α7 Integrin Expression Is Negatively Regulated by δEF1 during Skeletal Myogenesis*

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α7 integrin levels increase dramatically as myoblasts differentiate to myotubes. A negative regulatory element with putative sites for δEF1 is present in the α7 proximal promoter region. To define the role of δEF1 in regulating α7 integrin expression, we overexpressed δEF1 in C2C12 myoblasts. This resulted in a major down-regulation of α7 protein expression. Promoter assays revealed that C2C12 myoblasts transfected with δEF1 showed a decrease in activity of the 2.8-kb α7 promoter fragment, indicating regulation of α7 integrin at the transcriptional level. We have identified two E-box-like sites for δEF1 in the negative regulatory region. Mutation of these sites enhanced α7 promoter activity, indicating that these sites function in repression. MYOD, an activator of α7 integrin transcription, can compete with δEF1 for binding at these sites in gel shift assay. By using chromatin immunoprecipitation, we demonstrated a reciprocal binding of δEF1 and MYOD to this regulatory element depending on the stage of differentiation: δEF1 is preferentially bound in myoblasts to this region, whereas MYOD is bound in myotubes. The N-terminal region of δEF1 is necessary for α7 repression, and this region also binds the co-activator p300/CBP. Importantly, we found that the p300/CBP co-activator can overcome repression by δEF1, suggesting that δEF1 can titrate limiting amounts of this co-activator. These findings suggest that δEF1 has a role in suppressing integrin expression in myoblasts by displacing MYOD and competing for p300/CBP co-activator.

Integrins are heterodimeric molecules involved in cell-matrix binding and regulate diverse processes such as proliferation, survival, differentiation, and motility. Muscle myoblasts bind extracellular matrix proteins by using specific integrins, including the laminin-binding α7 integrin. Levels of α7 integrin increase over 50-fold during myodifferentiation. Tight regulation of α7 integrin ensures low levels of α7 integrin in myoblasts, which facilitates motility during embryogenesis and helps in muscle repair after injury in the adult (1–4). In contrast, myotubes and myofibers have high levels of the α7 integrin essential for stable costameric and myotendinous junctions (5). It is important to define how expression of α7 is regulated, not only for its role in myoblast movement, anchorage, and myotendinous junction formation but also because loss of α7 integrin is associated with certain types of muscular dystrophy/myopathy (6).

It is known that muscle-specific gene expression is regulated positively by the master regulators of the MRF family (myoD, myf5, myogenin, and MRF4) and negatively by Twist (7), Id (8), I-myf (9), δEF1 (10), and several other transcription factors (11–13). Although MYOD is expressed in undifferentiated myoblasts at modest levels, cells do not enter the terminal differentiation program. This failure to differentiate also reflects modulation of MYOD activity that is negatively regulated by several mechanisms, including the transcription factors mentioned above, by subcellular localization (14), and by association with the chromatin-regulating enzymatic activity of histone acetylase (p300/CBP)/deacetyase (15). Consequently, myoblasts remain in an undifferentiated state and are prevented from premature myodifferentiation. Modulation of α7 integrin expression may also be regulated by these mechanisms during skeletal myogenesis.

To understand the regulation of α7 integrin, a 2.8-kb promoter of the mouse α7 integrin promoter was cloned previously and shown to be positively regulated by myoD and myogenin but not by myf-5 or MRF4 (16). Recently, c-Myc, a repressor of myogenesis, was shown to negatively regulate α7 integrin promoter activity in myoblasts by directly binding to the −2.0- to −2.6-kb region of the promoter (17). c-MYC levels are high in proliferating myoblasts but decrease during myotube formation enabling α7 integrin levels to increase during differentiation. Deletion constructs of the α7 integrin promoter have shown that an additional negative regulatory region is present in the −401- to −292-bp region of the mouse α7 integrin promoter (16). Compared with the negative element between −2.1 and −2.8 kb, the region between −401 and −292 bp produces the dominant repression on α7 integrin promoter activity. Although this region is not responsive to c-MYC, it contains potential binding sites for δEF1, a zinc finger transcription factor with a homeodomain between the N- and C-terminal zinc fingers. δEF1 was first identified as a repressor of δ1-crystallin expression in lens (10). Because δEF1 binds E-boxes, the binding elements for basic helix-loop-helix activators such as MYOD, a role for this protein in myogenesis was postulated. Confirming this prediction, δEF1 was found to negatively regulate myogenesis, and overexpression of δEF1 inhibited MYOD-induced muscle differentiation in 10T1/2 cells and repressed MYOD-dependent activation of the muscle creatine kinase enhancer (10). Similar results were observed when the human homolog ZEB was overexpressed in mouse C2C12 myoblasts, where myotube formation was blocked and expression of myosin heavy chain and α1 integrin markers of differentiation were down-regulated (18, 19). Most interestingly, although δEF1/ZEB is a repressor of muscle differentiation, its levels are not down-regulated during muscle differentiation (18). It is thought that increased levels of MYOD displaces δEF1 from E-boxes and overcome δEF1-mediated repression.

In this study, we investigated the regulation of α7 integrin expression by δEF1. Multiple δEF1-like binding sites are present in the proximal
promoter regions that negatively regulate \( \alpha 7 \) promoter activity through interaction with \( \delta EF1 \). Further characterization revealed that this regulatory element also binds MYOD. Inhibition of the integrin promoter by \( \delta EF1 \) is dependent on myodifferentiation status.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—C2C12 myosatellite cells were maintained in growth medium (Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal bovine serum). For differentiation of C2C12 myoblasts to myotubes, cells were grown to 80% confluence in growth medium and subsequently differentiated for 8 days with DMEM supplemented with 2% horse serum (Invitrogen), by which time most of the cells had fused into myotubes (16). 10T1/2 cells were maintained in Earle’s minimum medium with 10% FBS.

**Plasmid Construction**—The deletion constructs of the \( \alpha 7 \) integrin promoter p300, p400, p600, p1.2 kb, and the full-length p2.8-kb promoter have been described previously (16). We have, however, changed the numbering of the promoter region. In this paper, +1 indicates the start site of translation. The p300 construct spans −292 to +3 and p400 spans −401 to +3. By using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), the AGGTG at −378 bp in the p400 construct was mutated to AACTT. The resulting construct was designated p400mut1. Additionally, the GACCTG site at −333 bp was mutated to GCCCGA and resulted in p400mut2. The PAGE-purified primers (IDT, Coralville, IA) used to generate these mutant constructs were the kind gifts from Dr. Yojiro Higashi (Osaka University, Japan). Deletion constructs of \( \delta EF1 \) were made by PCR amplification of the C-zinc-finger amplification region of the \( \alpha 7 \) integrin promoter (−2426 to 3243 bp), the C-zinc finger plus the homeodomain (CZF-HD) from 1357 to 3243 bp, and the C-zinc finger, homeodomain, and N-zinc finger regions (CZF-HD-NZF) from 454 to 3243 bp. The primers used are listed in TABLE ONE. These constructs were cloned at the BamHI and KpnI sites of pcDNA3.1A HisMyc(−). The vectors GAL4 Sp1 and the luciferase reporter vector GAL4Luc were the kind gifts from Dr. Reshma Taneja (Mount Sinai Medical School, New York). The expression vectors for MYOD, MEF2, CBP (CREB-binding protein), and the internal control vector for transfection efficiency, pRL-\beta-galactosidase, were the gifts from Dr. Rik Dernyck (University of California, San Francisco).

**Electrophoretic Mobility Shift Assays**—Nuclear extract was prepared from C2C12 myoblasts or myotubes grown in 10-cm plates according to the protocol of Schreiber et al. (20). The binding reaction (20 μl) included binding buffer (13 mM Hepes, pH 7.9, 60 mM KCl, 0.13 mM EDTA, 2 mM dithiothreitol, and 10% glycerol), 50,000 cpm of radiolabeled probe, and 3–4 μg of nuclear extract. The probes were end-labeled by using [γ\(^{32}\)P]ATP and polynucleotide kinase. DNA-protein binding reactions were carried out for 30 min on ice. Reactions were loaded onto a 5.0% polyacrylamide gel in 0.5× TBE (45 μM Tris, 45 μM boric acid, and 1 mM EDTA). Gels were dried and autoradiographed. For supershift assays, antibodies (1–2 μl) were added overnight to binding reactions. Antibody to \( \delta EF1 \) was obtained from Dr. Yojiro Higashi (Osaka University, Japan). Anti-MYOD antibody was from Novocastra (Newcastle upon Tyne, UK).

**Transient Transfections and Promoter Assays**—C2C12 cells were plated at 150,000 cells/well in 6-well plates (Nunc, Denmark) and transfected 16 h later using Lipofectamine Plus (Invitrogen) as described in the manufacturer’s protocol. Equal amounts of DNA were transfected in duplicate wells, and total DNA transfected per well ranged from 2 to 4 μg. Transfection efficiency was normalized by co-transfection of 80 ng of pRL-\beta-galactosidase as internal control. After 48 h, cells were rinsed with phosphate-buffered saline and lysed with 300 μl of lysis buffer supplied with the CAT enzyme-linked immunosorbent assay kit (Hoffmann-La Roche). For myotubes, 200,000 cells/well were plated in a 6-well plate and transfected as above. The day after transfection the medium was changed from DMEM with 10% FBS to DMEM with 2% FBS. Lysates were prepared after 96 h. Lysates from myoblasts or myotubes were collected, and either CAT or luciferase promoter assays were performed on a luminometer (Turner BioSystems, Sunnyvale, CA). CAT activity was determined from a standard curve using a standard CAT enzyme provided by the manufacturer and expressed as CAT activity relative to that of the empty vector, pCAT. Luciferase activities were determined using the luciferase assay system from Promega (Mad-

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**TABLE ONE**

| Primer name       | Sequence                                             |
|-------------------|------------------------------------------------------|
| CZF forward       | 5′-CCCGGATTCACCATGCGCATCTGAGCAGAACAGT-3′             |
| CZFHD forward     | 5′-CCCGGATTCACCATGCGCATCTGAGCAGAACAGT-3′             |
| CZFHDNZF forward  | 5′-CCCGGATTCACCATGCGCATCTGAGCAGAACAGT-3′             |
| EF1 reverse       | 5′-CCGCTGATCCGCTAGTACCATGATTACTGCTCCTTCTT          |
| P400MUT (−378)    | 5′-GTGCGACAGTGGCAACTCCGGGGGAGACCCGGAGGCGCG          |
| P400MUT (−333)    | 5′-GGCTGGCCGCCTGCTGACATAACGACCCGGGCTGCTGCT          |
| P300MUT (123)     | 5′-GAAGTGGGCAGGGTGGTGTCTGAGTACATGGAGAAGTTGGAAGACC   |
| P300MUT (−58)     | 5′-CCGAGAACACCTCCGGGAGATCTCCGGGGCTGCTGCTG          |
| −378 forward (gel shift) | 5′-GTAGCGACAGTGGCAACTCCGGGGGAGACCCGGAGGCGCG          |
| −333 forward (gel shift) | 5′-GGCTGGCCGCCTGCTGACATAACGACCCGGGCTGCTGCT          |
| 123 forward (gel shift) | 5′-CTGAGAAGGCGGCTGAAAGAGATGGGAAGACC                 |
| −58 forward (gel shift) | 5′-CCGGCCGGAGACCCTGGGCTGCTGCTGCTGCTG           |
| −378 F (PCR)      | 5′-CCGAGACCTGGCTAGGAGTTACCTCGATTGGGAGAAGCGCCAAGCT   |
| α7 F (PCR)        | 5′-CTAGGTAGGCTAGTTAGCTACACTGCAACGCTTAGGGA           |
| mGAPDH F          | 5′-CCCTGACACAGCAACTGCTTAA                         |
| mGAPDH R          | 5′-CTTACTCCTGGGAGCCCATGTA                         |
ison, WI) and expressed as arbitrary units. β-Galactosidase activity was determined with a kit from Tropix (Applied Biosystems, Foster City, CA). All transfection experiments were done in duplicate and repeated at least three times. Results are expressed as mean ± S.D.

Western Blotting—Whole cell lysates were prepared from C2C12 myoblasts or myotubes. In brief, cells were rinsed with several changes of cold cell rinse buffer (40 mM Hepes, pH 7.4, 1 mM EDTA, 0.15 M NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin). Cells were scraped into a cell rinse buffer using a rubber policeman and pelleted. The pellet was resuspended in chilled cell resuspension buffer (40 mM Hepes, pH 7.9, 0.4 M KCl, 1 mM dithiothreitol, 10% glycerol). Protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin) were added to the buffer just before use. The cells were subjected to three rounds of freezing and thawing. Debris was removed by spinning at 13,000 rpm at 4 °C. The supernatant was collected, frozen in aliquots, and stored at −70 °C. Protein levels were quantified by the BCA protein assay (Pierce). Equal amounts (20–50 μg) of total proteins from each sample were loaded onto

FIGURE 1. α7 Integrin promoter analysis. A, promoter activity of deletion constructs of mouse α7 integrin promoter. C2C12 myoblasts or 10T1/2 cells were transiently transfected with deletion constructs of mouse α7 integrin promoter. The deletion constructs used were p300 (−292 to +3 bp), p400 (−401 to +3 bp), p600 (−617 to +3 bp), p1.2 (−1160 to +3 bp), and p2.8 (−2809 to +3 bp). Cell lysates were prepared 48 h after transfection, and CAT promoter activity was measured and expressed as activity relative to that of empty vector pCAT. Transfection efficiency was normalized by using the β-galactosidase vector as an internal control. Negative regulatory elements were present between −401 and −292 bp and between −2809 and −1160 bp. The control 10T1/2 cells did not express α7 integrin and showed negligible α7 promoter activity. Data represent the mean of three separate experiments with error bars indicating S.D. B, promoter sequence analysis of the mouse α7 integrin gene. The proximal promoter region of the α7 integrin gene is shown. Potential E-box-like sites for EF1(a/g/T/CACCT) and MYOD (CANNTG) were identified at −378, −333, −123, and −58 bp (boxed region) upstream of the translation initiation site in the mouse α7 promoter. Several Sp1 sites as well as an AP1-binding site were present as reported previously. An initiator-like sequence at −174 bp and the translation start site at +1 bp are also indicated.
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an 8% SDS-polyacrylamide gel. Proteins were separated by SDS-PAGE, transferred onto polyvinylidene difluoride transfer membranes (Millipore, Billerica, MA), blotted with specific antibodies, and detected with an ECL Western blotting kit (Amersham Biosciences) according to the protocol provided by the manufacturer. Antibody to mouse δEF1 was a gift from Dr. Yojiro Higashi (Osaka University, Japan). To detect α7 integrin, polyclonal 1211 antibody against mouse α7 integrin was used (21). Anti-MYOD antibody (sc760) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and tubulin antibody from NeoMarkers (Fremont, CA).

**RT-PCR**—C2C12 myoblasts were transiently transfected with either empty vector or an equal amount of δEF1 expression vector using Lipofectamine Plus as mentioned above. Total RNA was prepared after 48 h using the RNeasy kit from Qiagen (Valencia, CA). Equal amounts of control or δEF1-transfected RNA were reverse-transcribed to cDNA using Stratascript RT enzyme (Stratagene, La Jolla, CA). PCR was carried out as described previously (17) by using primers against exons 1a and 1b of the EF1 expression vector. PCR products were analyzed on a 2% agarose gel.

**Chromatin Immunoprecipitation (ChIP)**—C2C12 myoblasts and myotubes were used for ChIP assay. A kit supplied by Upstate (Charlottesville, VA) was used following the manufacturer’s protocol. C2C12 myoblasts and myotubes were grown in 100-mm plates as described above. Proteins bound to DNA were fixed by the addition of formaldehyde (final 1%) to the medium. Cells were washed with cold phosphate-buffered saline and lysed with SDS-Lysis buffer supplied with the kit. The DNA in the lysate (from 1 × 10^6 cells) was sheared to between 500 and 1000 bp by sonication using a Vibracell sonicator (Sonics Materials, Inc., Newtown, CT) set to 25% output (four 10-s pulses with cooling between pulses). The lysate was further processed as per the manufacturer’s protocol. Immunoprecipitation was carried out by incubation overnight with antibodies against δEF1 (from Dr. Yohiro Higashi, Osaka University, Japan), MYOD (Santa Cruz Biotechnology), or control IgG antibody. DNA-antibody complexes were collected and processed as per the kit protocol. Eluted DNA was resuspended in 30 μl of TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). For PCR, primers that span the regulatory regions that control transcription of the EF1 expression vector were used (see TABLE ONE). PCR products were analyzed on a 2% agarose gel.

**RESULTS**

δEF1 Is a Negative Regulator of α7 Integrin Expression—Previous studies have shown that α7 integrin remains at basal levels in myoblasts, but mRNA and protein expression are strongly elevated following myo-differentiation (17, 22). To gain an understanding of the promoter regulatory elements that control α7 integrin levels during differentiation, several deletion constructs were analyzed for promoter activity in C2C12 myoblasts. As shown in Fig. 1A, the region from −401 to −292 bp displays significant negative regulation (compare p400 and p300). Two E-box sites are present in this region (Fig. 1B); however, we did not identify binding sites for any other known potential repressors. E-box sites are known to be involved in muscle gene activation through helix-loop-helix proteins of the MRF family (MYOD, myf-5, myogenin, and MRF). A search for E-box-binding myogenic repressors identified the δEF1 transcription factor. A consensus δEF1-binding site (CACC(T/A)GGTG) is present at −378 bp upstream of the translation start site (Fig. 1B), and a second potential binding site GACCTG is also present at −333 bp.

To test if δEF1 has a role in the regulation of α7 integrin, we transfected C2C12 mouse myoblasts with an expression vector for δEF1. Myoblasts transfected with the δEF1 expression vector showed a striking decrease in α7 integrin expression at the protein level by Western blot compared with cells transfected with a control vector (Fig. 2A). Tubulin levels indicate equal protein loading. Most interestingly, MYOD levels were unchanged following δEF1 overexpression, indicating that down-regulation in α7 integrin levels is not an indirect consequence of lower MYOD levels. To determine whether δEF1-induced loss of α7 expression resulted from transcriptional down-regulation, we transfected C2C12 myoblasts with a full-length 2.8-kb mouse α7-CAT promoter construct and performed promoter activity assays. Cells were co-transfected with either an empty expression vector or with increasing concentrations of δEF1 expression vector. As seen in Fig. 2B, in the

**FIGURE 2. δEF1 down-regulates α7 integrin expression. A**, effect of δEF1 on α7 integrin protein expression. C2C12 myoblasts were transiently transfected with either 4 μg of empty vector or the expression vector for δEF1. Cell lysates were analyzed by Western blotting for expression levels of α7 integrin, MYOD, and δEF1. Following overexpression of δEF1, α7 integrin expression was significantly decreased, whereas MYOD levels did not change. Tubulin levels indicate protein loading. **B**, overexpression of δEF1 represses α7 integrin promoter activity. C2C12 cells were transiently transfected with the full-length promoter p2.8 and co-transfected with either empty pcDNA vector or with increasing amounts of δEF1 expression vector. CAT activity was determined as in Fig. 1. A dose-dependent decrease in promoter activity was induced with increasing amounts of δEF1 vector. The data represent the mean ± S.D. of three independent experiments. **C**, effect of δEF1 on α7 integrin mRNA levels. C2C12 cells were transiently transfected with either empty vector or δEF1 vector. RNA was prepared after 48 h, and RT-PCR for α7 integrin and GAPDH was performed as described under “Experimental Procedures.” δEF1 induced a decrease in α7 integrin mRNA.
FIGURE 3. Functional analysis of the putative $\delta$EF1/MYOD-binding sites. A, mutation of putative $\delta$EF1-binding sites at −378 and −333 bp on the $\alpha 7$ promoter resulted in release of repression. C2C12 cells were transiently transfected with either the wild-type p400 construct or with p400mut1 (mutant −378-bp site) or p400mut2 (mutant −378- and −333-bp sites). CAT activity was determined and expressed as CAT activity relative to that of empty vector pCAT. Mutation of the E-box sites at −378 and −333 bp resulted in release of repression. To test if repression was DNA-binding dependent, C2C12 myoblasts were transiently transfected with wild-type p400 or p400mut2 and co-transfected with either control or expression vector for $\delta$EF1. Overexpression of $\delta$EF1 resulted in repression of the wild-type p400 as well as the mutant p400 construct, indicating the possibility of repression through non-DNA binding mechanisms or the presence of additional $\delta$EF1-binding sites. B, mutation of putative $\delta$EF1-binding sites at −123 and −58 bp resulted in minimal release of repression. CAT activity was determined as above. Mutation of the −123- and −58-bp sites resulted in a slight increase in promoter activity. To determine whether $\delta$EF1 could repress the wild-type p300 and mutant p300 constructs, C2C12 cells were transfected with the wild-type or p300mut2 construct and co-transfected with either control vector or expression vector for $\delta$EF1. Lysates were prepared, and CAT activity was determined as above. Overexpression of $\delta$EF1 resulted in a slightly greater repression of the wild-type p300 promoter as compared with the p300mut2 construct. Repression of both constructs indicated that $\delta$EF1 may bind the wild-type and mutant sites when overexpressed. C, the E-box-like sites at −123- and −58-bp function in activation. C2C12 myoblasts were transiently transfected with the wild-type p300mut1 or p300mut2 promoter fragments and co-transfected with either control vector or MYOD expression vector and processed as above. MYOD activated the wild-type promoter but mutation of the −123- and −58-bp sites prevented the MYOD-induced activation of the p300mut2 construct. D, transactivation by Sp1 was not blocked by $\delta$EF1. C2C12 cells were transiently transfected with the GAL4 reporter construct GAL4Luc and co-transfected with expression vectors for fusion proteins GAL4Sp1 or GAL4Sp1 and $\delta$EF1. Luciferase activity was measured as described under “Experimental Procedures.” Transfection efficiency was normalized by using the $\beta$-galactosidase vector as an internal control. Results reflect three independent experiments expressed as the means ± S.D. The results indicate that $\delta$EF1 does not modify transactivation of promoter activity by Sp1.
FIGURE 4. Binding of δEF1 and MYOD to the putative sites in the α7 promoter. A, δEF1 binds the −378-bp site, and MYOD binds the −378, −333, −123, and −58-bp sites. EMSA was performed as described under “Experimental Procedures” using C2C12 nuclear extracts and 32P-radiolabeled oligonucleotides based on sequence of the E-boxes at −378, −333, −123 bp, and −58 bp of the α7 integrin promoter (left panel). FP lane indicates free probe. Supershift analysis with δEF1 antibody indicated that δEF1 binds the −378-bp site in the α7 integrin promoter (left panel) and may also bind the −58-bp site (center panel). Supershift analysis with antibody to MYOD indicated that MYOD could bind the −378- and −333-bp sites (left panel) and the −123- and −58-bp sites (center panel). Binding of δEF1 and MYOD to the −378-bp site after myoblast differentiation (right panel) is shown. EMSA and supershift analysis were performed using nuclear extracts from C2C12 myotubes as described under “Experimental Procedures.” The results indicate that in differentiated C2C12 myotubes, δEF1 antibody fails to produce a supershift at the −378-bp site. B, MYOD and δEF1 have reciprocal effects on the α7 promoter in myoblasts; δEF1 loses repressive effect after myoblast differentiation to myotube. C2C12 myoblasts were transfected with the p400 construct or the full-length p2.8 α7 promoter. The cells were co-transfected with empty vector or with expression vector for δEF1, MYOD, δEF1 and MYOD, or δEF1 and MEF2. Transfection efficiency was normalized by using the β-galactosidase vector as an internal control.
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presence of increasing concentrations of δEF1, α7 promoter activity decreased progressively and plateaued with maximal inhibition near 70% with 3–4 μg of δEF1, indicating that δEF1 decreases α7 integrin expression through a transcriptional mechanism. In order to confirm repression at a transcriptional level, we also performed semi-quantitative RT-PCR for α7 integrin in C2C12 cells transfected with either empty vector or δEF1 expression vector. As seen in Fig. 2C, α7 integrin mRNA levels decrease when δEF1 is overexpressed, confirming that δEF1 exerts its effect at a transcriptional level.

Mutation of Putative δEF1 Sites Results in Release of Repression—In order to determine whether the two potential δEF1-binding sites at −378 and −333 bp function in negative regulation, we used site-directed mutagenesis to generate mutant binding sites and then performed CAT promoter activity assays. We used the p4000 (−401 to +3) construct to generate p4000mut1 having a mutant −378-bp E-box as well as a construct p4000mut2 with mutant E-box sites at −378 and −333 bp. C2C12 cells were transfected with wild-type or mutant constructs, and promoter activity was assayed. Mutation of the −378-bp site resulted in release of repression (activity increased over 2-fold), verifying the site’s function as a negative regulatory element (Fig. 3A). Mutation of the site at −333 bp resulted in only a small additional increase in promoter activity, indicating that this site may have a minor role in suppression of α7 integrin expression. These results indicate that endogenous δEF1 may be repressing the α7 promoter by binding at the −378-bp site and that the mechanism of repression by δEF1 depends on its targeting these sequences.

We were interested in determining whether DNA binding by δEF1 is the only mechanism of repression. To test this, C2C12 cells were co-transfected with δEF1 and either wild-type or mutant p400 constructs. If δEF1 repression requires DNA binding, then mutation of the δEF1-binding sites should prevent repression. As seen in Fig. 3A, δEF1 was able to repress the wild-type p400 as well as the mutant p4000mut2 construct, indicating that non-DNA binding mechanisms may be operative. It is also possible that additional unidentified δEF1-binding sites may be present in the p400 construct. We identified an AGGTC site located at −123 bp and a GACCTG site at −58 bp upstream of the ATG translation start site, respectively. We examined if these two sites are involved in repression by δEF1 by using a shorter promoter fragment, p300 (−292 to +3 bp), that has the −123- and −58-bp sites but lacks the two δEF1 sites at −378 and −333 bp. The site at −123 bp was mutated to give the construct p300mut1. A second construct p300mut2 contained mutations at −123 and −58 bp. C2C12 cells were transfected with the wild-type p300 or the mutant p300mut2 promoter constructs, and promoter activity was assayed. As seen in Fig. 3B, mutation of these two sites in the p300 fragment resulted in only a slight increase in promoter activity, indicating that these sites may not be important in negative regulation. We tested whether DNA binding is the exclusive mechanism of repression by transfecting C2C12 cells with δEF1 and wild-type p300 or mutant constructs. δEF1 could repress the wild-type p300 (activity decreased to ~50%) as well as the mutant p300mut2 construct (activity decreased to ~69%) (Fig. 3B). Because we did not identify any additional δEF1-binding sites in the p300 construct, we tested the possibility that, under conditions of overexpression, δEF1 is able to bind the mutant E-box. We found by electrophoretic mobility shift assay (EMSA) that the mutant site does not compete for binding δEF1, although with lower affinity than wild-type δEF1 (data not shown), and may explain the ability of δEF1 to repress the mutant p400 and p300 constructs. δEF1 may repress the p300 promoter through non-DNA binding mechanisms, but further work is needed to test this possibility.

Because the two E-box sites in the p300 construct did not appear to play a major role in repression, we next assessed whether these two sites might activate the α7 integrin through E-box binding by MYOD. C2C12 myoblasts were transfected with myoD and the wild-type p300 construct or mutant p300mut1 and p300mut2 constructs. We found that MYOD could activate the wild-type p300 construct (Fig. 3C); however, mutation of the −123-bp site (p300mut1) resulted in decreased activation by MYOD. Further mutation of both sites led to a complete loss of activation by MYOD (p300mut2). These results suggest that δEF1 represses the α7 promoter through the −378-bp site and that MYOD activates the promoter through E-box sites at −123 and −58 bp immediately upstream of the translation start site. MYOD is known to activate the full-length p28 α7 promoter (16, 17), and the current experiments have identified additional MYOD-binding sites in the α7 promoter.

To examine if a non-DNA binding mechanism of repression might also be operative, we investigated whether δEF1 interacts with other transcription factors that bind the α7 promoter within the −401- to +3-bp region. We used a GAL4 reporter assay to test the effect of δEF1 on transactivation by Sp1, as several Sp1 sites are present in this region. As seen in Fig. 3D, δEF1 had no effect on transactivation by Sp1. This result indicates that δEF1 does not repress through an effect on Sp1. It is possible that δEF1 may target other transcription factors, such as AP1, which we have not yet tested.

Identification of Potential δEF1-Binding Sites; Repression Correlates with δEF1 Binding—Functional studies on the α7 promoter identified the −378-bp site as responsible for repression and the −123- and −58-bp sites as involved in activation. We wanted to analyze the binding of δEF1 and MYOD to the various E-box-like sites in this region. The consensus binding site for δEF1 is a/g/T/CACCT or the palindromic AGGTG/A/c/t; the lowercase represents a lower frequency of occurrence of the nucleotide in the δEF1 consensus binding site (23). The AAGGTG site at −378 bp is a consensus δEF1-binding site (23). The GACCTG site at −333 bp may also bind δEF1. In order to check for δEF1 binding at the −378- and −333-bp sites, we used EMSA and supershift assay using C2C12 nuclear extracts. In these assays, δEF1 was found to bind to the −378-bp site, where a single band was shifted by the δEF1 but not to the −333-bp site (Fig. 4A, left panel). Similarly, EMSA also indicates that MYOD can bind the E-boxes at −378 and −333 bp. MYOD may thus compete with δEF1 for binding and displace δEF1 from the −378-bp site when MYOD levels are high, for example, in differentiating myotubes. Additionally, the results on binding correlate with the results on mutation of the −378-bp site which resulted in release of repression (Fig. 3A). Two additional sites that may bind δEF1 are present at −123 and −58 bp. We tested δEF1 and MYOD binding to

MYOD and MEF2 could overcome repression of α7 promoter by δEF1, indicating a reciprocal activity with δEF1. Similarly, C2C12 cells were transfected with the p400 or the full-length p28 α7 promoter and co-transfected with δEF1 following induction of differentiation as described under “Experimental Procedures.” Cell lysates were prepared from myotubes, and promoter activity was determined. The results show that δEF1 failed to repress the α7 promoter under physiological conditions of myoblast differentiation. C, δEF1 expression during myoblast differentiation to myotube. Equal amounts of whole cell lysates prepared from C2C12 myoblasts or differentiated myotubes were separated by SDS-PAGE and blotted with anti-δEF1 antibody. The results indicate that δEF1 levels did not change during myoblast differentiation, D, δEF1 binding using ChIP analysis. In vivo binding of δEF1 and MYOD to the α7 integrin promoter is shown. DNA-protein cross-linking was carried out as detailed under “Experimental Procedures.” Antibodies to δEF1 and MYOD were used to immunoprecipitate promoter-bound factors. Input is DNA not processed for immunoprecipitation. Negative control represents a sample that was immunoprecipitated by using normal IgG. PCR was carried out using primers (−378F and α7R) spanning the p400 region. The results indicate that δEF1 but not MYOD preferentially binds the α7 promoter in C2C12 myoblasts, whereas MYOD but not δEF1 binds to the α7 promoter after myoblast differentiation.
the putative binding site at −123 bp (GAGGGT) and the −58-bp site (GACCTG). We found that δEF1 appears to bind the −58-bp site but not the −123-bp site, whereas MYOD binds both sites (Fig. 4A, center panel). We reported earlier that mutation of the −123- and −58-bp sites indicated that these sites may be more relevant in activation by MYOD, rather than repression by δEF1 (Fig. 3C). These results correlate with the EMSA results that indicate that MYOD, rather than δEF1, binds at these sites.

**Role of δEF1 during Myodifferentiation**—The above results indicate that δEF1 and MYOD may compete for binding the −378-bp site in the α7 promoter. We tested whether MYOD and δEF1 have reciprocal effect on α7 promoter activity. We transfected C2C12 cells with the full-length p2.8 and p400 fragment of the α7 promoter. MYOD and MEF2 overcame repression by δEF1, indicating that δEF1 and MYOD have reciprocal effects on α7 promoter activity (Fig. 4B). We further studied this activity in the physiological context of myoblast-to-myotube differentiation by assessing repression of α7 promoter activity by δEF1 in differentiating C2C12 cells. We found that δEF1 failed to repress the p400 or p2.8 promoter constructs on myodifferentiation (Fig. 4B). This may be due to loss of δEF1 binding to the −378-bp site during myodifferentiation. We also used EMSA analysis of nuclear extracts prepared from differentiating myotubes to assess binding of δEF1 to the −378-bp site. δEF1 did not bind to this site after myogenic differentiation as evidenced by the absence of band shift by anti-δEF1 antibody; however, MYOD retained its binding to this site as shown by band shift with anti-MYOD antibody (Fig. 4A, right panel).

The possibility that MYOD may displace δEF1 from its binding site during myodifferentiation was further explored. Although it is well known that MYOD levels rise during myodifferentiation, the relative protein levels of δEF1 appear to be constant in C2C12 myoblasts and myotubes (Fig. 4C). We next used chromatin immunoprecipitation to further the relative interaction of δEF1 and MYOD with the proximal promoter region in vivo. ChIP assays were performed using anti-δEF1 and anti-MYOD antibodies to immunoprecipitate α7 promoter fragments bound by the two transcription factors. Antibody to δEF1 immunoprecipitated the α7 promoter fragment from myoblast (primers amplified a region between −378 and +3 bp) as identified by PCR (Fig. 4D). In contrast, in myoblasts, anti-MYOD failed to yield any detectable product in the −378- to +3-bp region of the α7 promoters. The situation was reversed after myoblast differentiation to myotubes, where antibody to MYOD effectively immunoprecipitated the region between −378 and +3 bp, but anti-δEF1 antibody failed to recover α7 promoter fragments in this region. This indicates that the mechanism by which α7 integrin levels increase during differentiation may be due to the reciprocal loss of δEF1 binding and an increase in MYOD binding to the α7 promoter in the negative regulatory region (−401 to +3 bp).

**Mechanism of Repression by δEF1**—ChIP assays indicated that δEF1 binds to the endogenous gene, suggesting a direct DNA-binding mechanism of repression and possibly a displacement of δEF1 by MYOD during muscle differentiation. We further investigated the domains of δEF1 required for repression of the α7 promoter by using the following three vectors expressing truncated forms of δEF1: construct CZF expressed the C-zinc finger region, construct CZF-HD expressed C-zinc fingers/homeodomain region, and construct CZF-HD-NZF expressed C-zinc fingers/homeodomain/N-zinc fingers. Only the full-length δEF1 protein was able to fully repress the α7 promoter (Fig. 5A). Vectors expressing CZF, CZF-HD, or CZF-HD-NZF were not able to efficiently repress the α7 promoter. This indicates that a crucial repression domain lies upstream of the N-zinc fingers in the region called the NR (negative region) domain. Most interestingly, the NR-containing region is known to bind p300/CREB-binding protein (co-activator proteins) (24). Subsequently, we tested whether overexpression of CBP could reverse repression because of δEF1. In promoter assays, we found that overexpression of MYOD could lead to increase activity, but this increase was lost in the presence of higher levels of δEF1 (Fig. 5B). Furthermore, CBP could reverse the repression by δEF1, indicating that δEF1 can compete for limited amounts of CBP. Similar results were obtained with full-length p2.8 promoter fragment (results not shown). The ability of CBP to overcome repression by δEF1 is comparable with that of MYOD (Fig. 5B).
DISCUSSION

Regulation of the α7 integrin during myodifferentiation is complex. Controlled expression of this integrin is important because it contributes to mechanical stability during muscle contraction, and its loss leads to congenital myopathy (6, 25). Importantly, α7 integrin expression in myoblasts remains low, facilitating cell motility needed during tissue remodeling and alignment of cells prior to fusion and myotube formation. Induction of differentiation to myotubes generates more than a 20-fold increase in α7 mRNA levels and a parallel increase in promoter activity (17, 22). Our previous studies have shown that the α7 promoter is tightly regulated, and gene expression is intricately modulated by MYOD family members as well as c-MYC (17). A negative regulatory element has been identified in the proximal promoter that contains E-box-like sequences that may be potential binding sites for δEF1. The current results indicate a role for δEF1 in suppression of α7 promoter activity through targeting this regulatory region, whereas during myotube differentiation this inhibitory activity is attenuated by increased levels of competing MYOD.

We found that forced expression of δEF1 in C2C12 myoblasts induced a major reduction in α7 integrin levels, suggesting a role for δEF1 as a selective negative regulator of α7 integrin gene transcription. MYOD levels were unchanged following δEF1 overexpression, indicating that δEF1 does not repress the α7 integrin gene by modulating MYOD levels. δEF1 may control α7 promoter activity by competing with MYOD for binding sites. We found by gel shift that δEF1 and MYOD compete for binding the α7 promoter in the negative regulatory region and that δEF1 and MYOD have opposite effects on α7 promoter activity. We tested for differential binding of δEF1 and MYOD during myogenic differentiation by ChIP analysis. We found that in myoblasts δEF1 preferentially binds the α7 promoter. However, in myotubes binding efficiency to the promoter shifts to MYOD, thereby releasing the repression and enhancing α7 gene transcription during differentiation.

However, simple displacement of MYOD by δEF1 does not seem to be the mechanism by which δEF1 represses the α7 promoter. MYOD binds E-boxes with the consensus sequence CAANNG, whereas δEF1 binds the E-box-like sequence a/g/T/CACCT. Because δEF1 binds only a subset of E-boxes, it is therefore not clear how it represses myogenesis. We found that only the full-length δEF1 construct could repress α7 integrin expression. We tested deletion constructs having the C-terminal zinc fingers (CFZ), the C-terminal zinc fingers plus the homeodomain CFZ-HD, or the C-terminal zinc fingers homeodomain and N-terminal zinc fingers CFZ-HD-NZF. All of these constructs failed to significantly repress α7 integrin expression, indicating that the N-terminal domain of δEF1 is needed for repression. Sekido et al. (23) also reported that an NR (negative region) domain upstream of the N-terminal zinc fingers is needed for repression of the δ-crystallin enhancer. However, ZEB, the human homolog of δEF1, represses the α4 integrin promoter in muscle through the region between the N-terminal zinc fingers and homeodomain. Neither the N-terminal nor the C-terminal zinc fingers were needed for repression of the α4 integrin (18). The same authors reported that the C-terminal domain of ZEB repressed muscle genes by targeting the trans-activation domain of MEF2 (19). This shows that δEF1 and ZEB are homologs that differ significantly in the domains needed for muscle-gene repression. However, both ZEB and δEF1 target MEF2, a gene essential for muscle gene expression.

In our study, the co-activator CBP could overcome repression by δEF1. It is known that δEF1 binds p300/CBP and may compete with MYOD for limited amounts of p300/CBP. p300/CBP has histone acetylase activity that opens up the chromatin to allow transcription. MYOD and MEF2 activate muscle genes by binding p300 and using the histone acetylase activity of p300/CBP to allow transcription (26, 27). Unfortunately, the exact position where p300/CBP binds δEF1 is not known, and it is currently not possible to test whether mutation of the CBP-binding sites of δEF1 might cause δEF1 to lose the ability to repress the α7 integrin gene. Postigo and Dean (19) also found that forced expression of p300 overcame the repression of c-myc and ets sites by ZEB in hematopoietic cells as well as the repression of MEF2 and NFκB. However, in their study, region 1 between the N-zinc fingers and the homeodomain was implicated as targeting p300 activity. Because δEF1/ZEB binds p300 at the extreme N-terminus upstream of the N-zinc fingers, it is not clear how region 1 may affect p300 activity. It seems that multiple domains in ZEB target the co-activator p300/CBP.

We reported earlier that c-Myc down-regulates α7 integrin expression (17). Normal levels of p300/CBP are needed to keep c-MYC repressed, and depletion of p300/CBP is known to result in raised c-MYC levels and inappropriate entry into S-phase (28). It is possible that titration of limited amounts of p300/CBP by δEF1 results in raised c-MYC levels and that c-MYC and δEF1/ZEB thereby cooperate to regulate muscle gene expression. It will be interesting in future studies to examine if δEF1 and c-MYC synergize to down-regulate α7 integrin expression in myoblasts.

In summary, this study shows that δEF1 controls α7 integrin expression in myoblasts by competing with MYOD for binding to the negative regulatory region of the α7 integrin promoter. During skeletal myogenesis increased levels of MYOD displace δEF1 from the promoter inducing elevated α7 integrin levels. The mechanism by which δEF1 represses the α7 integrin promoter is more complex than simply passive displacement of MYOD. Rather, it is likely that the ability of δEF1 to compete for limited amounts of the co-activator p300/CBP is responsible for repression of α7 integrin in myoblasts. Following differentiation into myotubes, sustained high levels of MYOD continue to override δEF1 repression, resulting in persistent elevated α7 integrin expression.

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REFERENCES

1. Boa, Z. Z., Lakonishok, M., Kaufman, S., and Horwitz, A. F. (1993) J. Cell Sci. 106, 579–589
2. Belkin, A. M., Zhidkova, N. I., Balzac, F., Altura, F., Tomatis, D., Maier, A., Tarone, G., Koteliansky, V. E., and Burridge, K. (1996) J. Cell Biol. 132, 211–226
3. Martin, P. T., Kaufman, S. J., Kramer, R. H., and Sanes, J. R. (1996) Dev. Biol. 174, 125–139
4. van der Flier, A., Gaspar, A. C., Thorstensdottir, S., Baudoin, C., Groeneveld, E., Mummery, C. L., and Sonnenberg, A. (1997) Dev. Biol. 201, 472–486
5. Mione, N., Klenczar, C., Herken, R., Willemin, M., and Mayer, U. (1999) Lab. Invest. 79, 1591–1599
6. Hayashi, Y. K., Chou, F. L., Engvall, E., Ogawa, M., Matsuura, C., Hiranayashi, S., Yokochi, K., Ziober, B. L., Kramer, R. H., Kaufman, S. J., Ozawa, E., Goto, Y., Nonaka, I., Tsukahara, T., Wang, J. Z., Hoffman, E. P., and Arakawa, K. (1998) Nat Genet. 19, 94–97
7. Zhao, P., and Hoffman, E. P. (2004) Dev. Dyn. 229, 380–392
8. Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L., and Weintraub, H. (1990) Cell 61, 49–59

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9. Chen, C. M., Kraut, N., Groudine, M., and Weintraub, H. (1996) Cell 86, 731–741
10. Sekido, R., Murai, K., Funahashi, J., Kamachi, Y., Fujisawa-Sehara, A., Nabeshima, Y., and Kondoh, H. (1994) Mol. Cell. Biol. 14, 5692–5700
11. Li, L., Chambard, J. C., Karin, M., and Olson, E. N. (1992) Genes Dev. 6, 676–689
12. Lassar, A. B., Thayer, M. J., Overell, R. W., and Weintraub, H. (1989) Cell 58, 659–667
13. Lemercier, C., To, R. Q., Carrasco, R. A., and Konieczny, S. F. (1998) EMBO J. 17, 1412–1422
14. Vandromme, M., Carnac, G., Gauthier-Rouviere, C., Fesquet, D., Lamb, N., and Fernandez, A. (1994) J. Cell Sci. 107, 613–620
15. McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2002) Curr. Opin. Cell Biol. 14, 763–772
16. Ziober, B. L., and Kramer, R. H. (1996) J. Biol. Chem. 271, 22915–22922
17. Xiao, J., Jethanandani, P., Ziober, B. L., and Kramer, R. H. (2003) J. Biol. Chem. 278, 49780–49788
18. Postigo, A. A., and Dean, D. C. (1997) EMBO J. 16, 3935–3943
19. Postigo, A. A., and Dean, D. C. (1999) Mol. Cell. Biol. 19, 7961–7971
20. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) Nucleic Acids Res. 17, 6419
21. Yao, C. C., Breuss, J., Pytela, R., and Kramer, R. H. (1997) J. Cell Sci. 110, 1477–1487
22. Burkin, D. J., and Kaufman, S. J. (1999) Cell Tissue Res. 296, 183–190
23. Sekido, R., Murai, K., Kamachi, Y., and Kondoh, H. (1997) Genes Cells 2, 771–783
24. Postigo, A. A., Depp, J. L., Taylor, J. J., and Kroll, K. L. (2003) EMBO J. 22, 2453–2462
25. Pegoraro, E., Cepollaro, F., Prandini, P., Marin, A., Fanin, M., Trevisan, C. P., El-Messlemani, A. H., Tarone, G., Engvall, E., Hoffman, E. P., and Angelini, C. (2002) Am. J. Pathol. 160, 2135–2143
26. Sartorelli, V., Huang, J., Hamamori, Y., and Kedes, L. (1997) Mol. Cell. Biol. 17, 1010–1026
27. McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2001) Curr. Opin. Genet. Dev. 11, 497–504
28. Rajabi, H. N., Baluchamy, S., Kolli, S., Nag, A., Srinivas, R., Raychaudhuri, P., and Thimmmapaya, R. (2005) J. Biol. Chem. 280, 361–374
