1 binding to their cognate sites in the proximal myogenin promoter but not with binding of Myf5 or MyoD to the E-box. The importance of MEF2 activity was further demonstrated by expression of antisense MEF2 RNA which repressed MEF2 and Myf5-mediated MEF2 site-dependent reporter gene activation and the synergistic transactivation of a myogenin CAT reporter by Myf5 and MEF2. Adenovirus E1A which has previously been shown to specifically interfere with myogenin gene transcription also inhibited the cooperative transactivation by Myf5/MEF2 and MEF2. Consistently, coimmunoprecipitation studies revealed impaired MEF2/Myf5 protein-protein interactions. These results support a model of transcriptional activation and stabilization of myogenin expression in which DNA-bound MEF2 recruits myogenic bHLH factors into an active but E1A-sensitive transcription factor complex.

Regulation of skeletal muscle determination and differentiation in vertebrates involves two important families of transcription factors, the myogenic regulatory basic helix-loop-helix (bHLH) proteins MyoD, Myf5, myogenin and Myf-6/MRF4 (1–2) and the myocyte enhancer factor 2 (MEF2) group of MADS-box regulators (3). In a variety of nonmuscle cell types, each of the four myogenic bHLH proteins can activate the program for skeletal muscle differentiation when overexpressed (4–7). All established muscle cell lines express MyoD or Myf5 and activate transcription of the myogenin gene only when induced to differentiation. Functional analysis of the different myogenic factors in tissue cultures cells and gene disruption studies in mice led to the model that Myf5 and MyoD define the myoblast stage and myogenin initiates terminal differentiation. Thus, Myf5 and MyoD double knockout mice fail to activate the myogenin gene (8), while myogenin null mice are deficient in differentiated skeletal muscle, although fairly normal populations of myoblasts exist (9–13). Direct activation of the myogenin gene by MyoD and Myf5 in cultured myoblasts has been documented and supports the epistasis model of these genes (14, 15).

Muscle differentiation is not only associated with expression of myogenin but also with a rapid upregulation of myocyte enhancer factor 2 (MEF2) (16). The MEF2 genes (A to D) encode nuclear phosphoproteins which belong to the MADS (MCM1, Agamous, Deficiens, Serum Response Factor) family of DNA-binding proteins (3). The four mef2 gene products share about 85% amino acid identity within the MADS domain and an adjacent 27 amino acid spanning region referred to as MEF2 domain (17). This homology is also seen in the single D-MEF protein in *Drosophila melanogaster* (18–20). Early lethality of MEF2C knock-out mice with severe cardiac defects and the functional redundancy of the four mouse MEF2 genes so far prevented to document the role of MEF2 in skeletal muscle devel-

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0006-291X/99 $30.00

opment (21). Several lines of genetic and biochemical evidence suggest that skeletal muscle differentiation is dependent on the presence and function of MEF2 proteins. During embryogenesis, MEF2 transcripts are expressed in precursor cells of the cardiac and skeletal muscle lineages and are subsequently upregulated when these cells differentiate (22–26). Functional inactivation of D-MEF protein gives rise to correct spatio-temporal development of myoblasts, but, myoblast differentiation is inhibited (27–29). However, the contribution of MEF2 protein to activate the myogenic program is still controversial. MEF2A protein was shown to convert 10T1/2 fibroblasts to the muscle phenotype similar to the myogenic bHLH proteins (30). In contrast, it was demonstrated that MEF2 proteins lack myogenic activity but rather augment the myogenic activity of bHLH proteins (31). The cooperation is based on physical interactions of MEF2 factors with heterodimers of myogenic bHLH factors and E-proteins (31–32).

Activation of many muscle specific genes including the myogenin gene appears to be dependent on the DNA consensus sequence CANNTG, referred to as E-box, which constitutes the binding site for bHLH transcription factors. In addition, the consensus sequence [(C/T)TA $(A/T)_4$TA G] for MEF2 protein binding is required (32–36). Interestingly, the MEF2 binding site but not the E-box appears to be necessary for MyoD or Myf5 mediated transcriptional activation of the myogenin promoter in tissue culture (15) although, as we show here, MEF2 transcription factors alone do not confer transactivation of the myogenin gene. Faced with this paradoxon, our purpose was to determine whether and how MEF2 activity is required for Myf5 or MyoD regulated myogenin expression.

To approach this question, we analyzed the protein interactions taking place on the myogenin promoter by *in vivo* footprinting. Secondly, we constructed an antisense MEF2 expression vector, which, when coexpressed with Myf5 in 10T1/2 fibroblasts would abrogate the function of endogenous MEF2 and thus, indicating its indispensibilty for Myf5 activity. Given that adenovirus E1A protein specifically inhibits myogenin gene expression and the transactivating function of Myf5 (37) without *de novo* protein (38), we used E1A as a further tool to dissect the myogenic bHLH/MEF2 interactions. Here we show that, though Myf5 acts as a transcriptional activator of the myogenin promoter *in vivo*, transcriptional activation does not take place through E-box binding. Conversely, MEF2 alone does not activate myogenin expression, though MEF2-DNA interaction is indicated by hypermethylation of its respective binding site in the proximal promoter region. We further show that expression of antisense MEF2 RNA interferes with MEF2 and with Myf5 mediated transactivation of a myogenin reporter gene and of an artificial MEF2 dependent reporter gene, respectively. According to these data, MEF2 protein recruits Myf5 to the MEF2 site and thus generates an active transcription factor unit for myogenin gene expression. The physical interaction of MEF2 with the myogenic bHLH factor/E-protein was shown to be impaired by the E1A onco-protein.

MATERIALS AND METHODS

Cell culture and transfections. Mouse C2C12 myoblasts, rat L6 cells, and 10T1/2 fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). To induce differentiation, 10T1/2 cells and the muscle cell lines were grown in DMEM supplemented with 2% and 10% horse serum, respectively. MM14 myoblasts (39) were cultured on gelatine coated tissue culture dishes in Ham's F10 medium containing 15% donor horse medium and 2 ng/ml–8 ng/ml FGF-2 depending on the cell density. FGF-2 was added every 12 hr. Differentiation was induced in Ham's F10 medium containing 2% donor horse medium and 1 μM insulin for 24 to 48 hr. Transfections were performed using calcium phosphate precipitation as described previously (40). In transient transfection assays, cells were shifted to differentiation medium 24 hr after transfection and cultured for additional 24 hr. Chloramphenicol acetyltransferase (CAT) and β-galactosidase activities were determined in cell extracts according to standard procedures (41). CAT activities were standardized by cotransfection of RSV βGal plasmid (5 μg).

Plasmid constructs. The pEMSV vectors expressing MyoD and Myf5 and the Gal4-VP16 chimeric gene were described previously (37, 40). The full-length chicken MEF2A cDNA was isolated by screening an embryonic (E12) chicken heart library and cloning into the EcoRI site of the pEMSV expression vector (42) and into pBluescript (43). As reporter constructs in transient transfections we used −211 Myf4CAT (myogenin-CAT) containing 211 bp proximal promoter region of the Myf4 gene (15), pE102MEF 2x2 CAT (23), and pG5E1b CAT (44). Expression vectors encoding MEF2C wild-type, MEF2C-VP16, MEF2C-1-117, E12bHLH, MyobHLH, and pEMSV-E12Δ were kindly provided by E. Olson (31). Ad5E1A plasmids were published previously (38). The pcDNAI/Amp expression vector for antisense MEF2C was obtained by replacing the EcoRI/XbaI MEF2C fragment encoding aa 1-117 (31) with a reverse orientated 1.4 kb encompassing MEF2C HindIII/EcoRI cDNA fragment derived from the MEF2C full length cDNA cloned into pcDNAI/Amp vector (31). Plasmids pT7myf4, pBS-ATG-E12, and pT7E2.5 used for immunoprecipitation were described previously (37). pT7E1A was constructed by cloning a HincII PCR fragment encoding 12S E1A sequences (aa 2 to 249) into the HincII linearized pT7βSal. The plasmid pcDNAI/Amp MEF2A1-118 was obtained by generating an EcoR/XbaI MEF2A PCR fragment encoding aa 1-118 of the chicken MEF2A cDNA which was then cloned into the EcoRI/XbaI sites of the pcDNAI/Amp vector (Invitrogen).

Reverse transcriptase-PCR analysis. Total RNA was extracted by the guanidinium thiocyanate method from tissue culture cells. 2 μg of total RNA pretreated with 1.5U RQ1 DNase (Promega) was transcribed using 0.5 μg oligo p(dT) primer and 15U AMV reverse transcriptase for 1 hr at 42°C in the presence of 50 mM Tris–HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM DTT, 1 mM dNTPs, and 25U RNase inhibitor in a total volume of 25 μl. Reactions were stopped at 100°C for 2 minutes and diluted with 75 μl water. One tenth of the cDNA reaction mix was subjected to the polymerase chain reaction (PCR) in a final volume of 50 μl containing 50 pMol of each primer, 0.2 mM dNTP and 2.5 U Taq polymerase. Oligonucleotide sequences used in PCRs were as follows: 5′ MEF2C: 5′-CATGCCGCCATCTGCCCTCAG-3′; 3′ MEF2C: 5′-CCCTTTCG-TCCGGCGAAGGTC-3′; 5′ ribosomal L6 protein: 5′-GAAGCTCATC-TATGAGAAGGC-3′; 3′ ribosomal L6 protein: 5′-AAGACGAAGG-

AGCTGCAGAAC-3′. The cDNAs were amplified in 30 cycles, and one half of the reaction mix was separated on a 1.8% agarose gel. The size of PCR fragments was determined according to DNA standard markers (Boehringer-Mannheim).

Immunocytochemical staining. To determine E1A and MEF2 expression, transfected 10T1/2 cells were fixed in 100% ice-cold methanol, washed extensively with phosphate-buffered saline (PBS), incubated in PBS containing 1% bovine serum albumin and 3% horse serum for 30 min at room temperature, followed by washing in PBS. Co-localization of E1A and MEF2 protein was performed with the mouse monoclonal M73 antibody (45) and rabbit polyclonal MEF2 antiserum (Santa Cruz), respectively. As secondary antibodies, TRITC-conjugated goat anti mouse IgG and Flourescein-conjugated sheep anti rabbit IgG were used. Immunostaining for E1A protein in rat L6 cells was performed as described above except that bound primary antibody was visualized using the Vectastain ABC kit.

Methylation protection experiments. C2C12 and MM14 cells were incubated in DMEM and Ham's F10 medium containing 0.04% dimethyl sulfate (DMS), respectively, for 2 min at 37°C. Cell culture dishes were washed once in medium and three times in phosphate buffered saline (PBS). Prior to isolation of the *in vivo* methylated DNA from C2C12 and MM14 mytube cultures, cells were trypsinized and resuspended in 15 ml PBS. 30 ml of a Percoll stock solution (30% of a stock solution containing 90% Percoll and 10% 10× PBS) containing 0.1 g/l phenol red was covered with the cell suspension and centrifuged at 400× g for 20 min. Purified myotubes (>95%) were isolated from the interphase, diluted in 50 ml PBS, and centrifuged again for 5 min. Following resuspension in 5 ml PBS, genomic DNA was isolated. An aliquot of the cells was reseeded, cultured as described above, and inspected for the presence of myoblasts. Control DNA (50 μg genomic DNA diluted in 200 μl Tris-EDTA) was methylated by addition of 1 μl DMS, either undiluted, diluted 1:3, 1:10 or 1:20. After 20 sec the reaction was stopped by adding 50 μl of a stop solution containing 1.5 M sodium acetate, 1 M β-mercaptoethanol, and 100 μg/ml yeast tRNA. Genomic DNA was chemically cleaved with 10% (v/v) piperidine at 95°C for 30 min, followed by a four-fold ethanol precipitation, yielding single stranded DNA fragments which ranged in a size of 400 to 2000 bases.

Ligation mediated PCR (LM-PCR) was carried out as described (46) with some minor modifications. PCR and primer extention reactions were performed using Vent exo$^-$ DNA polymerase (New England Biolabs). The linker primer was generated by annealing the oligonucleotides 5′-GCGGTGACCCGGGAGATCTGAATTC-3′ and 5′-GAATTCAGATC-3′ in 250 mM Tris–HCl, pH 7.7 at a final concentration of 20 μM. Oligonucleotide sequences used for extension and amplification reactions were as follows: "Coding 1": 5′-TAGAAGTGGGGCTCCTG-3′, "Coding 2": 5′-ATGTCTCATACAGCTCCATCAGGTCGG-3′, and "Coding 3": 5′-CCATCAGGTCGGAAAAGGCTTGTTCCTGCC-3′. Primers used for the non-coding strand were "Non-Coding 1": 5′-AATCAAATTACAGCCGACG-3′, "Non-Coding 2": 5′-AGGGAAGGGGAATCACATGTAATCCAC), "Non-Coding 3": 5′-AGGGAAGGGGAATCACATGTAATCCACTGG-3′. A promotor-exon 1 fragment (basepairs −318 to +115) of the mouse myogenin gene was generated by PCR and sequenced in order to adjust methylated guanine residues with the authentic guanine bands. Therefore, 200 ng of genomic DNA of NIH3T3 fibroblasts was amplified using the oligonucleotides 5′-AGGAGCAGATGAGACGGGGGAATG-3′ and 5′-GGTAGTTTTCCCCATCATAGAAGTGG-3′ and cloned into EcoRV sites of pKS II+ (Stratagene).

In vitro translation and coimmunoprecipitation. Protein interactions between MEF2A, myogenin, E12/E2.5, and E1A were analyzed by immunoprecipitation of the *in vitro* translation products MEF2A1-118, myogenin, E12/E2.5, and E1A. Proteins were derived from *in vitro* transcription of their corresponding linearized plasmid templates pcDNAI/Amp chickMEF2A-1-118 (aa 1-118), pT7myf4, pBS-ATG-E12, pT7E2.5 and pT7E1A and following *in vitro* translation using rabbit reticulocyte lysate (Promega) as suggested by the supplier. Equal volumes of translated proteins were incubated in 200 μl EBC (50 mM Tris–HCl, pH 8.0, 180 mM NaCl, 0.1% NP-40) containing 10 μg/ml protease inhibitors aprotinin, leupeptin, PMSF and 25 μl of a 1:1 slurry of protein A-Sepharose (Pharmacia) in 5% BSA for 30 min at 4°C. Following preclearing, the supernatant was incubated on a rocker for 1 hr at 4°C with 20 μl of mouse myogenin monoclonal antibody (47). Immunocomplexes were collected by adding 25 μl of protein A-Sepharose beads and further rocking for 30 min. Finally, the beads were washed five times in NET-N (10 mM Tris–HCl, pH 8.0, 250 mM NaCl/500 mM NaCl, 1 mM EDTA, 0.5% NP-40) and, after boiling in SDS-sample buffer (2% SDS, 10% glycerol, 62 mM Tris–HCl at pH 6.8, 1% β-mercaptoethanol), were analyzed on SDS–polyacrylamide gels.

RESULTS AND DISCUSSION

Active transcription of the myogenin gene in differentiated myotubes correlates with footprints corresponding to sites that can bind MEF2 and NF-1. We have previously shown that 96 bp of the promoter-proximal region of the Myf4 gene, the human myogenin homologue, encompassing a MEF2 site and an E-box suffices to direct muscle-specific gene expression. The presence of the MEF2 site but no Myf5 or MyoD-DNA interactions appears to be relevant for the active transcription of the Myf4 gene in tissue culture cells (15).

To define the *in vivo cis*-acting DNA sequence elements and the status of transcription factors participating in this regulatory process in more detail, we examined the DNA-protein interactions at the human Myf4 promoter in myoblasts and in differentiated myotubes by methylation protection experiments. Mouse C2C12 and MM14 muscle cells were incubated with dimethyl sulfate (DMS) and the myotube containing cultures were sorted by density gradient centrifugation in order to obtain >95% enriched myotube populations. After chemical cleavage of the genomic DNA with piperidine the coding and non-coding DNA strand of the resulting DNA-fragments were analyzed by linker mediated PCR. In differentiated myotubes two guanine residues neighbouring the MEF2 binding sequence of the coding strand of the Myf4 promoter region displayed hypermethylation (Fig. 1, coding strand, lanes +; left, footprint derived from 75% myogenin positive cells, middle and right, footprints derived from 100% myogenin positive cells) compared to DNA of undifferentiated cells (Fig. 1, coding strand, lanes −; 0% differentiated cells) and to control DNA which was methylated *in vitro* (Fig. 1, coding strand, lanes c). In addition, three guanine residues within the putative binding sequence of nuclear factor-1 (NF-1) transcription factor were partially protected against DMS methylation (Fig. 1, coding strand, lanes +). At position −43/44 in the non-coding strand of the myogenin promoter we found two guanine residues being almost completely protected against methylation suggesting NF-1 binding in differentiated but not in undifferentiated muscle cells (Fig. 1, non-coding strand, lanes +). Methylation specific effects within the E-box motif comprising nucleotides CAGTTG due to binding of endogenous Myf5 or MyoD was not

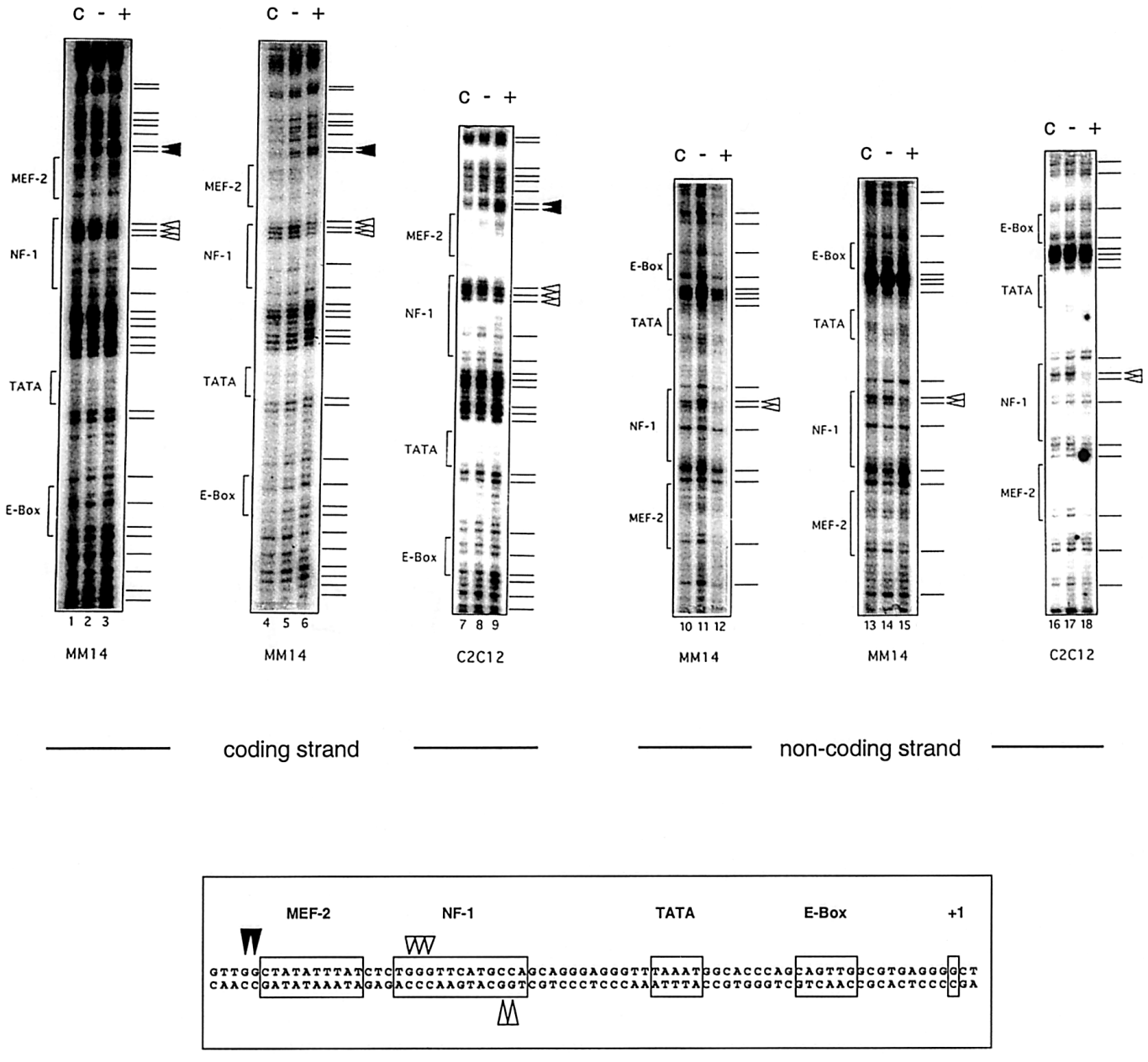

FIG. 1. Methylation protection analysis of the coding and non-coding strand of the myogenin promoter. Genomic DNA of DMS-treated mouse MM14 and C2C12 myoblasts (lanes −; 0% myogenin positive nuclei), of myotubes (lanes +; left 75%, middle and right 100% myogenin positive cells), and of *in vitro* methylated genomic DNA (lanes c) was analyzed by ligation mediated PCR. One representative autoradiogram derived out of three independent experiments is shown. Horizontal lines indicate the positions of guanine residues determined by sequencing. Open triangles denote protected guanines, and closed triangles point to hypermethylated guanines. Positions of transcription factor binding sequences are indicated. Below, the nucleotide sequence of the myogenin promoter region is given. Binding positions for the indicated transcription factors are marked by open boxes. Open and closed arrows summarize protected and hypermethylated guanines in differentiated myotubes, respectively.

observed, neither in myotubes nor in myoblasts (Fig. 1, lanes + and −). According to these results, binding of ubiquitously expressed transcription factors, MEF2 and NF-1, to the promoter-proximal region of the myogenin gene characterizes the active myogenin gene in differentiated muscle cells. Myogenic bHLH transcription factor binding to the E-box *in vivo* as reported for the MCK enhancer (48) and for a variety of muscle structural genes *in vitro* (2) however, seems to be absent or to be a short-lived interim step during the activation process of the myogenin gene, which then will be barely detectable *in vivo*.

Comparably to the footprint results, a myogenin promoter-LacZ transgene in mice lacking the proximal

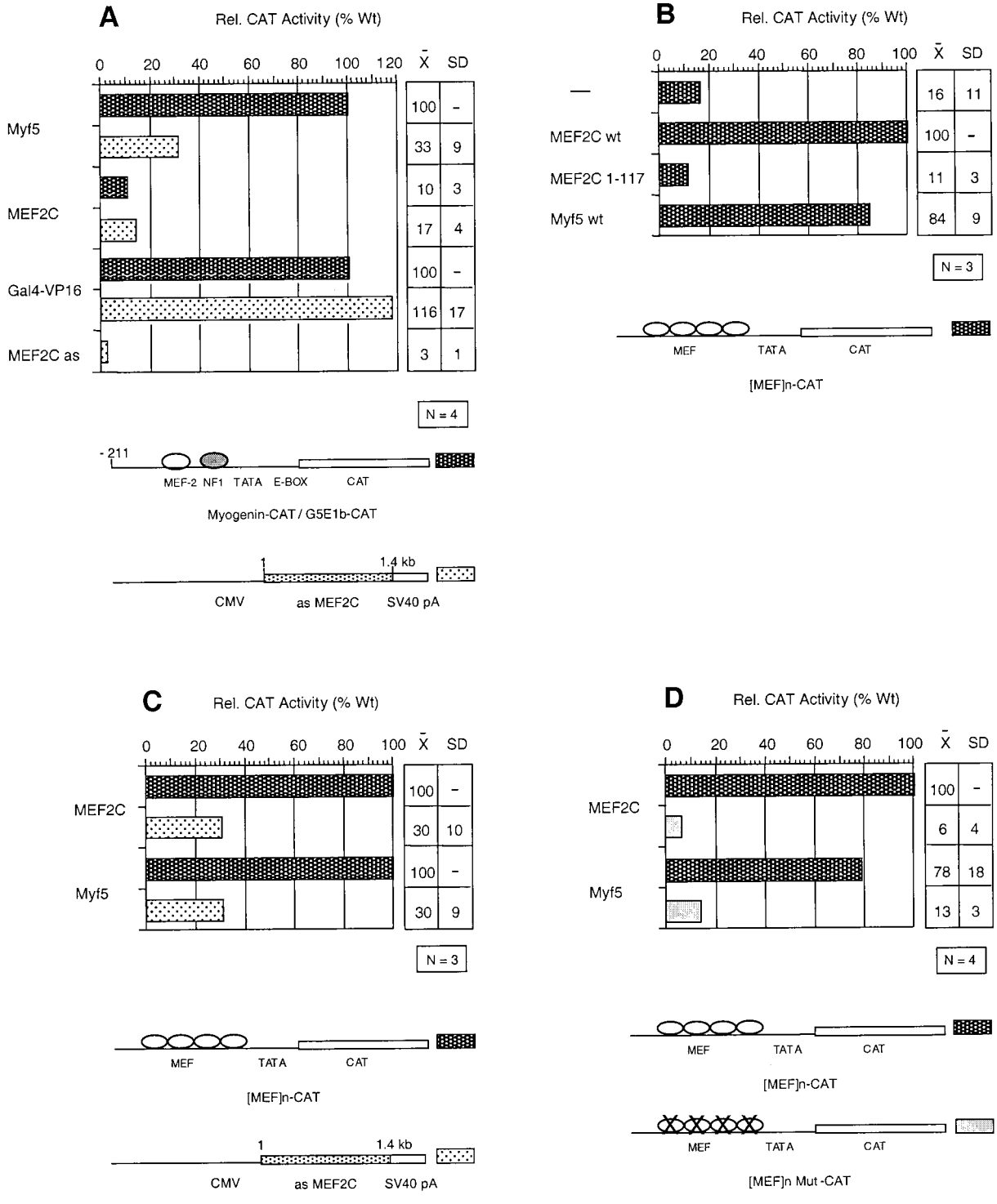

FIG. 2. Overexpression of a vector encoding antisense MEF2 RNA inhibits Myf5-mediated transactivation of a −211 bp myogenin promoter CAT construct and the MEF2-dependent reporter gene pE102MEF2x2CAT. (A) The −211 bp myogenin promoter CAT construct (4 μg), RSV β-gal plasmid (4 μg), and pEMSV Myf5 (lanes Myf5) or MEF2C (lanes MEF2C) expression vector (1 μg) were transfected into 10T1/2 cells either along with 10 μg of an expression vector encoding antisense MEF2 RNA (bright stippled bars) or along with 10 μg of an empty vector (dark stippled bars). As controls, transfections were performed with the expression vector encoding antisense MEF2C alone (lane MEF2C as), and with Gal4-VP16 (1 μg) transcribing the Gal4-dependent reporter gen pG5E1b-CAT (4 μg) (lanes Gal4-VP16). (B) MEF2C and Myf5 activate transcription of the MEF2-dependent reporter gene pE102MEF2x2CAT (MEFn-CAT). As indicated, 10T1/2 cells were transiently transfected with the reporter construct MEFn-CAT, RSV β-gal plasmid, along with the activator plasmids pEMSV-Myf5, or MEF2C, or the MEF2C deletion mutant 1-117 lacking the C-terminal activation domain. Of each of the plasmids 6 μg were transfected. (C) Expression vectors for MEF2C or Myf5 were cotransfected with the MEF2 reporter plasmid, the construct encoding antisense MEF2 RNA

E-box is properly expressed in somites and shows a delayed, nevertheless cell type specific activation in limb buds and visceral arches (49). In the absence of the MEF2 site however, transgene expression in the limb buds and dorsal regions of the somites is lost. In the ventral regions of the somites myogenin expression is not dependent on the presence of the MEF2 site (24, 49). An interpretation of these data could be that different myotomal cell lineages exist with different needs for stable Myf5/MyoD and MEF2 binding to the myogenin promoter (13). Although inspection of the nucleotide sequence of the myogenin promoter reveals only three basepairs spacing between the MEF2 and NF-1 binding site, a function of NF-1 in myogenesis has not been analyzed yet. Parallel binding of both factors to their binding sites provides circumstantial evidence for a physical interaction of MEF2 and NF1. Alternatively, NF1 and the Myf factor which is essential for myogenin gene transcription (8) could interact although, the E-box binding protein does not contact the DNA. Indeed, transcription factor complexes containing Myf protein and NF1 could be demonstrated (47) which should lend to a further analysis of the combinatorial activity of muscle restricted proteins and more widely expressed transcription factors in myogenesis.

Transactivation of the myogenin depends on MEF2-mediated recruitment of Myf5 into an active transcription factor complex. In the light of the experiments described above and of those done previously, showing that Myf5 and MyoD directly transactivate the myogenin gene without apparent binding to the promoter (15, 50), we reasoned that MEF2 might bind to its cognate binding sequence and then recruits Myf5 into an active muscle-specific transcription factor complex thus initiating or stabilizing gene transcription. Transient transfections of either Myf5, chicken MEF2A (43), or cotransfection of both along with a −211 Myf4-CAT reporter encompassing a MEF2 and an E-box binding site into 10T1/2 fibroblasts confirmed that MEF2 cannot activate the myogenin promoter by itself and that coexpression of Myf5 and MEF2 jointly elevates transactivation of the myogenin gene compared to the activation by Myf5 alone (Fig. 3A). To find out as to whether MEF2 which is present in 10T1/2 fibroblasts (31) is needed for Myf5 mediated transcriptional activation of the myogenin gene, we cotransfected expression plasmids for Myf5 and antisense MEF2C into 10T1/2 cells, thus attempting to circumvent endogenous MEF2 activity. As shown in Fig. 2A, transactivation of the −211 bp myogenin CAT reporter by Myf5 was significantly repressed in the presence of an expression vector encoding 1.4 kb full-length MEF2C antisenseRNA (compare dark and light spotted bars). The specificity of myogenin promoter repression was controlled in transfections using the chimeric Gal4-VP16 transcription factor. Gal4-VP16 mediated transactivation of the Gal4 dependent reporter gene pG5E1b-CAT reporter was not inhibited in the presence of antisense MEF2C RNA (Fig. 2A). Transactivation of the myogenin reporter stimulated by expression of MEF2C, antisense MEF2C or both together revealed only background CAT activities (Fig. 2A). To show that Myf5 activity does not depend on E-box binding but rather on an interaction with DNA-bound MEF2, we tested whether Myf5 could activate transcription of a MEF2 dependent reporter in the presence or absence of MEF2 protein. Comparably to MEF2 wild-type protein, Myf5 was able to transcriptionally activate the reporter pE102MEF2x2CAT (MEFn-CAT) which contains two tandem copies of the MEF2 site of the MCK enhancer upstream of the embryonic MHC basal promoter but no functional DNA-binding site for myogenic bHLH proteins (Fig. 2B). As shown previously, truncated MEF2-1-117 protein (aa 1-117) missing the C-terminal transactivator domain did not to transactivate the MEFn-CAT reporter (Fig. 2B; 31). Setting up the same experiment with the pE102MEF2x2CAT reporter which contains a single mutation within the MEF2 site (MEFnMut-CAT) abrogates both, MEF2 and Myf5 mediated transactivation (Fig. 2D), and thus proves that Myf5 does not operate via artificial E-box like motifs within the reporter gene. Consistenly, cotransfection of antisense MEF2 led to a 3-fold repression of MEF2 and Myf5 mediated transactivation of the MEFn-CAT reporter (Fig. 2C). Expression of the vector encoding antisense MEF2C did not completely block transactivation of the reporter genes, but this is reasonable since functional active MEF2 protein is already present at the point of transfection (15). However, the MyoD induced expression of MEF2 DNA-binding activity (51, 52) should be repressed by anti-sence MEF2C molecules and lead to the observed decrease of CAT activities.

According to these data, Myf5 relies on MEF2 protein in order to form an activation complex which then directly initiates myogenin gene transcription presumably via MEF2-DNA interaction. Arguing from this point of view, it is MEF2 which requires Myf5 or MyoD

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or empty vector as described under A. (D) Transient transfection assay as described in B. Transactivation of the MEF2-dependent wild-type reporter (dark stippled bars) was compared to transactivation of the pE102MEF2x2 CAT (MEFn Mut-CAT) reporter containing single mutations within the MEF2 site abrogating MEF2 binding (bright stippled bars). The expression vector encoding antisense MEF2 RNA, the myogenin promoter, and MEF2-dependent reporter constructs are schematically outlined. CAT conversion was normalized for β-gal activity. The results are expressed relative to the activity of Myf5 or MEF2 (100% values). Means ($\bar{X}$) and ± standard deviation (SD) of the indicated number of transfections (N) are given right to the panels.

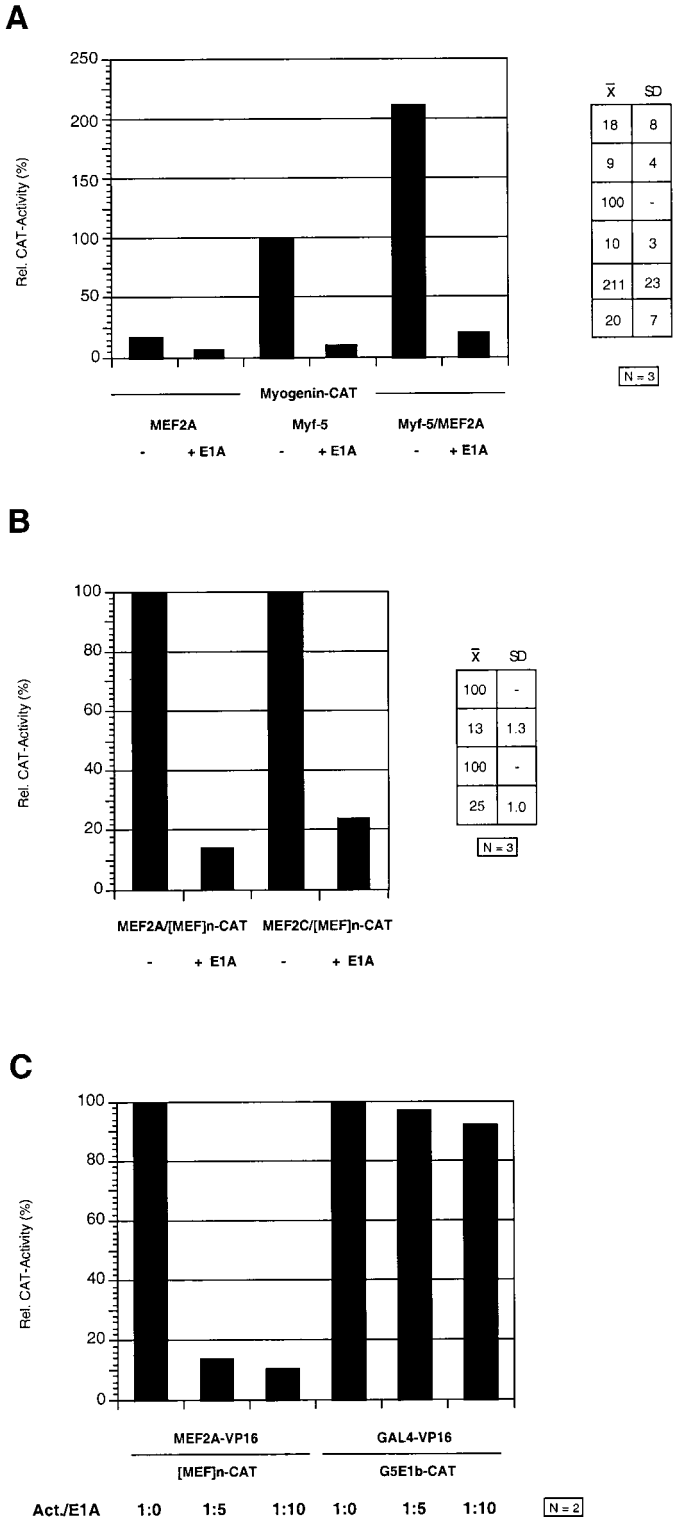

FIG. 3. Myf5 and MEF2 mediated synergistic transactivation of the −211 bp Myogenin promoter CAT construct (Myogenin-CAT) and MEF2/MEF2-VP16-mediated activation of the MEF2-dependent reporter gene pE102MEF2x2CAT (MEFn-CAT) is inhibited by E1A. (A) 10T1/2 were transiently transfected with the MEFn-CAT reporter (5 μg), RSV β-gal plasmid (5 μg), and with either pEMSV-MEF2A, pEMSV-Myf5 (5 μg), or with both (2.5 μg of each activator). When indicated, 5 μg of an expression vector encoding 13S E1A was cotransfected. The results are expressed relative to the activity of Myf5 (100% value). Means ($\bar{X}$) and ± standard deviation (SD) of the number of transfections (N) are given. (B and C) The transactivator plasmids (1 μg) MEF2A, MEF2C, and MEF2-VP16 were cotransfected along with the reporter plasmid MEFn-CAT (5 μg). To determine the specificity of E1A inhibition Gal4-VP16 (1 μg) transcribing the pG5E1b-CAT reporter plasmid (5 μg) was transfected in a parallel assay. Vector expressing 13SE1A was cotransfected in a 1:5 ratio compared to the activator (B) or as indicated in C. The results are expressed relative to the activity of MEF2 and chimeric MEF2-VP16 and Gal4-VP16 proteins (100% values).

as a critical coregulator for myogenin gene expression. In this context, it is further interesting to mention that MEF2C and myogenin gene expression coincide in the developing myotome at embryonic day 8.5 (E8.5) whereas Myf5 is already present a few hours before at embryonic day 8 (E8) (53). Further striking evidence for an initiative role of MEF2 activity in myogenin gene expression has come from several studies. Ornatsky *et al.* (1997) showed that the amino terminus of MEF2A (amino acids 1-131) acts as a DNA binding trans-dominant inhibitor of MEF2 and thus, impairs on myogenin expression in Myf5 and MyoD expressing L6 and C2C12 muscle cells, respectively. Consistenly, MyoD induced myogenic conversion of 10T1/2 was also inhibited in the presence of trans-dominant MEF2 protein (54) and the MyoD homolog Nautilus (55) was shown to be incapable of transactivating muscle structural genes in mef2 mutant Drosophila embryos (27–29). Although artificial, multimerized MEF2 and E-box reporter were used in *in vitro* transfection assays to demonstrate a physical interaction of MEF2 factors with heterodimers formed between myogenic bHLH and E proteins permitting either factor to activate transcription through the DNA binding site of the other (31, 56), this provides a likely mechanistic explanation for Myf5 mediated transactivation of the MEF2 reporter as well as for MEF2 protein dependent (present study) but E-box independent activation of muscle specific 15, 50, 57).

E1A abrogates MEF2 activity and interaction between MEF2 and myogenic bHLH proteins. We have previously shown that the expression of the myogenin gene and transactivating function of Myf5 is specifically inhibited by constitutive expression of the E1A onco-protein (37). As our results here indicate that Myf5 requires MEF2 as a DNA-binding partner for transcriptional activation of the myogenin gene, we asked whether E1A might impair on MEF2 function.

As shown in Fig. 3A, transactivation of the −211 myogenin-CAT reporter exerted by pEMSV-Myf5 alone or in conjunction with pEMSV-MEF2A which resulted in an enhanced CAT conversion was repressed in the presence of an expression vector encoding 13SE1A when equal amounts of E1A and MEF2/Myf5 (ratio of inhibitor:activators = 1:1) were transfected.

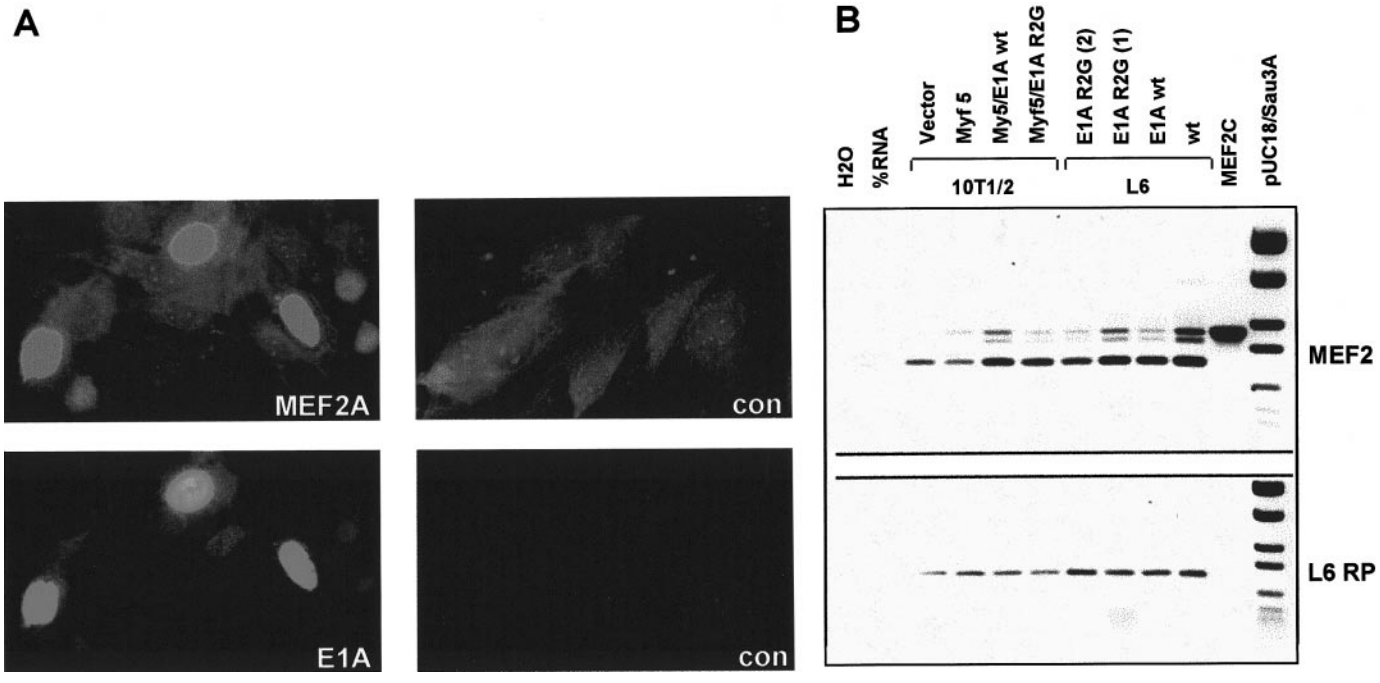

FIG. 4. E1A protein does not interfere with MEF2 transcription, protein expression, and translocation to the nucleus. (A) Coimmunofluorescence staining of MEF2 and E1A protein in 10T1/2 cells which were transiently transfected with 10 μg pEMSV-MEF2A and 10 μg vector encoding 13S E1A. Magnification $\times$400. Non-immune controls were negative in all cases (con). One representative experiment is shown. (B) RT-PCR analysis of endogenous MEF2 gene transcription in 10T1/2 cells transiently transfected with pEMSV-Myf5 (10 μg) and cotransfected with 13SE1A wild-type (wt) or E1A mutant R2G lacking p300 binding (15 μg) as well as in L6 cells stably expressing E1A wild-type or mutant R2G (clones 1 and 2). RT-PCR for L6 ribosomal protein served as the gel-loading control. Control transfections with 10 ng plasmid MEF2C, pEMSV-αscribe (V, 10 μg), PCR with water (H_2O), and RT-PCR without RNA (%RNA) are shown.

MEF2A and MEF2C were equally capable of activating the MEFn-CAT reporter but failed to do so in the presence of E1A (Fig. 3B). E1A repression was also observed for the activity of a chimeric MEF2-VP16 protein encoding amino acids 1-117 of MEF2C fused to the E1A insensitive VP16 activation domain (37). Transactivation of the pG5E1b-CAT reporter by Gal4-VP16 protein was not affected by E1A (Fig. 3C). As determined so far, MEF2 and in particular, the N-terminus of MEF2 protein mediating DNA-binding and heterodimerization domain constitutes a specific target for E1A repression.

To ascertain that E1A does not impair on the transcriptional activation of endogenous MEF2 genes, RT-PCR was performed on total RNA from 10T1/2 cells transiently transfected with Myf5 alone, or together with wild-type E1A or mutated E1A, lacking p300 binding activity. The assay was also performed on three different rat L6 cell clones, stably expressing E1A wild-type or the mutated E1A protein (data not shown). In both cell types, 10T1/2 fibroblasts and rat L6 cells, transcriptional activation of endogenous MEF2 was observed independent of whether Myf5 or E1A protein was present (Fig. 4B, top). The same results were seen using an E1A mutant deficient in binding to pocket proteins (data not shown). L6 ribosomal protein (L6RP) cDNA was amplified to control the integrity of RNA samples (Fig. 4B, bottom). Furthermore, immunocytochemical co-localization of transiently transfected MEF2A and E1A into 10T1/2 cells proved that the MEF2 protein expression and its translocation to the nucleus is unaffected by E1A (Fig. 4A).

To further determine whether E1A specifically disturbs an interaction between the myogenic bHLH domain and the MEF2 transcription factor we used the GAL4DBD-dependent reporter system, in which the bHLH region of E12 and myogenin, referred to as E12bHLH and myobHLH, were fused to the DNA binding domain (DBD) of the yeast GAL4 transcription factor (31). When intoduced into 10T1/2 cells solely, the chimeric proteins failed to activate expression of the Gal4 dependent reporter pG5E1b-CAT (Fig. 5, lanes 1 and 2). Coexpression of Gal4DBD-myobHLH fusion protein and E12, however resulted in a $\sim$8-fold CAT activation of the Gal4 reporter above background (Fig. 5, lane 4). Consistent with the previously described synergism of MEF2 and MyobHLH factors (31) and comparably to the data shown in Fig. 3A, the Gal4DBD-myobHLH/E12 activation was much enhanced when Gal4DBD-myobHLH and E12 were expressed together with MEF2C wild type protein or with MEF2C-VP16 (Fig. 5, lanes 9 and 11). Cotransfection of the deletion mutant MEF2C1-117 lacking the activation domain also resulted in an activation of the Gal4

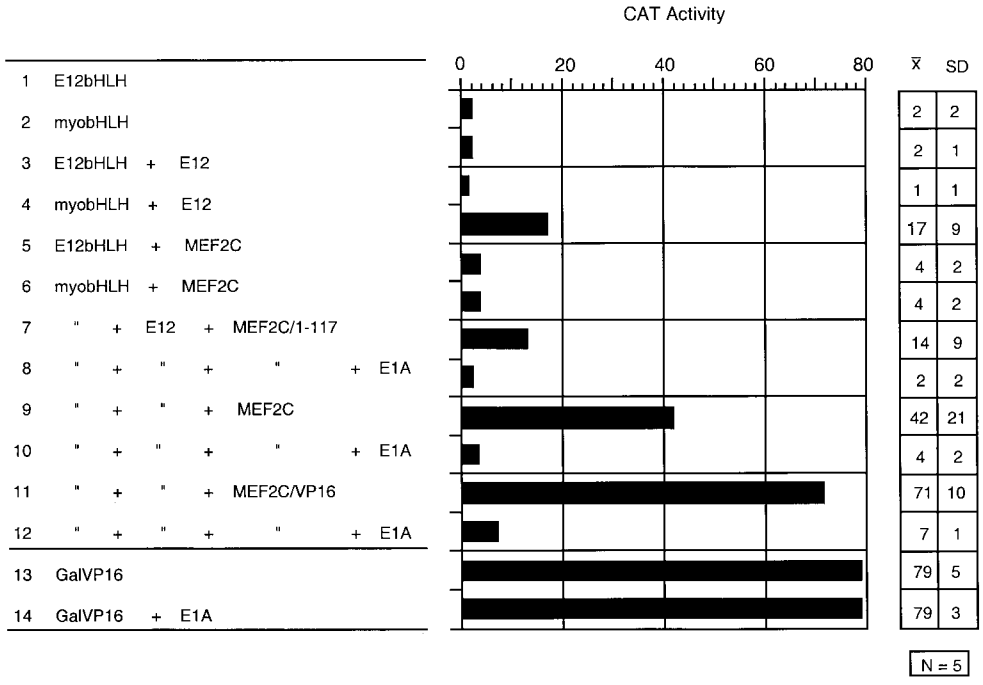

FIG. 5. E1A protein inhibits interaction between MEF2C, Myogenin, and E12. 10T1/2 cells were transiently transfected with pG5E1b-CAT reporter plasmid, expression vectors encoding chimeric proteins for the Gal4 DNA-binding domain-E12bHLH (E12bHLH), Gal4 DNA-binding domain-myogenin bHLH (myobHLH), an N-terminal deletion mutant of E12 (E12), the various MEF2C expression vectors and 13SE1A plasmid as indicated. 5 μg of each of the plasmids were transfected. The specificity of E1A inhibition was controlled by transfection of Gal4-VP16 activator with and without E1A. CAT activities were normalized to transfection efficiency determined as β-gal activity. The percentage of substrate conversion represents the average of four independent transfections ($\bar{X}$). Standard deviation $\pm$ (SD) are given right to the panels.

reporter comparably as to when Gal4DBD-myobHLH and E12 were coexpressed alone (Fig. 5, lane 7). These weak but significant activations could be due to endogenous MEF2 protein recruited into the myogenin/E12 heterodimeric complex or to MEF2C1-117 mutant protein which retains the ability to dimerize (31). It is interesting to note, that in these two assays we observed very diverse CAT conversions being reflected in high standard deviation values. An explanation could be that due to slight differences in tissue culturing conditions different levels of endogenous MEF2 protein could become critical for interaction with myobHLH and MEF2C1-117 proteins. Independent of the intensity of myogenin/E12 and MEF2C induced activation of the Gal4 reporter, if equal amounts of expression vector encoding 13SE1A were added to the assay, CAT activities decreased to background levels (Fig. 5, lanes 8, 10, and 12). Transactivation of the Gal4 reporter plasmid mediated by Gal4-VP16, however was not subject to E1A inhibition (Fig. 5, lanes 13 and 14).

The data reported here suggest that E1A might specifically inhibit a direct interaction of myogenin/E12 protein with MEF2C by disturbing one or more of the interactive surfaces distributed throughout the myogenic bHLH region and the MEF/MADS box. To support that E1A could interfere with the assembly and/or activity of a myogenic bHLH/MEF2 complex, we tested whether the previously demonstrated physical interaction between MEF2C, myogenin and E12 (31) is abrogated in the presence of E1A protein. E1A, Myf4, chicken MEF2A or MEF2A1-118 deletion mutant and E12 or E2.5 templates were translated in rabbit reticulolysate either separately and mixed before immunoprecipitation or were cotranslated prior to immunoprecipitation with an anti-myogenin antibody (47). 35S-labeled proteins were then resolved by SDS–polyacrylamide gel electrophoresis. In Fig. 6A is shown that MEF2A1-118 and a myogenic/E2.5 heterodimer do physically interact if both, myogenin and E2.5 proteins are present. In the presence of E1A, however, this protein-protein interaction was not evident. The result of this study supports that MEF2 factors collaborate with bHLH proteins to activate myogenin gene transcription. The cooperativity is based on the assembly of a tri-partite transcription factor complex in which MEF2 protein functions as a DNA-binding partner and co-factor of Myf5/E-protein, thus making the myogenic bHLH factor available for transcriptional activation of the myogenin promoter and probably sensitive for E1A repression. The protein-protein interaction is distributed throughout the myogenic bHLH and the MADS/MEF domain (31; present study). This has already been indicated by previous experiments showing that myogenin CAT reporter transactivation by Myf5-VP16

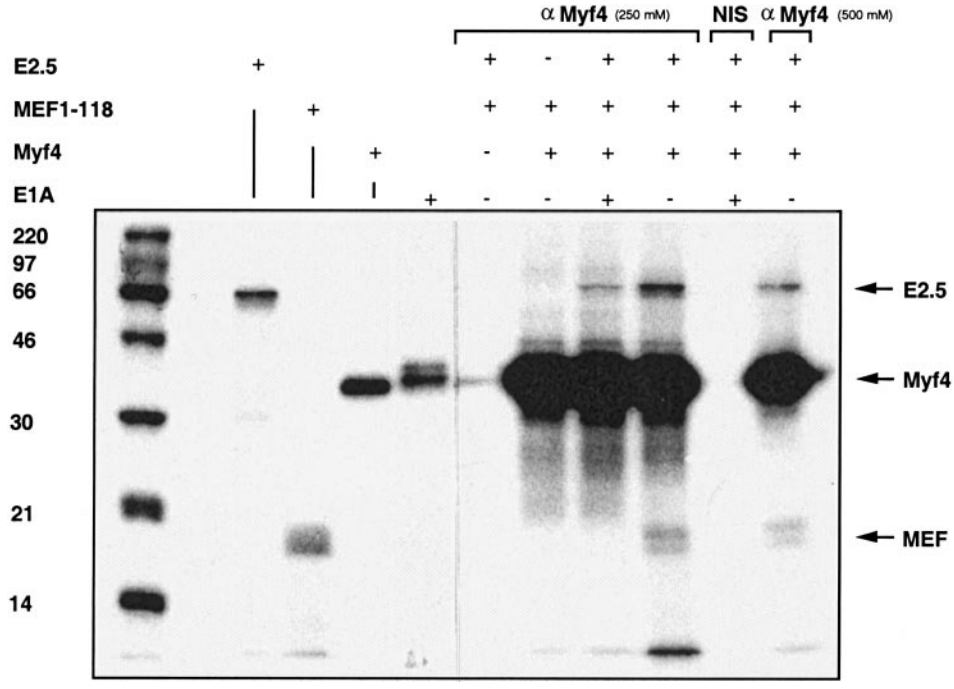

FIG. 6. Coimmunoprecipitation of MEF2 with myogenin and E12 is abrogated in the presence of E1A protein. Equal volumes of *in vitro* translated C-terminal truncated MEF2A protein (aa 1-118), myogenin, E2.5, and 12SE1A protein were mixed in the various combinations as indicated and immunoprecipitated with a mouse myogenin monoclonal antibody (αMyf4). To detect putative unspecific binding of [^{35}S]methionine-labeled proteins to the beads, hybridoma culture medium was used as a control (NIS). Immunocomplexes were washed either with 250 mM or 500 mM NaCl prior to resolving protein-protein interactions by SDS–PAGE analysis. The *in vitro* translation products mixed in the indidual assay are pointed out left hand.

hybrid protein encoding an active DNA-binding bHLH region (aa 1-149) of Myf5 fused to VP16 is inhibited by E1A. Transactivation of an artificial MCK4R-CAT reporter containing a tetrameric minimal E-box, however, is not inhibited (37). It is reasonable to assume that the mechanism of E1A repression is depending on the promoter architecture. E-box mediated binding and transactivation by Myf5 is not inhibited by E1A (37). MEF2 site and MEF2/E-box mediated transactivation by MEF2 alone or by MEF2/Myf protein complexes, respectively, is repressed. Interestingly, the first hint for E1A repression of myogenic differentiation was delineated to the RSRF consensus sequence within the α-actin muscle-specific promoter, thus substantiating the role of MEF2 as a regulator of skeletal muscle differentiation and as a target for oncogenes (58). E1A imposes on the assembly and transactivation capacity of this complex and was also shown to selectively repress the DNA-binding function of MEF2. It remains to be clarified whether E1A hinders MEF2 DNA binding resulting in loss of complex formation capability or rather inhibits complex formation which then results in loss of DNA-binding, and thus activation of the myogenin gene promoter. One attempt to answer this question is to analyze whether MEF2/Myf5 tethered transcription factors are resistent to the E1A challenge. In addition to that, selective mutations of E1A that relieve the putative disruption of the tripartite transcription factor complex *in vitro* can test the inhibition pathways individually.

ACKNOWLEDGMENTS

We thank Eric N. Olson for kindly providing the Gal-MyobHLH, Gal-E12, and MEF2C constructs. We also thank E. Harlow for providing the M73 antibody. This work was supported by the Deutsche Forschungsgemeinschaft.

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