Expression of the laminin-binding $\alpha_7$ integrin is tightly regulated during myogenic differentiation, reflecting required functions that range from cell motility to formation of stable myotendinous junctions. However, the exact mechanism controlling $\alpha_7$ expression in a tissue- and differentiation-specific manner is poorly understood. This report provides evidence that $\alpha_7$ gene expression during muscle differentiation is regulated by the c-Myc transcription factor. In myoblasts, $\alpha_7$ is expressed at basal levels, but following conversion to myotubes the expression of the integrin is strongly elevated. The increased $\alpha_7$ mRNA and protein levels following myogenic differentiation are inversely correlated with c-Myc expression. Transfection of myoblasts with the c-Myc transcription factor down-regulated $\alpha_7$ expression, whereas overexpression of Madmyc, a dominant-negative c-Myc chimera, induced elevated $\alpha_7$ expression. Functional analysis with site-specific deletions identified a specific double E-box sequence in the upstream promoter region (−2.0 to −2.6 kb) that is responsible for c-Myc-induced suppression of $\alpha_7$ expression. DNA-protein binding assays and supershift analysis revealed that c-Myc forms a complex with this double E-box sequence. Our results suggest that the interaction of c-Myc with this promoter region is an important regulatory element controlling $\alpha_7$ integrin expression during muscle development and myotendinous junction formation.

The $\alpha_7$ integrin is the dominant laminin-binding integrin in muscle and plays diverse roles during the different stages of development. Myoblasts and satellite cells express relatively low levels of the integrin, which promotes their rapid cell movement during development and after injury (1–4). As differentiation proceeds, the elevation of $\alpha_7$ expression facilitates myoblast immobilization, fusion, and terminal differentiation. In mature muscle, as the $\alpha_7$-rich myotendinous junctions are formed, the muscle fiber uses extra copies of the integrin for formation of stable adhesion contacts as it inserts into the tendon. It is therefore important to understand how $\alpha_7$ is regulated, not only for its role in modulating cellular functions but also because defects in its expression are associated with certain types of muscular dystrophy (5, 6). Our goal in the present study was to identify regulatory promoter elements of $\alpha_7$ expression and understand how they cooperate to fine-tune expression in myoblasts and terminally differentiated myotubes.

The expression of $\alpha_7$ integrin during skeletal muscle differentiation is regulated primarily at the transcriptional level (20). The expression of this gene is driven by an upstream promoter (>2.8 kb) that lacks both TATA and CCAAT boxes but contains multiple consensus protein-binding sites. Deletion analysis indicates that the promoter consists of both positive and negative regulatory elements, which are believed to be important for tissue-specific expression of the integrin. Although the promoter activity is up-regulated by the muscle-specific transcription factors MyoD and myogenin and although the expression levels of the $\alpha_7$ mRNA and protein increase following myogenic differentiation, the mechanism governing the regulation of tissue- and differentiation-specific expression of $\alpha_7$ is unclear. Even less is known about how this gene is tightly controlled in undifferentiated myoblasts. It is important to identify specific signaling mechanisms involved in this regulation process. The $\alpha_7$ promoter contains multiple putative protein-binding sites that are highly conserved in the mouse and the human, suggesting that these protein-binding sites are essential for regulating expression of the $\alpha_7$ gene at the transcriptional level.

Analysis of the human and mouse $\alpha_7$ promoters has revealed multiple consensus E-box sequences that are binding sites for the basic helix-loop-helix (bHLH) transcription factors. One potential consensus binding site has been identified for c-Myc, which is known to be a strong inhibitor of myogenic differentiation (7–9). c-Myc exerts its regulatory functions by binding either directly to the CACGTG core sequence (in complex with members of the Max and Mad family proteins) or indirectly to protein-binding sites through other c-Myc-binding proteins (10–17).

Because there are at least eight consensus E-box sequences in the $\alpha_7$ promoter region, it is possible that c-Myc and other bHLH proteins may be involved in regulating $\alpha_7$ expression through these sequences (7). As reported, the mRNA level of c-Myc is down-regulated when myoblastic C2C12 cells are induced to differentiate into multinucleated myotubes (18, 19). Consistent with this is the finding that levels of $\alpha_7$ protein expression are strongly elevated as C2C12 cells undergo terminal differentiation (20). To understand the mechanisms that regulate the tissue- and differentiation-specific expression of $\alpha_7$, i.e., whether c-Myc is important for control of $\alpha_7$ gene expression in undifferentiated C2C12 myoblasts, we further analyzed the function of the upstream regulatory element of the $\alpha_7$ promoter and explored the potential role of c-Myc in down-regulating $\alpha_7$ expression.
EXPERIMENTAL PROCEDURES

Cell Culture—C2C12 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 20% fetal bovine serum (Sigma). Differentiation of these cells was induced by replacing fetal bovine serum with 2% horse serum. Murine breast carcinoma HtLM2, were grown in Dulbecco’s modified Eagle’s medium (Cell Culture Facility, University of California at San Francisco) supplemented with 10% fetal bovine serum. To preserve cells in an undifferentiated state, they were cultured in fresh growth medium containing 20% fetal bovine serum every 24 h. Cell differentiation was induced as previously described (20) by maintaining cells in growth medium supplemented with 2% horse serum.

DNA Transfection—A calcium phosphate precipitation method (21) was used for DNA transfections. Briefly, plasmid DNA was prepared using the Qiagen plasmid Maxi kit. The cells were plated into 10-cm cell dishes using the Qiagen plasmid Maxi kit. The cells were plated into 10-cm cell dishes 16–20 h before transfection at 5 × 10^5 cells/plate. For each transfection, a total of 25 μg of plasmid DNA was diluted in 300 μl of 300 mM CaCl₂ solution and then mixed with 500 μl of 2× HBS (280 mM NaCl, 1.5 mM Na₂HPO₄, 100 mM HEPES, pH 7.12). Cell medium was replaced with fresh growth medium (7 ml/plate) 30 min before transfection. The DNA-CaPO₄ mixture was then added to each plate and cultured for 48–96 h or selected for stable expression using G-418 (Geneticin; Invitrogen). For chloramphenicol acetyltransferase (CAT) assays, a plasmid expressing the β-galactosidase protein was cotransfected with other expression vectors in each transfection to be used as an internal control for transfection efficiency. For stable expression of transfected genes, transfectedants were selected with 500 μg/ml G-418. For transient transfection, a plasmid expressing the mouse c-Myc was kindly provided by Dr. Steven Hann (22). The dominant-negative c-Myc plasmid Madmyc was a generous gift from Dr. Rene Bernards (The Netherlands Cancer Institute). The CAT reporter vector pCAT3-promoter (Promega, Madison, WI) containing the CAT gene driven by the simian virus 40 (SV40) promoter without a downstream enhancer was used as a cloning vector for analyzing regulatory functions of the α₂ integrin promoters. To analyze the functions of different regions of the α₂ promoter, the full promoter was amplified as five individual fragments by PCR using specific primers (Table I). The PCR fragments were then cloned into the plasmid pCAT3 promoter between MluI and XhoI restriction sites. Five constructs were created: pCAT0–0.5, pCAT0.5–1.0, pCAT1.0–1.5, pCAT1.5–2.0, and pCAT2.0–2.6. Deletion of protein-binding sites AτC-rich (AT), E-box-A/T-rich (EAT), and double E-box (E-E) was carried out using site-specific mutagenesis method originally described by Rashtchian et al. (23) and modified by Xiao and Buehring (24). Briefly, two sets of DUPT-containing primers (Table I) were used to amplify the entire plasmid DNA in two separate reactions. Each PCR fragment was obtained by using a universal primer (which anneals to the vector sequence) and a mutagenic primer (which contains the desired mutation). Overlapping sequences of 14–16 bp were used for both the mutagenic and universal primers. After treating the PCR fragments with uracil DNA glycosylase to digest the DU-containing primer sequences, the PCR fragments were then reannealed to form a circularized plasmid, which was then used directly to transform competent Escherichia coli DH5α cells. The template plasmid DNA was digested with multiple restriction enzymes so that no colonies would result from the parental plasmid DNA after transformation. The three mutants were constructed: pCATΔAT, pCATΔEAT, and pCATΔE-E. CAT Assay—The cells were rinsed once with phosphate-buffered saline and lysed with 500 μl of lysis buffer/dish (Roche Applied Science), collected, and clarified by microcentrifugation. The supernatants were stored at −80°C until use. CAT activity was detected and measured by assaying 10–50 μl of each cell lysate, using an enzyme-linked immunosorbent assay kit according to the manufacturer’s protocol (Roche Applied Science). CAT activity in cell lysates was determined from a standard curve (obtained for each experiment using the method provided by the manufacturer). Transfection efficiency and CAT activity between transfections were normalized by cotransfection of the β-galactosidase plasmid DNA and by detection of β-galactosidase activity in each cell lysate using a Turner luminometer and a β-galactosidase assay kit (Promega).

Super Electrophoretic Mobility Shift Assays—The nuclear extracts were prepared according to the method of Dyer and Herzog (25) with some modifications. Briefly, monolayer cells were trypsinized and washed once with ice-cold phosphate-buffered saline, resuspended in sucrose buffer I (0.32 M sucrose, 3 mM CaCl₂, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5% (v/v) Nonidet P-40), and centrifuged at 500 × g for 5 min at 4°C. The nuclear extracts were obtained according to the published protocol (25). The probes were labeled with [γ-32P]ATP (Amersham Biosciences) using T4 polynucleotide kinase according to a standard protocol (26). For DNA-protein binding, 10 μl (50 μg) of each nuclear extract was incubated with 50,000 cpm of radioactive probe in 50 μl of binding buffer (25 mM Tris-HCl, pH 8.0, 50 mM KCl, 6.25 mM MgCl₂, 1 mM EDTA, 20% glycerol, and 1 mM dithiothreitol) at 4°C for 30 min; 5 μl of antibody or 5 μl of phosphate-buffered saline was then added to the DNA-protein mix, and the reaction mix was incubated further at 4°C for 12 h or at room temperature for 1 h. The DNA-protein complex was separated by electrophoresis on an 8% nondenaturing polyacrylamide gel in Tris-borate-EDTA buffer at room temperature for 2 h. The gel was vacuum-dried onto Whatman 3MM filter paper. Then DNA-protein binding was detected by autoradiography for 24–48 h. The following probes are synthesized as double-stranded oligonucleotides (the consensus E-box is underlined; only the forward strands are shown here): AτC-rich, GGGACCTCTCTCAAATTATTTGGGCAAGAG; E-box-AT-rich, GAAGCGAGCTTTCGGAAGGCTGGT; double E-box, GGGAGGTCCTGGAGAGGACGAGGACGAGGCTTGAATGAGCTGGCGAGGTC; scrambled double E-box, AAGCGTAGTTGAGAGGAGGAAAGGGAGGTGTTAAGCAAGACGAGAAGACGAGGCTTGAATGAGCTGGCGAGGTC; and consensus c-Myc, GGAACCAGAACGACGACGACGACTTCC.

Southwestern Blot Analysis—Nuclear extracts (100 μg/sample) were denatured at 100°C for 5 min in 4% SDS and 50 mM DTT and then separated by SDS-PAGE. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The filters were washed in denaturing buffer (25 mM HEPES, pH 7.5, 25 mM Tris-HCl, 5 mM MgCl₂, 0.5 mM dithiothreitol) with 6 M guanidine HCl and then renatured by successive washing (five times for 10 min each at 4°C) in 2-fold dilutions of guanidine HCl in denaturing buffer, with a final incubation in denaturing buffer without guanidine HCl. The membrane was then blocked by 5% nonfat dry milk in binding buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, and 1 μM

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Table I

| Primer name           | Construct | Sequences                        |
|-----------------------|-----------|----------------------------------|
| Aτp0.5–3’             | pCAT 0–0.5| ATCTCTCAGAAATGTTGGAGCAGCCTGGCC  |
| Aτp0.5–3’             | pCAT 0.5–1.0| ATACAGGTGTTACCCTCTGAACTTCACCCGTTGTG |
| Aτp1.0–3’             | pCAT 1.0–1.5| TCCCTCAGAGGACTCCAGGAAGAGCAAGAGGAAG |
| Aτp1.5–3’             | pCAT 1.0–1.5| ATACAGGTGTTACCCTCTGAACTTCACCCGTTGTG |
| Aτp1.5–5’             | pCAT 1.5–2.0| CCCTCAGAGGACTCCAGGAAGAGCAAGAGGAAG |
| Aτp2.0–5’             | pCAT 2.0–2.6| CCCTCAGAGGACTCCAGGAAGAGCAAGAGGAAG |
| Aτp2.6–5’             | pCATΔAT   | AGGAGGAGGACAGCAATTC                |
| Aτp3’                 | pCATΔEAT  | ACCUCUCAAGGACCCGAACAGG            |
| dEAT’                 | pCATΔE-E  | AAGCAUCUGCTUGCATGACCTCTCT          |
| dEAT’                 | pCATΔE-E  | AGGAGGAGGACAGCAATTC                |
| dEAT’                 | pCATΔE-E  | AGGCCAGAAACUGCCATTGTTCAGCT         |
| dEAT’                 | pCATΔE-E  | AGTGGCTTGGUCUCGTGTCGACCT           |
dithiothreitol) for 1 h at room temperature and followed by overnight incubation with 5 × 10⁶ cpm/ml of labeled probes in binding buffer containing 0.25% nonfat dry milk. Competition was performed by adding 1 μg/ml of unlabeled probe (about 200-fold in excess). The membranes were then washed with binding buffer with 0.25% nonfat dry milk at 4 °C for 30 min. Autoradiography was carried out at −80 °C with Kodak X-Omat AR film (Eastman Kodak Co.).

Western Blotting—For detection of protein expression, the cells were rinsed once with 1× phosphate-buffered saline, lysed with 0.5 ml/10-cm dish of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1% Triton X-100 or 1% Nonidet P-40), collected, and clarified by microcentrifugation at maximum speed. Fifty micrograms of total proteins (10 μl) of each sample was mixed with 10 μl of 2X sample buffer and loaded onto a 10% SDS-polyacrylamide gel. The proteins were separated by SDS-PAGE, transferred onto polyvinylidene difluoride transfer membranes (Millipore), blotted with specific antibodies, and detected with an ECL-Western blotting kit (Amersham Biosciences) according to the manufacturer's protocol. Antibodies included mouse anti-c-Myc monoclonal antibody, rabbit anti-human β, cytodomin (polyclonal antibody 22778), and rabbit anti-mouse α, light chain (polyclonal antibody 1211) (27, 28). For sequential blotting, the antibodies were stripped using reblot buffer (Amersham Biosciences) after each ECL detection according to the manufacturer's protocol.

Quantitative PCR—High pressure liquid chromatography-purified salt-free primer for target gene mouse α, (forward primer, AACCAGT-GGCTGGCGAGTCTCG; reverse primer, ATCCCGAGTCTCCAAGAGGCTT) were synthesized by the Bimolecular Resource Center (University of California at San Francisco). These primers were designed so that the melting temperature was 10 °C lower than the specific probes and were optimized with regard to AmpliTaq Gold (ABI) and MgCl₂ concentration at 60 °C. Total RNA was extracted from C2C12 myoblasts (ATCC) as described previously. Total RNA (5 μg) was reverse transcribed with 250 units of murine leukemia virus reverse transcriptase (Invitrogen) in a volume of 100 μl, using 5 μm random hexamer primers (Invitrogen) according to the following protocol: 25 °C for 10 min, 48 °C for 40 min, 95 °C for 5 min, with 1 mM dNTP, 7.5 mM MgCl₂, and RNase inhibitor (Ambion). The efficiency of probes was checked over a range of cDNA concentrations for quantitative PCR. FAM(5'-client) and TAMRA(3'-client) tagged probes were purchased from ABI (Athens, GA), the probe for α, was CAAGAT-TGTTACGTGTGACACCCGGATATG. Relative expression values were obtained in reference to mouse Gus gene expression. Each sample was assayed in triplicate on an ABI PRISM 7700 with Sequence Detection Software version 1.6. For the determination of relative expression from assayed in triplicate on an ABI PRISM 7700 with Sequence Detection TGTTACGTGTGCACACCGATATG. Relative expression values were determined by the software automatically.

RESULTS

α, Expression Is Suppressed in Myoblasts but Is Up-regulated during Myogenic Differentiation—Using quantitative real time PCR, we found that as C2C12 cells differentiate into myotubes, α, mRNA expression increased linearly to more than five times that of undifferentiated myoblasts (Fig. 1A). This increase continued up to 9–10 days when the α, mRNA reached a plateau with more than a 20-fold increase compared with myoblasts (data not shown). To confirm that the increased transcription was responsible for this change in mRNA levels, we detected promoter activity in myoblasts using the CAT reporter gene driven by the full-length α, promoter. In differentiated compared with undifferentiated C2C12 cells, CAT activity of the α, promoter increased by about 1.5- and 3-fold at 48 and 96 h, respectively (Fig. 1B).

Localization of Positive and Negative Regulatory Elements in the α, Promoter—To study the mechanisms involved in regulation of α, integrin expression, we tested various regions of the α, promoter (Fig. 2A) for silencer and enhancer activities by using the pCAT3-promoter vector in which DNA fragments are inserted upstream of the SV40 promoter driving the CAT gene. We dissected the 2.6-kb α, promoter into five fragments, cloned them into the pCAT3-promoter vector (Fig. 2B), and assayed enhancer and silencer activities by transient transfection and

CAT-enzyme-linked immunosorbent assays in C2C12 cells. As shown in Fig. 2C, the −1 to −500-bp DNA fragment increased the activity of the SV40 promoter nearly 4-fold, indicating that it functions as an enhancer for the SV40 promoter. However, the 2.0–2.6-kb fragment decreased CAT activity by 80%, suggesting that this region may act as a silencer for the SV40 promoter. In contrast, no change in promoter activity was found for the other three fragments, pCAT0.5–1.0, pCAT1.0–1.5, and pCAT1.5–2.0, suggesting that these fragments lack enhancer or silencer activity over the SV40 promoter. Because we were interested in the mechanisms involved in down-regulation, we focused on the 2.0–2.6-kb fragment, which is called the upstream regulatory element (URE). As shown in Fig. 2D, more than 90% of the URE sequence is highly conserved between the mouse and the human α, integrin gene, suggesting that this region and the partially conserved motifs may be important for regulating expression of this gene. Of particular interest is the conserved CAGGGTG E-box that is similar to the consensus sequence of c-Myc.

Deletion Analysis of the α, Promoter in Myoblasts and Differentiated Myotubes—As shown in Fig. 2D, the URE (−2 to −2.6 kb) contains two A/T-rich regions and three E-box regions that are potential binding sites for bHLH family proteins including c-Myc. To explore the possibility of involvement of c-Myc in regulation of α, expression, we detected endogenous c-Myc expression in C2C12 cells undergoing differentiation. We found that c-Myc expression rapidly decreased to undetectable levels 96 h after the cells were cultured in differentiation medium (Fig. 3A). Our data are
consistent with the literature showing that c-Myc is down-regulated during myodifferentiation.

To investigate the mechanisms of gene regulation of the \( \alpha_7 \) integrin involving this region, we constructed the wild-type pCAT2.8 and three mutated CAT reporter plasmid vectors: pCATdAT, pCATdEAT, and pCATdEE. In these plasmids, the CAT gene is driven by mutated forms of the \( \alpha_7 \) promoter, each with a specific deletion at one of the three consensus protein-binding sites (Fig. 3B).

To test whether MyoD and c-Myc can regulate promoter function through these protein-binding sites, we cotransfected each of the reporter constructs into undifferentiated C2C12 myoblasts, with the empty pcDNA3 vector, MyoD, c-Myc, dominant-negative Madmyc, MyoD and c-Myc (MyoD/Myc), or c-Myc and Madmyc (c-Myc/Mad). Promoter activities were then detected by CAT-enzyme-linked immunosorbent assay. Cotransfection of MyoD enhanced the wild-type \( \alpha_7 \) promoter activity in myoblasts by about 2-fold (Fig. 3C). Conversely, the c-Myc oncoprotein inhibited expression of the CAT gene in myoblasts by over 50%. Deletion of the A/T-rich sequence produced no change in promoter activity in response to either MyoD or c-Myc, indicating that this region of the promoter is probably not relevant for these transcription factors (Fig. 3C, pCATdAT). However, deletion of the E-box-A/T-rich region resulted in a loss of response to MyoD but not c-Myc (Fig. 3C, pCATdEAT), suggesting that this region may be important for the MyoD function in regulating integrin promoter activity.

Next, deletion of the double E-box resulted in a loss of response to c-Myc (Fig. 3C, pCATdEE). To determine whether low levels of \( \alpha_7 \) expression are due to the effect of endogenous c-Myc in C2C12 myoblasts, we cotransfected these cells with the dominant-negative c-Myc (Madmyc) construct with the wild-type or the mutant \( \alpha_7 \) promoter constructs into C2C12 myoblasts and analyzed the promoter activity by CAT assay.

Interestingly,
FIG. 3. c-Myc and myodifferentiation. A, expression of endogenous c-Myc in C2C12 cells undergoing myodifferentiation. Total cellular proteins from cell lysates made at the indicated time points were subjected to Western blotting to detect c-Myc expression, as described under "Experimental Procedures." B, schematic view of the wild-type and three mutant a2 promoter constructs expressing the CAT reporter protein. Protein-binding sites are indicated as follows: AT, the A/T-rich region (-2259 to -2249, CAAATATTG); EE, the double E-box region (-2155 to -2129, CTGCAGGTGGAGAAAGGAAGGCAGCTGGAG); and EAT, the A/T-rich and E-box region (-2221 to -2191, TGCCATCTGATTTCAGC-CTTGGATTGTGG). C, CAT activity of the wild-type and mutant a2 promoters in C2C12 cells cotransfected with the indicated plasmids; C2C12 myoblasts were maintained in growth medium with 20% fetal bovine serum, and the cells were placed in fresh medium every 24 h until 48–72 h after transfection for CAT assays. CAT activities were determined as described in the legend to Fig. 2 and are expressed as pg/10 mg of total cell lysates. MyoD/Myc, cotransfection of MyoD and c-Myc plasmids; Myc/Mad, cotransfection of c-Myc and Madmyc plasmids.
Madmyc increased the promoter activity of the wild-type α7 promoter and two of the mutant α7 promoters (pCATdEAT and pCATdAT) but not the mutant with deletion at the double E-box. Furthermore, cotransfection with c-Myc and Madmyc (Myc/Mad) tended to neutralize the inhibitory activity of c-Myc. In cotransfection experiments with MyoD and c-Myc, the stimulating effects of MyoD on the α7 promoter were repressed by c-Myc, indicating that high levels of MyoD cannot completely override the effects of c-Myc. When the double E-box deletion mutant (pCATdEE) was tested with MyoD/Myc, strong stimulation of the promoter was detected. This is consistent with the conclusion that the double E-box is the primary sequence in the promoter where c-Myc exerts its repressor activity.

Following differentiation of myoblasts into myotubes, transfection of MyoD failed to further enhance α7 gene promoter activity (Fig. 4, pCAT2.8, right panel). This is explained by the fact that endogenous MyoD expression is already elevated in myotubes and is sufficient to drive maximum α7 expression. However, c-Myc overexpression strongly decreased wild-type promoter activity in the differentiated myotubes. Deletion of the E-box-A/T-rich region resulted in loss of response to MyoD but not c-Myc, which is also especially evident in myotubes. Because this mutant also failed to respond to c-Myc in myotubes, we speculate that c-Myc may also function at this site through complex protein-protein interaction (Fig. 4, pCATdEAT). Both in myoblasts and in myotubes, deletion of the double E-box resulted in a loss of response to c-Myc (Fig. 4, pCATdEE), and this was particularly relevant in differentiating myotubes (Fig. 4, pCATdEE, right panel). This suggests that the double E-box may be important in mediating the down-regulation of α7 expression by the c-Myc oncoprotein and implicates this region as the binding site for c-Myc-like protein complexes.

Expression of c-Myc Down-regulates α7 Expression—As detailed above, there are multiple consensus E-box sequences including potential c-Myc-binding sites in the α7 promoter region. Because c-Myc and α7 expression levels are inversely correlated during differentiation, we speculated that α7 may be down-regulated by c-Myc. To test this possibility, we stably transfected C2C12 cells with the plasmid vector expressing the mouse c-Myc protein. c-Myc and α7 expression in C2C12 cells were then detected by immunoblotting using specific antibodies. As expected, α7 expression was strongly suppressed in the transfected cells expressing high levels of c-Myc (Fig. 5A, third lane) but not in cells transfected with the empty pcDNA3 vector (Fig. 5A, second lane). Next we tested the effect of the dominant-negative Madmyc on α7 expression. Compared with control (Fig. 5B, first lane), α7 expression did not change in c-Myc-expressing cells cotransfected with Madmyc (Fig. 5B, second lane). However, in cells transfected with Madmyc alone, α7 expression increased significantly (Fig. 5B, fourth lane). These results suggest that c-Myc is counteracted by the dominant-negative Myc protein and that c-Myc may play a role in down-regulating α7 gene expression in myoblasts.

The Double E-Box Sequence Binds to a Nuclear Protein of ~64 kDa—To confirm that the double E-box serves as a protein-binding site for c-Myc-like complexes, we performed Southwestern and gel supershift analysis. Southwestern anal-

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**Fig. 4.** CAT activity of the wild-type and mutant α7 promoters in C2C12 myoblasts and myotubes transfected with the indicated plasmids. For C2C12 myoblasts, the cells were maintained in growth medium with 20% fetal bovine serum, and the cells were placed in fresh medium until 48–72 h after transfection. For C2C12 myotubes, the cells were maintained in 2% horse serum (to induce myogenic differentiation) and harvested at 96 h after transfection for CAT assays. The CAT activities were determined as described in the legend to Fig. 2. For comparison, the CAT activity from cotransfection of each promoter construct with pcDNA3 was adjusted to 1.0.
Discussion

The expression of α7 during muscle development follows a complicated stepwise process that is tightly regulated. Muscle differentiation is controlled by the MyoD family of bHLH transcription factors that regulate muscle-specific genes. Our results support the hypothesis that the regulation of α7 by MyoD...
family members is modulated by c-Myc at the level of the integrin promoter. In myoblasts, α7 expression remains at basal levels, but after differentiation α7 mRNA increases more than 20-fold. It is important to understand how this dramatic differentiation-specific change in α7 is regulated at the level of the promoter, because expression of the integrin is known to modulate muscle function.

Our results indicate that c-Myc is a potential negative regulator of the α7 promoter in myoblasts. On the basis of the following observations, we postulate that increased expression of α7 in myoblasts is due to a loss of c-Myc in combination with increased expression of muscle-specific transcription factors such as MyoD. The level of α7 mRNA expression markedly increases upon differentiation of myoblasts into myotubes (Fig. 1). In contrast, c-Myc levels are high in proliferating myoblasts but dramatically decrease during myotube formation, consistent with the findings of previous studies (9). Thus, c-Myc expression inversely correlates with α7 mRNA expression. Importantly, the URE in the α7 promoter contains several E-box sequences that serve as putative binding sites for bHLH transcription factors, including c-Myc.

Further analysis showed that exogenous c-Myc protein was able to inhibit activity of the wild-type α7 promoter in undifferentiated C2C12 myoblasts by over 50%, with the inhibition being more dramatic (75%) in cells undergoing differentiation into myotubes (Fig. 4). Moreover, deletion of the double E-box located between positions −2155 and −2129 led to a loss of the promoter response to c-Myc but not to MyoD, and deletion of the E-box-A/T-rich region located between positions −2221 and −2191 resulted in loss of response to MyoD but not to c-Myc. However, deletion of the A/T-rich region located between positions −2259 and −2249 showed no effects on the promoter response to either protein. These results indicate that the double E-box may be responsible for c-Myc-specific down-regulation of the α7 gene, whereas the E-box-A/T-rich element is involved in MyoD-specific up-regulation of α7. Because the deletion of the A/T-rich region did not affect the promoter activity in response to either c-Myc or MyoD, we conclude that the changes in promoter activity by the deletion of the double E-box (pCATdEE) or the E-box-A/T-rich region (pCATdEAT) were probably not due to changes in the overall structure of the promoter. Finally, Southwestern and gel supershift analysis revealed that c-Myc can bind directly to the α7 promoter through the identified double E-box sequence.

Using the double E-box oligonucleotides as a probe, we detected two protein bands on Southwestern gels, with the 64-kDa band having the same molecular mass as that of c-Myc (Fig. 7). The identity of the 64-kDa protein is not known, but further experiments are planned. Importantly, when we used a consensus c-Myc-binding probe, a 64-kDa protein was again detected. We also found that one of the protein-DNA complexes formed between the double E-box and nuclear extracts from C2C12 cells could be supershifted by anti-c-Myc antibody, indicating the presence of the c-Myc protein in the complex.

Although c-Myc plays central roles in the regulation of cell proliferation, differentiation, and apoptosis, its exact function in regulating expression of other genes and the mechanisms involved has remained elusive (7, 14, 29, 30). As a transcription factor, c-Myc binds to gene promoters containing a binding sequence with a palindromic E-box (CACGTG) in its center (11, 15, 31). A number of factors, including flanking nucleotides, chromatin structure, DNA methylation, and interaction with other DNA-binding proteins (32), may influence Myc/Mad binding in addition to the primary DNA sequence. It is well known that Myc-Max heterodimers preferentially bind the E-box sequence CACGTG, but the complex can also interact with related noncanonical sequences that may be recognized more frequently in vivo (33).

The two E-boxes identified in the URE of the α7 promoter, CAGGTG and CAGCTG, vary from the consensus c-Myc-binding E-box, CACGTG. However, there are other examples of c-Myc binding to variations of the c-Myc consensus E-box sequence (32). It is likely that c-Myc or its heterodimeric complex with Max can bind to these variant E-box sequences. Another possibility is that this double E-box is not recognized directly by c-Myc, which may bind to the promoter through association with other muscle-specific bHLH transcription factors such as MyoD (Fig. 6). This multiple protein-protein and protein-DNA interaction may be required for transcriptional suppression of the α7 promoter. This can be explained by our observation that the protein-DNA complexes not supershifted by anti-c-Myc antibody were detected only in nuclear extracts from myoblastic C2C12 cells and not in those from the α7-negative HLM2 epithelial cells.

Our data strongly suggest that c-Myc represses α7 integrin promoter activity by directly binding to specific DNA-binding sites containing the double E-box sequence. This is evidenced by the finding that deletion of the double E-box results in a loss of responsiveness to both c-Myc and Madmyc. Such a result does not seem to be due to changes in promoter conformation, because deletion of the AT-rich region did not affect Myc responsiveness. The dominant-negative Madmyc differs from c-Myc in the transactivation domain but can also bind to E-box sequences. If c-Myc or Madmyc were acting through non-DNA-binding mechanisms, then deleting the E-boxes would not be expected to lead to a loss of responsiveness to either c-Myc or Madmyc. Finally, additional evidence in support of the role of the identified E-box sequence in c-Myc-mediated repression is provided by the finding that the double E-box can bind c-Myc or a related protein in gel shift experiments.

Of the four known members of the myogenic regulatory factor family proteins (Myf5, MyoD, myogenin, and MRF4) that are important for initiating myogenic differentiation in myoblasts (36, 37), MyoD was shown to be the major effector protein that can activate α7 expression (20). Because MyoD activates gene expression by binding to the promoter region of its target genes at E-box sequence CANNNTG, it may interact with multiple E-box sequences in the α7 promoter. In the present study, however, we found that deletion of the E-box-A/T region containing the sequence CACTTG followed by an A/T-rich sequence, but not deletion of the double E-box, abolished the response of the α7 promoter to MyoD. This suggests that the E-box-A/T region, not the EE region, is necessary for the function of MyoD in up-regulating α7 expression, although it may not be sufficient. MyoD may have a dominant effect over c-Myc, because c-Myc can only partially block the positive effect of MyoD on the α7 promoter if both proteins are introduced into the same cells (Fig. 3C). This may reflect the fact that MyoD can bind to multiple E-boxes in the α7 promoter, whereas c-Myc may only function by interaction with the double E-box region to repress α7 expression.

α7 is also strongly expressed in smooth muscle and cardiac muscle following differentiation (2, 27). However, because MyoD is not believed to mediate the regulation of gene expression following differentiation in these specialized tissues, there must be other transcription factors that are important for regulating α7 promoter activity. Whether c-Myc also plays a role in the modulation of α7 expression in smooth muscle and cardiac muscle, as it does in skeletal muscle, remains to be determined. However, in cardiac tissue, c-Myc levels can fluctuate and have been shown to correlate with cardiac myocyte hypertrophy and cell proliferation (38).
Our findings are consistent with the current knowledge about c-Myc as an important regulator of gene transcription. Binding of the c-Myc protein to its DNA targets either enhances or inhibits transcription of its target genes (8, 9, 12, 13, 31, 34, 35). The idea that Myc can act as a repressor has remained controversial until recently. Currently, it is believed that Myc/Max may repress activity through their interaction with other transcription factors that promote target gene expression (36). Although there is no evidence that c-Myc and MyoD can interact directly, it is possible that each may interact through a common transcription factor. It has been reported that c-Myc induces transcriptional repression of myoD in quail myoblasts and inhibits terminal differentiation (37). Further studies are necessary to explore the exact mechanisms involved in c-Myc-related down-regulation of the α7 gene in C2C12 myoblasts, but the possibility deserves further study.

In summary, our data indicate that α7 expression is under the negative regulatory control of c-Myc in the early stage of myodifferentiation, when c-Myc levels are relatively high and levels of myogenic transcription factors are low. As myoblasts undergo differentiation, they gradually lose expression of c-Myc and up-regulate expression of myogenic regulatory factors, including MyoD, leading to a shift from loss of negative regulation to positive regulation of the promoter. α7 integrin protein is therefore expressed at high levels, as required for the formation of myotendinous junctions during generation of differentiated myotubes. Further studies are necessary to explore the exact mechanisms involved in c-Myc-related down-regulation of α7 gene expression in myoblastic cells.

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