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A Role for the ETS Domain Transcription Factor PEA3 in Myogenic Differentiation

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Activation of adult myoblasts called satellite cells during muscle degeneration is an important aspect of muscle regeneration. Satellite cells are believed to be the only myogenic stem cells in adult skeletal muscle and the source of regenerating muscle fibers. Upon activation, satellite cells proliferate, migrate to the site of degeneration, and become competent to fuse and differentiate. We show here that the transcription factor polyomavirus enhancer activator 3 (PEA3) is expressed in adult myoblasts in vitro when they are proliferative and during the early stages of differentiation. Overexpression of PEA3 accelerates differentiation, whereas blocking of PEA3 function delays myoblast fusion. PEA3 activates gene expression following binding to the *ets* motif most efficiently in conjunction with the transcription factor myocyte enhancer factor 2 (MEF2). In vivo, PEA3 is expressed in satellite cells only after muscle degeneration. Taken together, these results suggest that PEA3 is an important regulator of activated satellite cell function.

In mature muscle, activation of adult myoblasts called satellite cells is one mechanism for the maintenance of skeletal muscle mass. Although quiescent in normal muscle, these cells are activated to proliferate and differentiate in response to muscle damage or degeneration, thereby regenerating muscle fibers (5). To identify regulatory molecules in satellite cells that respond to external signals and control gene expression during satellite cell activation, we analyzed the regulation of the human β-enolase gene, which belongs to a relatively small group of muscle-specific genes expressed in proliferating, undifferentiated myoblasts from adult muscle (42). We showed that expression of the β-enolase gene in undifferentiated myoblasts is independent of the basic helix-loop-helix (bHLH) family of transcription factors, including MyoD, myogenin, myf-5, and MRF4 (42). Through comparative deletion-transfection analyses of myoblast and fibroblast cell lines, we identified an enhancer from the human β-enolase gene that promoted high-level myoblast-specific expression of a reporter gene (49). After myoblast differentiation into myotubes, the activity of the enhancer declined dramatically, suggesting that other *cis*-regulatory elements are responsible for maintaining β-enolase gene expression in myotubes. Electrophoretic mobility shift assays and mutational analysis indicated that proteins present in myoblast nuclear extracts specifically bound to an *ets* motif within the enhancer required for high-level activity in myoblasts (49). ETS proteins comprise a family of transcription factors that share a conserved 85-amino-acid domain necessary for specific binding to purine-rich DNA sequences with a GGA core consensus (22, 38). They have been shown to be involved in regulating gene expression and controlling cell growth, differentiation, and migration in a variety of biological systems (21, 47, 52).

A feature of many ETS domain proteins is that they form complexes with transcription factors of unrelated families, thereby strengthening their relatively weak inherent transactivating abilities and/or modifying DNA-binding activity (6). One of the best-characterized examples is the interaction of the MADS domain protein serum response factor with the ETS domain proteins Elk-1 (19) and SAP-1 (7) that bind to the c-*fos* promoter. In this study, we show that the ETS domain protein polyomavirus enhancer activator 3 (PEA3) is expressed in proliferating adult myoblasts and binds to the *ets* motif in a complex with the MADS domain protein myocyte enhancer factor 2 (MEF2, also referred to as RSRF, for "related to SRF" [40, 43]). MEF2 DNA-binding activity has been shown to be important for the expression of numerous muscle-specific genes, and MEF2 has recently been demonstrated to act as a coregulator to potentiate the myogenic activities of the bHLH proteins independent of direct MEF2 DNA binding (23, 32, 36). MEF2 activity is encoded by four genes (MEF2A to -D), all four of which are expressed in differentiated myotubes (40). MEF2B and -D are also expressed in undifferentiated myoblasts.

Our results also show that MEF2 potentiates the transactivating ability of PEA3. Furthermore, overexpression of PEA3 alone accelerates myoblast differentiation upon withdrawal of serum, whereas blocking PEA3 function inhibits differentiation. Taken together with the observation that PEA3 expression is induced during muscle degeneration and satellite cell activation in vivo, these results suggest that PEA3 is involved in regulating myogenesis in adult muscle.

MATERIALS AND METHODS

Library screening. A λgt11 expression library was generated from C2C12 myoblast poly(A)$^+$ RNA by Clontech Laboratories, Inc. The probe for library screening was generated starting with 5 μg of the two complementary, synthetic oligonucleotides 5′-CATCCGT**TTCCT**GTCCCCTAACT-3′ and 3′-AGGCA**A AGGA**CAGGGGATTGAGT-5′. The *ets* motif is highlighted in boldface. The oligonucleotides were phosphorylated, annealed, catenated, and labeled exactly as described in reference 51. Library screening (approximately 10^6 plaques) was also performed as described by Vinson et al. (51), except that Magna NT membranes (Micron Separations, Inc.) were used, membranes were wiped vigorously after addition of guanidine hydrochloride, and 1 μM ZnCl was added to the binding reaction mixture. In the mutant oligonucleotide, the underlined AG was mutated to CT as shown in the sequence above.

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Cell culture and transfections. The C2C12 myoblast cell line was grown in the following growth medium (GM): Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 10% defined bovine serum (Hyclone) at 37°C in a humidified 10% CO_2–90% air atmosphere. To induce differentiation, cells were washed with serum-free DMEM and maintained in differentiation medium (DM [DMEM plus 2% horse serum]) (Hyclone). COS-1 cells were cultured in DMEM containing a high concentration of glucose plus 10% fetal calf serum at 37°C in a humidified 5% CO_2–95% air atmosphere.

For generation of stably transfected clones, introduction of expression constructs into C2C12 cells was performed by the calcium phosphate coprecipitation method as previously described (46). Approximately 10^6 cells were transfected with 15 μg of a plasmid. The DNA was removed 24 h later with the addition of fresh GM. After an additional 24 h, cells were split 1:4 and refed with medium to which 400 μg of G418 (Geneticin; GIBCO BRL) per ml had been added. G418-resistant clones were picked 14 days later and analyzed individually or as pools of several hundred clones.

Transient transfections were performed with Lipofectamine (GIBCO BRL) according to the manufacturer's recommendations. Specifically, 7 μl of Lipofectamine and 2 μg of total DNA were incubated for 45 min in serum-free medium, after which serum-containing medium was added before the mixture was applied to approximately 10^5 COS-1 cells. Cotransfection of different plasmids was performed with 0.4 μg of reporter and 0.8 μg of each effector. Empty vector (pcDNA3 or pRc/RSV; Invitrogen) was added where necessary to ensure a constant amount of input DNA. Cotransfection with a vector constitutively expressing the *lacZ* gene verified that differences in luciferase reporter gene expression were not due to differences in transfection efficiency. Cells were incubated for 5 h with the DNA mixtures, at which time additional medium was added. Twenty-four hours after transfection, the medium was changed, and cells were harvested 48 h after transfection. The total protein content in cell extracts was determined (Bio-Rad protein assay), and β-galactosidase activity (Galacto-Light kit; TROPIX, Inc.) and luciferase activity (Promega luciferase assay kit) were quantitated with a TD-20e luminometer (Turner Designs). Absolute light emission generated from the luciferase enzyme reaction was determined, and the value obtained for each transfection was presented as a ratio over that of the reporter alone. Data were analyzed by Student's *t* test. Values are shown as means ($n = 6$) ± standard errors. Significance is assumed at $P < 0.05$.

Plasmid constructs. A full-length cDNA (2.4 kb) encoding mouse PEA3 was cloned into the *Hin*dIII site of pRc/RSV (Invitrogen) downstream of the Rous sarcoma virus (RSV) long terminal repeat. The resulting plasmid was sequenced to verify insert orientation. For in vitro transcription or translation, the 2.4-kb PEA3 cDNA was cloned into the *Eco*RI site of the pSPORT vector (GIBCO BRL) such that the T7 promoter generated sense RNA. To generate a dominant negative ETS protein, a 775-bp fragment was isolated from the original phage vector by digestion with *Eco*RI, which cut within the vector, and *Sac*I, which cuts within the 3′ untranslated region of the PEA3 cDNA. This fragment was subcloned into pET30A (Novagen) to generate a cDNA in frame with the SV5 tag (16) after BamHI-XhoI digestion of sites flanking the insert. A *Hin*dIII-*Eco*RI fragment encoding SV5 was excised from pCMV19-SV5 and cloned into pcDNA3 (Invitrogen), which contains the enhancer-promoter sequences from the immediate-early gene of human cytomegalovirus. This plasmid was subsequently digested with *Bam*HI (present 3′ of the SV5 tag from the PUC19 polylinker) and *Xho*I within the pcDNA3 polylinker and ligated to the PEA3 cDNA to generate the SV5–truncated-PEA3 expression vector, which was sequenced to verify cloning strategy.

The reporter plasmid in transient transfections, 3x*ets*-luciferase (39), contained three copies of the *ets* motif (CAGGAAGTGAC) upstream of a synthetic TATA box corresponding to the adenovirus 2 major late promoter controlling luciferase in the pGL3-basic vector (Promega). Expression constructs encoding MEF2A and MEF2C were under the control of the cytomegalovirus enhancer-promoter sequences and were provided by Brian Black and Eric Olson.

DNA binding assays. Oligonucleotide sequences for all DNA binding assays are described above for library screening. Nuclear extracts were isolated, and gel shift assays were performed as described previously (49). Oligonucleotides were synthesized with a 381A DNA synthesizer (Applied Biosystems), annealed, and end labeled, and 10,000 cpm (approximately 1 ng of DNA) was incubated with the indicated amount of nuclear protein in a mixture of 10 mM HEPES (pH 7.9), 75 mM KCl, 1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 0.15 μg of poly(dI-dC) · (dI-dC) per μl in a reaction volume of 20 to 25 μl for 30 min at room temperature or for several hours at 4°C. Reaction products were separated on a nondenaturing 6% acrylamide gel (75:1 acrylamide-bisacrylamide) containing 0.5× Tris-borate-EDTA at 15°C. For competition experiments, various concentrations of unlabeled DNA (ranging from 1- to 100-fold molar excess [see "Library screening" for oligonucleotide sequences]) were added to the reaction mixture prior to addition of extract. Supershift assays were performed as described with antibodies to PEA3 (58); MyoD (8); myogenin (F5D; provided by Woody Wright, University of Texas Southwest Medical Center, Dallas); c-Jun (Santa Cruz, Inc.); MEF2D, MEF2B, and MEF2A (15); and MEF2C (provided by John J. Schwarz, University of Texas Medical School at Houston) and an antibody recognizing MEF2A strongly and MEF2C weakly (Santa Cruz, Inc.).

DNA binding assays were also performed with wild-type and mutant annealed oligonucleotides (see "Library screening" for oligonucleotide sequences) in which the sense strand had been biotinylated at the 3′ end (Ransom Hill Bioscience, Inc.). Binding reactions were performed as described above, except that 500 ng of double-stranded, biotinylated oligonucleotide was incubated with 20 μg of myoblast nuclear extract in a 30-μl reaction volume. The reaction mixture was then incubated with gentle mixing for 30 min at 4°C with an equal volume of avidin-agarose beads (4%; Sigma) that had been pre-equilibrated with 500 μg of bovine serum albumin per ml and 200 μg of carrier DNA in binding buffer (described above). The reaction mixture was pelleted gently (1,000 rpm, 1 min) in an Eppendorf Microfuge, and the procedure was repeated for the pellet and supernatant separately. The resulting pellets were combined and then washed three times with binding buffer, and the final pellet was resuspended in 30 μl of SDS loading buffer for Western blot analysis as described below.

RNA isolation and Northern analysis. RNA was isolated, and Northern blotting was performed as previously described (41, 46). The following cDNAs were labeled by the random primer method with an Ambion kit to generate probes: a 300-bp fragment from the 3′-untranslated region of mouse PEA3; 549- and 612-bp *Eco*RI fragments specific for mouse ERM (34) and ER81 (35), respectively; for β-enolase, a 1.6-kb *Bam*HI fragment from pCP2A (42); a 1.1-kb *Eco*RI fragment from mouse myogenin (41); and a 500-bp *Eco*RI fragment recognizing all actin transcripts (42).

Western analysis. Western blots were performed exactly as described previously (46) with 30 μg of protein extract or 5 μl of TNT T7 quick coupled transcription-translation reaction mixture (Promega) with the PEA3 cDNA. A monoclonal antibody directed to amino acids 256 to 333 (exons 8 and 9) of PEA3 (Santa Cruz, Inc.) and a cocktail of polyclonal MEF2 antibodies recognizing all four MEF2 isoforms (MEF2A, -B, and -D [15], MEF2C [provided by John J. Schwarz, University of Texas Medical School at Houston], and MEF2A and -C [Santa Cruz, Inc.]) were used. Immunoreactive proteins were detected with the Renaissance chemiluminescence reagent (DuPont, NEN).

Immunohistochemistry. Cultured cells were washed twice with phosphate-buffered saline (PBS) and fixed with 1% formaldehyde in PBS for 1 h at room temperature, followed by treatment with ice-cold methanol for 20 min. Muscle myosin heavy chain (MyHC) was detected with 4A.1025 antibody (46). Cells were incubated with primary antibody for 1 h at room temperature, followed by incubations with biotinylated anti-mouse immunoglobulin G (H + L) (Vector), diluted 1:400 in PBS plus 0.1% Tween 20, and avidin-biotin complexed with horseradish peroxidase (Vector ABC kit). Horseradish peroxidase reactivity was visualized with 1 mg of diaminobenzidine per ml in 50 mM Tris-HCl (pH 7.2)–0.03% H_2O_2–0.03% $CoCl_2$. For detection of the nuclear antigens PEA3 and MyoD (8) in muscle tissue, 10-μm-thick cryostat sections were fixed in 2% paraformaldehyde and permeabilized with 1% Nonidet P-40. Immunoreactive proteins were detected as described above, except that 0.1% Nonidet P-40 was present in all incubations to allow accessibility of the antibody to the nucleus.

RESULTS

Cloning and characterization of PEA3 expression during myogenic differentiation. To identify the ETS domain transcription factor in myoblasts that regulates β-enolase gene expression, we used the catenated *ets* motif from the β-enolase enhancer as a probe to screen a λgt11 expression library constructed from C2C12 mouse myoblast mRNA. Three positive cDNAs were isolated, all of which encode the ETS domain protein PEA3, originally cloned from a mouse mammary epithelial cell line (55). The specificity of the PEA3-DNA interaction was demonstrated by direct comparison of the binding activity of protein expressed from the three purified plaques to wild-type *ets* oligonucleotide versus that to a mutated oligonucleotide in which the GA within the core *ets* motif had been mutated to CT (data not shown).

We examined PEA3 mRNA accumulation during myoblast differentiation in cell culture (Fig. 1). Northern blot analysis of RNA isolated from C2C12 myoblasts maintained in high-serum-containing GM and after 24 and 48 h in low-serum-containing DM showed only one major PEA3 mRNA at 2.4 kb that accumulated to its highest levels in undifferentiated myoblasts (Fig. 1, GM lane). PEA3 mRNA abundance decreased 50% after 24 h in DM and was less than 15% of the levels in GM by 48 h. PEA3 mRNA levels remained low after longer exposure to DM (data not shown). PEA3 mRNA accumulated in myoblasts to significantly higher levels than mRNAs encoding related ETS domain proteins such as ER81 (6.8 and 3.1 kb) and ERM (3.6 kb) (4, 34, 35), which were expressed at relatively low constitutive levels during differentiation. β-Enolase mRNA accumulated to high levels at all stages tested, indicat-

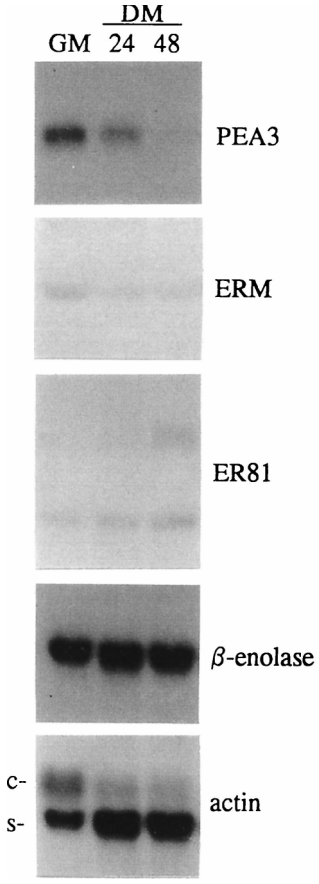

FIG. 1. PEA3 mRNA preferentially accumulated in undifferentiated myoblasts. Total RNAs (10 μg) from undifferentiated C2C12 myoblasts in GM and after 24 and 48 h in DM were analyzed by Northern blotting with the indicated probes. The C2C12 myoblasts in GM were confluent at the time of RNA isolation. The differentiation state of the cells was monitored with an actin coding region probe that recognized both cytoskeletal (c [a marker of undifferentiated myoblasts]) and sarcomeric (s [a marker of muscle differentiation]) isoforms of actin mRNAs.

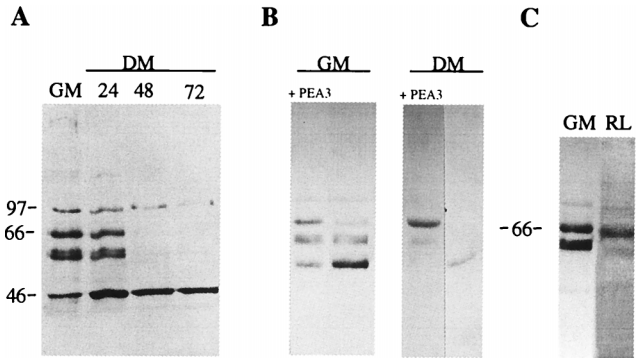

FIG. 2. PEA3 abundance decreased after myogenic differentiation. Protein extracts (30 μg) from C2C12 myoblasts cultured under different conditions were analyzed by Western blotting after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis through 7.5% gels. (A) Extracts from undifferentiated C2C12 myoblasts in GM and after 24, 48, and 72 h in DM are shown. Molecular masses are indicated in kilodaltons. (B) After stable transfection of C2C12 myoblasts with an RSV-PEA3 expression construct, protein extract was isolated from pooled clones maintained in GM (+PEA3, lane 1) or after 48 h in DM (+PEA3, lane 3), and PEA3 levels were compared to those in C2C12 cells stably transfected with empty vector and cultured under the same conditions (lanes 2 and 4). A protein with a size of 66 kDa is expressed from the RSV-PEA3 plasmid. (C) PEA3 present in myoblast extract (GM) was compared to PEA3 expressed from the PEA3 cDNA in a reticulocyte lysate (RL).

ing that regulatory factors other than PEA3 control β-enolase gene expression after differentiation. Recently, an enhancer that promotes expression of the β-enolase gene in myotubes was identified within the first intron of the gene (10).

PEA3 protein accumulation also decreased with differentiation demonstrated by Western analysis (Fig. 2A). Five proteins with sizes of 92, 66, 60, 58, and 48 kDa reacted strongly with the PEA3 monoclonal antibody only in extracts from proliferating myoblasts and from myoblasts undergoing the early stages of differentiation (Fig. 2A, GM and DM 24-h lanes, respectively). The 66-kDa protein corresponds to the size of cloned PEA3 (55) and is the approximate size of protein expressed from the full-length cDNA in vitro in a reticulocyte lysate (Fig. 2C, RL). A PEA3-like protein with a size of 91 kDa has also been described (30). The doublet at 60 and 58 kDa likely results from alternative splice events, with the 58-kDa isoform translated from an RNA splice variant lacking exon 7 and the 60-kDa isoform derived from an RNA lacking exon 2 (16a). These splice variants were not detectable by Northern analysis because of the small size of the alternatively spliced exons. The abundance of these proteins, particularly the 66-, 60-, and 58-kDa forms, dropped dramatically after longer exposure to DM (Fig. 2A, DM 48-h lane) and remained low in fully differentiated myotubes (Fig. 2A, DM 72-h lane). The identity of the 48-kDa protein is unknown, and the intensity of this band was highly variable between extracts and experiments. The 66-kDa protein accumulated upon stable expression of the full-length PEA3 cDNA in cells (Fig. 2B, +PEA3 lanes). This is most apparent after exposure of cells to DM for 48 h, when endogenous PEA3 abundance drops dramatically.

Analysis of PEA3 DNA-binding activity in myoblasts. Consistent with PEA3 abundance, *ets* motif DNA-binding activity was highest in myoblast nuclear extracts, as demonstrated by gel shift assays (Fig. 3). Myoblast nuclear extract generated one major shifted complex on the *ets* motif from the β-enolase myoblast-specific enhancer (Fig. 3A, solid arrow). Unlabeled *ets* motif DNA competed for protein binding efficiently, whereas an unlabeled oligonucleotide in which the *ets* motif was mutated did not compete even when in vast molar excess, demonstrating the specificity of this protein-DNA interaction. *Ets* motif binding activity decreased after differentiation (Fig. 3B, compare the GM and DM lanes). Pooled clones of C2C12 cells stably transfected with a full-length PEA3 cDNA that overexpressed the 66-kDa PEA3 relative to vector-transfected cells (see Fig. 2B) demonstrated increased *ets* motif DNA-binding activity that was most apparent in the transfectants after exposure to DM (Fig. 3B, DM + PEA3).

To identify the protein components of the shifted complex, supershift assays were performed (Fig. 3C). The PEA3 antibody interacted with proteins present in the shifted complex, decreasing migration in the gel (Fig. 3C, open arrows) compared to the migration of the complex in the absence of antibody (Fig. 3C, closed arrow). Antibodies to MyoD, myogenin, and c-Jun, as well as nonimmune serum, had no effect on the mobility of the complex, whereas antibodies to all MEF2 isoforms tested (Fig. 3C, MEF2C and A and MEF2D [data not shown]) also supershifted the complex, suggesting that the protein complex on the *ets* motif contained both PEA3 and MEF2.

To confirm that PEA3 and MEF2 form a complex on the *ets* motif, an additional DNA binding assay was performed. Biotinylated oligonucleotides encoding either the wild-type or mu-

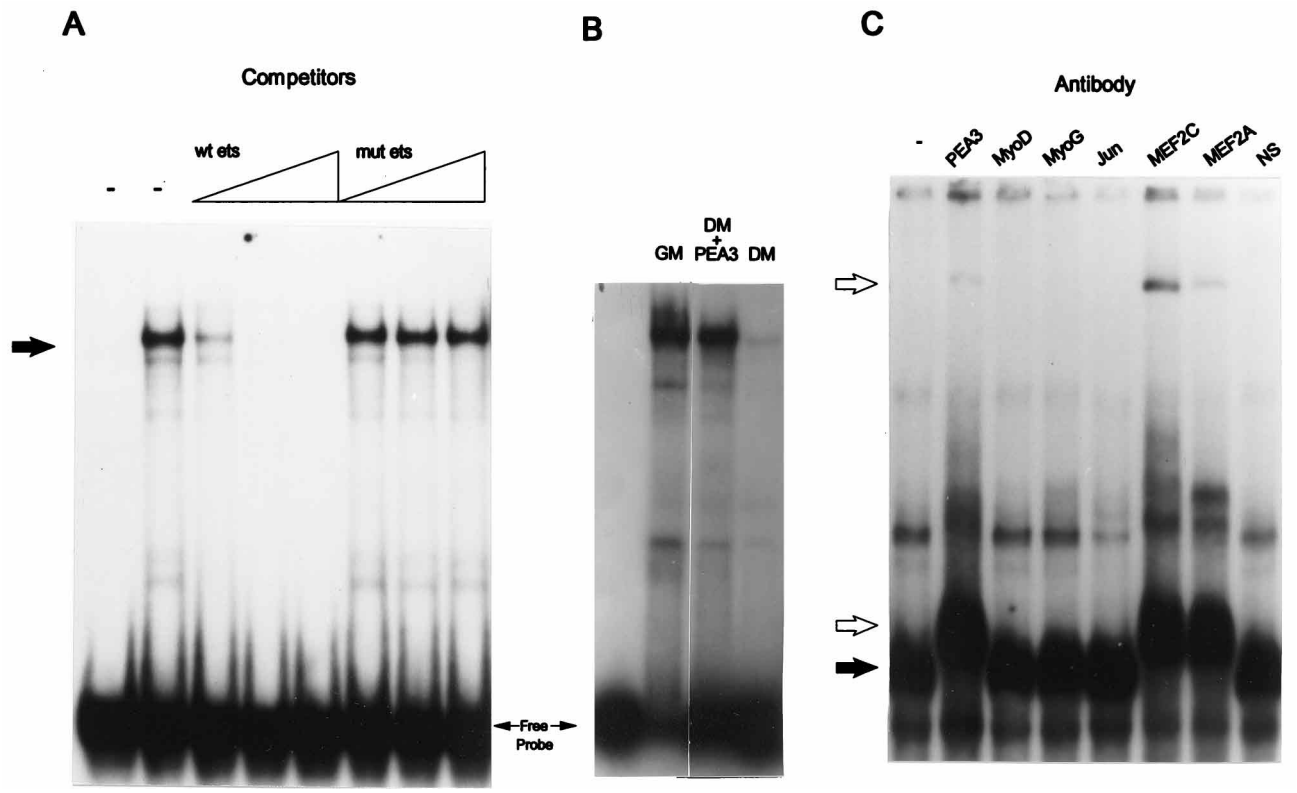

FIG. 3. PEA3 present in muscle cell nuclear extracts specifically binds to the β-enolase *ets* motif. Gel shift assays were performed with 4 μg of nuclear protein isolated from C2C12 cells cultured as described in the text and with a 25-bp radiolabeled double-stranded oligonucleotide encompassing the *ets* motif (see Materials and Methods). (A) Nuclear extracts from myoblasts in GM generated one major shifted complex (solid arrow). Binding reactions were carried out with increasing concentrations (1-, 10-, and 100-fold molar excess) of two different unlabeled oligonucleotides as competitors: a 25-bp oligonucleotide containing the wild-type *ets* motif (wt ets) and a 25-bp oligonucleotide in which 2 bp within the core *ets* motif were mutated from GA to CT (mut ets). (B) Gel shift assays were performed with nuclear extracts isolated from myoblasts in GM and after 48 h in DM and from C2C12 cells stably transfected with an RSV-PEA3 expression construct after 48 h in DM (DM + PEA3). (C) Supershift assays were performed with nuclear extracts from C2C12 cells in GM with the indicated antibodies or nonimmune rabbit serum (NS). The gel was electrophoresed twice as long as those shown in panels A and B to resolve shifted complexes (solid arrows) from supershifted complexes (open arrows). The gel was also exposed to film for 3 days, as opposed to overnight for panels A and B, so that less-abundant, nonspecific complexes are more readily visible.

tated *ets* motif were incubated with myoblast nuclear extract, and the protein-DNA mixtures were precipitated with avidin-agarose beads. After extensive washing, proteins interacting on the DNA were identified by Western blotting (Fig. 4). The 66-kDa protein reacting with the PEA3 antibody was specifically enriched with the wild-type *ets* DNA, but not the mutant DNA (Fig. 4, compare lanes 1 and 2). Proteins at 60 and 58 kDa were precipitated with both the wild-type and mutant DNAs, suggesting that these protein-DNA interactions were not dependent on the intact *ets* motif. A protein with a size of approximately 64 kDa recognized by a mixture of MEF2 antibodies was also specifically precipitated with the wild-type *ets* DNA (Fig. 4, compare lanes 3 and 4). This is consistent with the electrophoretic mobility reported for all MEF2 isoforms, including MEF2B and -D, the MEF2 isoforms expressed in proliferating myoblasts (33, 40).

MEF2 expression affects PEA3-dependent transactivation. Results of DNA-binding assays suggested that MEF2 interacts with PEA3 on DNA. To determine if MEF2 expression affects PEA3 activity, cotransfection experiments were performed with COS-1 cells that lack significant endogenous PEA3 DNA-binding activity (39). The luciferase reporter construct used was controlled by three copies of a canonical PEA3 binding site in a head-to-tail arrangement upstream of a basal promoter (3x*ets*), but it lacks a putative MEF2 binding site. Figure 5 shows that under these transfection conditions, PEA3 acti-

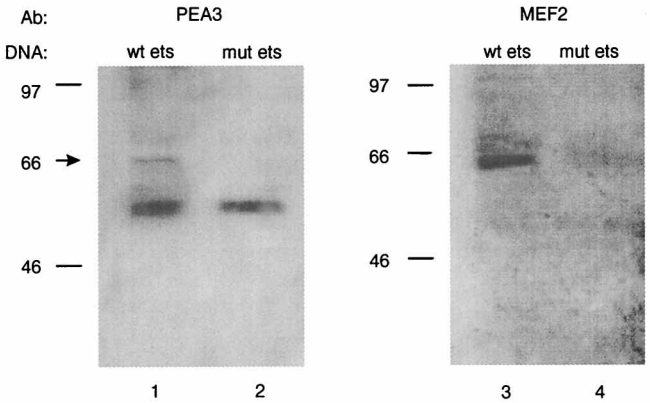

FIG. 4. PEA3 and MEF2 form a complex on the *ets* motif. Binding assays were performed with myoblast nuclear extracts and biotinylated wild-type (wt ets [lanes 1 and 3]) and mutant (mut ets [lanes 2 and 4]) *ets* motif double-stranded oligonucleotides. Proteins complexed with the DNA were enriched by precipitation with avidin-agarose beads and analyzed by Western blotting with PEA3 (lanes 1 and 2) or MEF2 (lanes 3 and 4) antibodies (Ab). The PEA3 antibody identified one protein with a size of 66 kDa that interacted specifically with the wild-type DNA. The MEF2 antibodies identified a protein with a size of 64 kDa present only in the complex formed on wild-type *ets* DNA. Molecular masses are indicated on the left in kilodaltons.

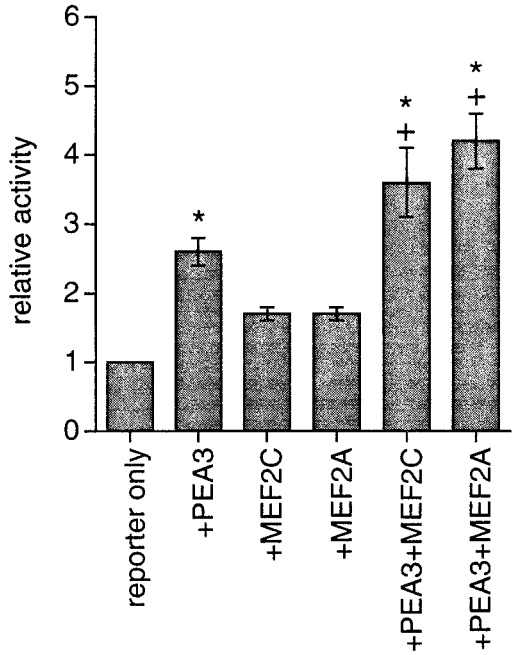

FIG. 5. MEF2 increases PEA3 transactivation in transient transfection assays. COS-1 cells were transfected with the indicated plasmids, and relative luciferase activity from the 3x*ets*-luciferase reporter was determined as described in Materials and Methods. Data were analyzed by Student's *t* test. Values are shown as means ($n = 6$) ± standard errors. Significance is assumed at $P < 0.05$. *, statistically different from reporter alone; +, statistically different from reporter plus PEA3.

vated expression from the 3x*ets*-luciferase reporter approximately 2.5-fold. MEF2C and MEF2A alone had no significant effect. Cotransfection of PEA3 and MEF2 together with the reporter increased reporter gene expression four- to fivefold, suggesting that MEF2 increases the activity of PEA3.

Overexpression of PEA3 promotes myogenic differentiation. To determine the role of PEA3 in controlling myoblast function, the effect of PEA3 overexpression on growth rate and differentiation was determined. The proliferation rates in pooled clones, measured by counting cells after trypsinization with a Coulter counter, were not significantly different in PEA3- and vector-transfected myoblasts (data not shown). Unexpectedly, a time course of differentiation determined with pooled clones of PEA3- and vector-transfected myoblasts indicated that myoblasts overexpressing PEA3 differentiated more readily than myoblasts expressing normal levels of PEA3. Cells were plated near confluency, allowed to attach in GM, and switched into DM, and the formation of myotubes was monitored (Fig. 6). By 24 h in DM, small myotubes that reacted with a monoclonal antibody to MyHC were readily detected in PEA3-transfected (Fig. 6B) compared to vector-transfected (Fig. 6A) clones. After 36 h, MyHC-positive myotubes could also be observed in vector-transfected cells (Fig. 6C). At this time, cells overexpressing PEA3 had abundant, fully formed myotubes that began to detach from the plate (Fig. 6D).

To attempt to quantitate the differentiation process more carefully in response to PEA3 overexpression, accumulation of myogenin mRNA, a gene product activated during differentiation, was analyzed in pooled as well as individual clones (Fig. 7). In control C2C12 cells, myogenin mRNA abundance was low in proliferating myoblasts (Fig. 7, lane 1) and increased progressively with increasing exposure to DM (Fig. 7, lanes 2 to 5). In all cases, accelerated differentiation in PEA3 overexpressers upon withdrawal of serum was accompanied by elevated myogenin mRNA expression. The results shown in Fig. 7 are representative of all individual clones tested, as well as pooled clones. Myogenin mRNA was abundant at all stages in PEA3 overexpressers, even in proliferating myoblasts maintained in GM (Fig. 7, PEA3 overexpresser, lane 1), but the absolute levels of myogenin mRNA were not tightly correlated to the absolute levels of PEA3 (data not shown). The maximum induced level of myogenin mRNA did not differ between clones. These results suggest that PEA3 promotes morphological and biochemical differentiation.

Blocking of PEA3 function inhibits myogenic differentiation. To further test the role of PEA3 in myogenic differentiation, we blocked PEA3 function by overexpression of a dominant repressor of ETS-dependent transcription. For these experiments, a truncated PEA3 cDNA, approximately 775 bp, that encodes the ETS domain of the protein (amino acids 242 to 480), but lacks the putative acidic and glutamine-rich activation domains (55), was cloned downstream of the enhancer-promoter sequences from the immediate-early gene of the human cytomegalovirus CMV. This expression construct also encodes the 14-amino-acid SV5 tag 5′ to the cloning site so that an SV5-truncated PEA3 fusion protein is produced (16). Protein expressed from this plasmid should compete with endogenous ETS domain proteins for DNA binding sites, thereby blocking ETS-dependent gene expression. Following stable transfection of the truncated PEA3 expression construct into myoblasts, pooled clones demonstrated nuclear localization of the fusion protein visualized immunocytochemically with an SV5 antibody (Fig. 6E). Myoblasts that accumulated SV5–truncated-PEA3 fusion protein appeared impaired in their ability to form myotubes. Even at 48 h in DM, only rare, small, MyHC-positive myotubes were observed (Fig. 6F). Figure 7 shows that myogenin gene expression was delayed in cells expressing truncated PEA3. This observation is consistent with that described above, in which overexpression of PEA3 in myoblasts resulted in precocious differentiation.

Nuclear extracts from cells expressing truncated PEA3 were assayed by gel shift to analyze *ets* DNA-binding activity (Fig. 8). Although the major shifted complex was still produced in extracts from cells expressing truncated PEA3 (Fig. 8A, solid arrow), a complex that migrated further in the gel was also detectable. However, the most dramatic change was observed in supershift assays with both PEA3 and MEF2 antibodies (Fig. 8A and B, respectively). Although both antibodies reacted with shifted complexes, the mobility of the supershifted complexes (Fig. 8, open arrows) differed between control cells and those expressing the truncated PEA3, suggesting that the introduced gene product was binding to DNA in place of endogenous PEA3, thereby interfering with normal protein function.

PEA3 expression is induced in muscle after satellite cell activation. Previous analysis of adult mouse tissues indicated that PEA3 mRNA was not detectable in muscle (55). This is not surprising given that satellite cells are quiescent in normal adult muscle. To determine whether PEA3 is associated with satellite cell activation in vivo, we induced muscle degeneration and satellite cell proliferation in adult rat soleus muscle by denervation. We first monitored MyoD accumulation, an indicator of satellite cell activation (11, 13, 25). Abundant MyoD-immunoreactive nuclei were detected in soleus muscle after complete transection of the sciatic nerve (Fig. 9A, solid arrows) compared to the contralateral innervated soleus (Fig. 9B). Denervation also results in increased MyoD expression in myofiber nuclei (11, 13, 25), and MyoD-positive nuclei closely

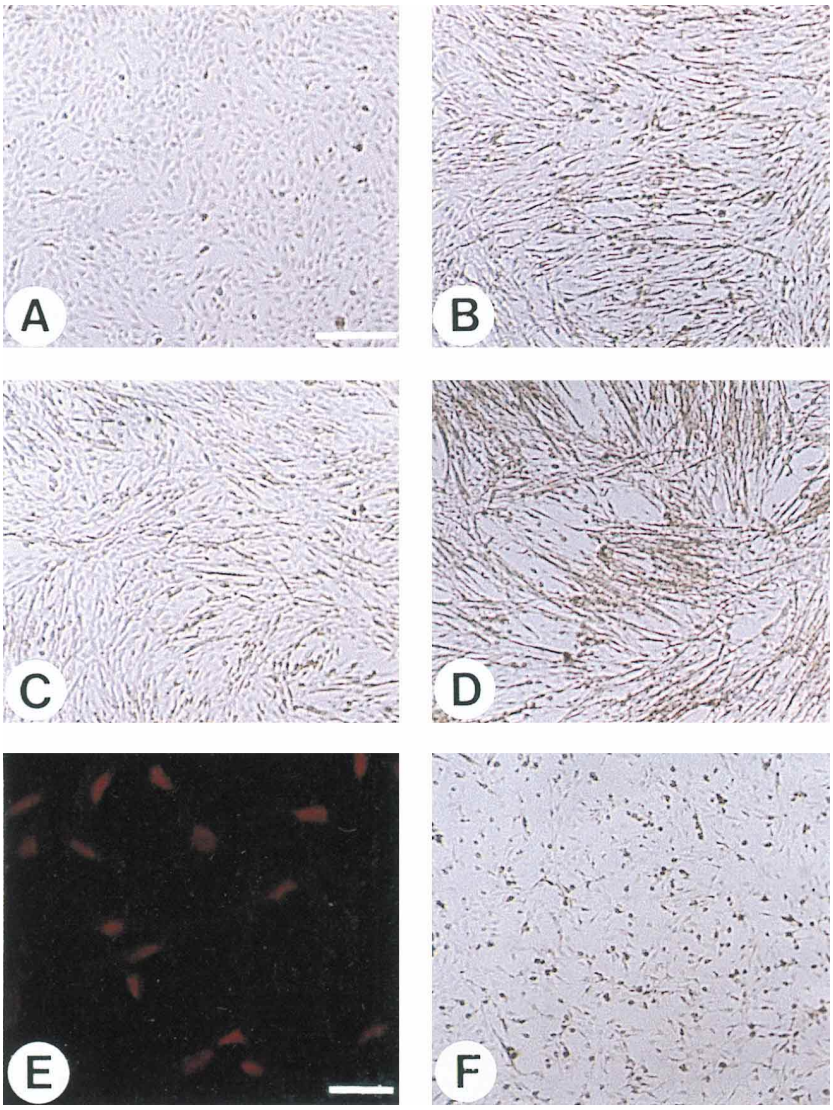

FIG. 6. Manipulating PEA3 activity in C2C12 cells alters their ability to differentiate. The extent of myogenic differentiation was assessed by reactivity with MyHC antibody in transfected (B, D, and F) and control (A and C) C2C12 cells after different times in DM. Pooled clones of cells stably transfected with RSV-PEA3 compared to control cells after 24 h (B and A, respectively) and 36 h (D and C, respectively) in DM are shown. In panels E and F, cells were stably transfected with a plasmid encoding SV5–truncated-PEA3 fusion protein. (E) Accumulation of the fusion protein in the nucleus was demonstrated immunocytochemically with an SV5 antibody. (F) Cells expressing SV5-truncated PEA3 were analyzed for MyHC expression after 48 h in DM. Bar in A to D and F, 50 μm. Bar in E, 200 μm.

associated with myofibers were detected after denervation (Fig. 9A, open arrows). Analysis of serial sections showed that PEA3-immunoreactive nuclei were also present in denervated muscle (Fig. 9C, solid arrows) compared to control muscle (Fig. 9D); however, these appeared to be a subset of the MyoD-positive nuclei. The location and frequency of PEA3-positive nuclei suggest that PEA3 preferentially accumulated in activated satellite cells in the muscle undergoing atrophy.

DISCUSSION

We cloned the ETS domain transcription factor PEA3 from a myoblast cDNA expression library based on its affinity for the *ets* motif from the β-enolase enhancer. Loss of PEA3 expression after myoblast differentiation is consistent with the loss of activity of the β-enolase enhancer (49) and implicates PEA3 in enhancer function. However, PEA3 appeared to activate transcription relatively weakly via binding to the *ets* motif in these

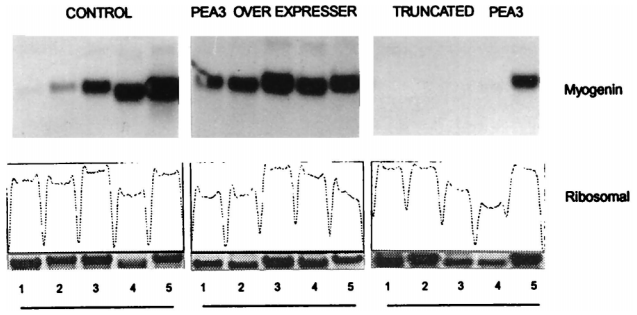

FIG. 7. Myogenin mRNA accumulation was altered in response to PEA3 activity. Total RNA (10 μg) from C2C12 cells, a clone overexpressing PEA3, and pooled clones expressing truncated PEA3 in GM (lane 1) and after 8, 16, 24, and 32 h in DM (lanes 2 to 5, respectively) were analyzed by Northern blotting with a probe specific for myogenin mRNA. Quantitation of gel loading was performed by scanning the 18S rRNA bands.

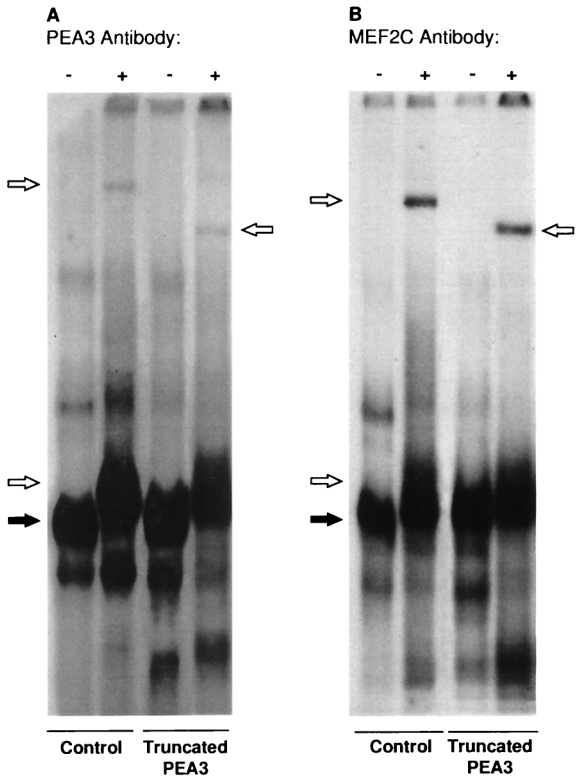

FIG. 8. Expression of truncated PEA3 in myoblasts alters *ets* motif DNA-binding activity. Gel shift and supershift assays were performed with myoblast nuclear extracts as described in the legend to Fig. 3 with (+) or without (−) antibodies to PEA3 (A) and MEF2C (B). Solid arrows indicate shifted complexes in the absence of antibodies, and open arrows indicate supershifted complexes.

transfection experiments. Transactivation by PEA3 was augmented by cotransfection with MEF2A and MEF2C expression plasmids in the absence of apparent MEF2 binding sites within the reporter gene construct. It has recently been shown that MEF2A to -D can synergize with myogenic bHLH proteins to activate gene expression via direct protein-protein interaction (23, 32, 36). Amino acids within the MADS and MEF2 domains appear to mediate this interaction (32). Similarly, MEF2 isoforms may interact directly with PEA3, thereby increasing transactivation. Although the nature of this putative protein-protein interaction has yet to be determined, it is likely to involve the ETS domain of PEA3 or regions immediately surrounding it, in that a truncated version of the protein lacking the N-terminal 242 amino acids still appeared to interact with MEF2 isoforms.

PEA3, as well as other ETS domain proteins, has been shown to cooperate with transcription factors from other families to stimulate transcription (6). Because there was no MEF2 binding site in the reporter gene construct, as mentioned above, nor were there any within the oligonucleotides used in DNA-binding assays, our data suggest that PEA3 and MEF2 interact directly. Although we did not identify the specific MEF2 isoforms associated with PEA3 in myoblasts and MEF2A to -D (3, 28, 29, 31, 33, 43, 59) have been shown to have similar transactivating abilities, changes in the expression of specific MEF2 isoforms that occur during myogenic differentiation may modify PEA3 activity and specificity. Direct interactions between ETS proteins, including ERM, and AP-1 transcription factors have also recently been reported (1, 37). Although we were unable to demonstrate direct interaction of Jun and PEA3, because AP-1 is only expressed at significant levels in proliferating myoblasts, it may influence PEA3-dependent transcription prior to differentiation. Recently, an AP-1-like protein, MafB, has been shown to interact directly with Ets-1 and repress its ability to transactivate transcription in myelomonocytic cells (48).

PEA3 accumulates to high levels both in proliferating myoblasts and during the initial stages of differentiation and is down-regulated in fully differentiated myotubes. That PEA3 expression is maintained for 24 h after serum withdrawal in vitro raises the possibility that loss of mitogenic stimulation may modify PEA3 function by altering the phosphorylation state and/or interactions with other proteins. Transcriptional transactivation by PEA3 is enhanced by Ha-ras (27, 53) and the mitogen-activated protein kinase (MAPK) signal transduction pathways involving both the extracellularly regulated and stress-activated MAPKs (39). Phosphorylation of Elk-1 and SAP-1 complexed with serum response factor has been shown to affect transactivation by the ternary complex (12, 17, 18, 50, 57). Thus, during mitogen stimulation of myoblasts, PEA3 may regulate a unique set of genes, including β-enolase.

Overexpression of PEA3 in myoblasts accelerated myogenic differentiation only upon withdrawal of serum. No changes in morphology were detected in the myoblasts maintained in high concentrations of serum, which is unexpected, given that myogenin mRNA was expressed in these myoblasts, supporting the idea that transcription factor function is modified by cellular growth conditions. That absolute levels of myogenin and PEA3 mRNA were not tightly correlated implies that once a threshold level of PEA3 is reached, no effect of further accumulation is readily observed. Consistent with results given above, overexpression of truncated PEA3 appeared to delay myogenic differentiation. Although this process was not analyzed clonally because of considerable clonal variation and the appearance of spontaneous nonfusing variants in both vector-transfected and truncated PEA3-transfected C2C12 cells, analysis of several hundred pooled clones demonstrated altered *ets* DNA-binding activity, a lag in myoblast fusion, and delayed accumulation of myogenin mRNA in cells expressing truncated PEA3. Thus, PEA3 appears to be involved in regulating the early stages of myogenic differentiation.

PEA3 binding sites have been shown to be required for the expression of many genes important for cell proliferation, migration, and metastasis, including genes encoding proteins involved in extracellular matrix turnover, such as the metalloproteinases, both collagenases and stromelysins (14, 54), tissue inhibitor of metalloproteinases-1 (9), and urokinase (26, 45). Activated satellite cells are migratory (20), and several lines of evidence suggest that myoblast fusion also involves metalloproteinase-sensitive mechanisms (24). Moreover, a putative metalloproteinase, meltrin-α, with homology to fertilin, a membrane protein involved in sperm-egg fusion (2, 44), has recently been cloned from a muscle cell line and appears to be important for myoblast fusion (56). Although the array of genes controlled by PEA3 in myoblasts is unknown, loss of normal PEA3 activity appears to delay myoblast fusion, and overexpression of the protein accelerates the differentiation process, suggesting that the observed induction of PEA3 expression in vivo in activated satellite cells is likely to be an important step in muscle regeneration.

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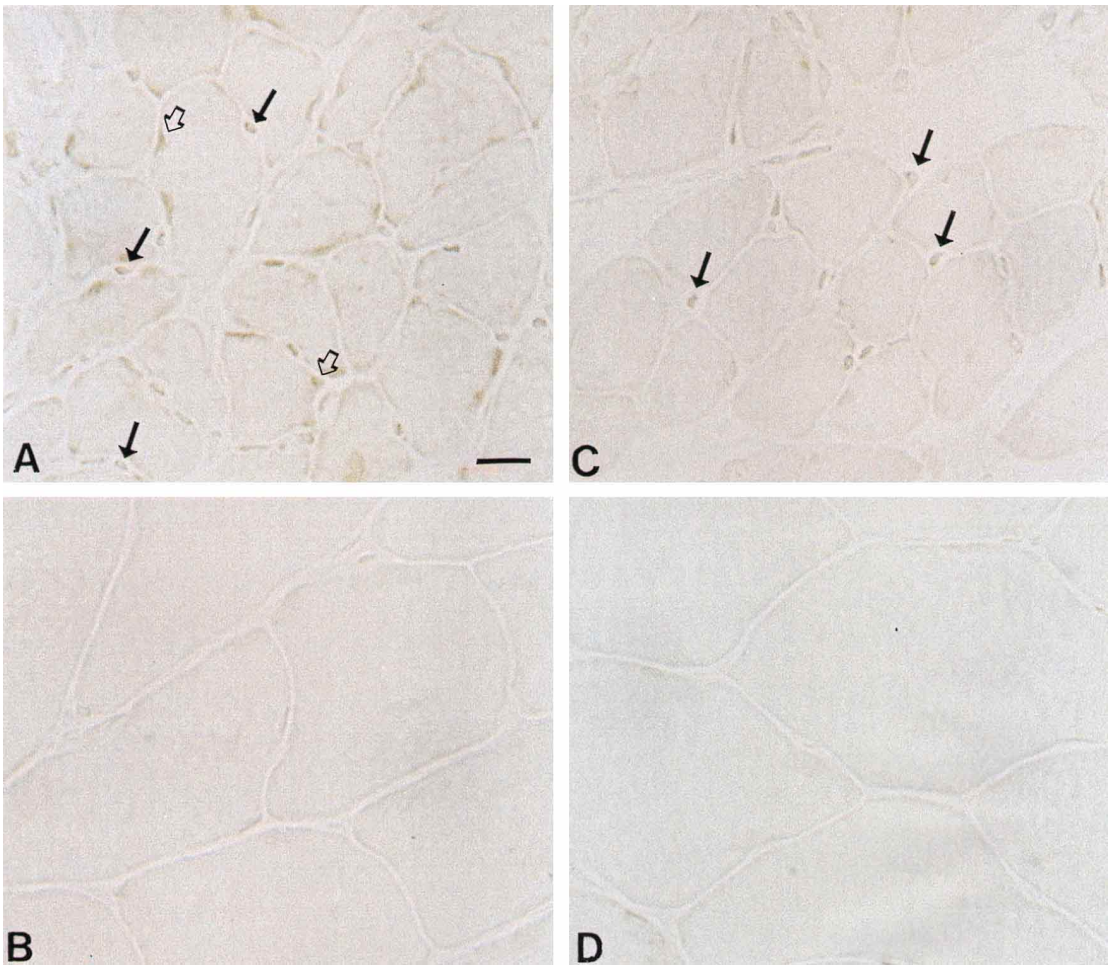

FIG. 9. PEA3 expression in adult rat muscle increased during muscle degeneration. Three weeks after complete unilateral transection of the sciatic nerve, serial cryostat sections of denervated (A and C) and contralateral control (B and D) soleus muscles were analyzed immunocytochemically with monoclonal antibodies specific for MyoD (A and B) and PEA3 (C and D). MyoD and PEA3 are expressed by satellite cells in denervated muscle (representative satellite cells are indicated by black arrows). MyoD is also detectable in putative myofiber nuclei, examples of which are indicated with open arrows. Bar, 50 μm. Animals were cared for and used in accordance with institutional guidelines.

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