Identification and Characterization of the Cell Type-specific and Developmentally Regulated \( \alpha7 \) Integrin Gene Promoter\(^*\)

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Expression of \( \alpha7 \) is mainly confined to skeletal and cardiac muscle in which it appears to be the major laminin-binding integrin. When myoblasts differentiate to myotubes, \( \alpha7 \) mRNA and protein expression is up-regulated. To explore the mechanisms involved in the tissue-specific and developmentally regulated expression of \( \alpha7 \), we isolated and characterized a genomic clone containing \( \sim 2.8 \) kilobase pairs (kb) of the \( 5' \)-flanking region of the murine \( \alpha7 \) gene. The \( 5' \)-flanking region lacks both TATA and CCAAT boxes but contains five putative Sp1 binding sites located in a CpG island. Two transcription start sites, located near an initiator-like sequence, are \( 176 \) and \( 170 \) base pairs upstream of the translation start site. There are numerous binding sites for developmental and cell type-specific trancription factors, including AP-1, AP-2, GATA, and several AT-rich sites. There are also eight consensus E-boxes that bind the basic helix-loop-helix family of muscle-specific transcription factors. The \( \sim 2.8 \)-kb \( 5' \)-flanking region was an active promoter in C2C12 skeletal myoblasts and exhibited increased expression upon conversion to myotubes but was inactive in HlLM2 cells, a mouse breast carcinoma epithelial cell line that does not express \( \alpha7 \). Deletion analysis identified both positive and negative regulatory elements within the \( \sim 2.8 \)-kb fragment. In 10T1/2 fibroblasts the \( \sim 2.8 \)-kb \( \alpha7 \) promoter was trans-activated by the myogenin basic helix-loop-helix proteins myogenin and MyoD but not by MRF4 and myf5. These results suggest that muscle-specific transcription factors play a role in regulating the cell-type expression of the \( \alpha7 \) gene during development.

The integrin superfamily is made up of a number of transmembrane heterodimeric receptors that mediate cell-extracellular matrix and cell-cell interactions (1). Each integrin is composed of noncovalently paired \( \alpha \) and \( \beta \) subunits. Regulation of the genes expressing these extracellular matrix receptors is important in a number of processes including development, migration, and invasion (2–4).

Previously, we have reported that human and mouse melanoma cells express an integrin complex designated \( \alpha7 \beta1 \) (5, 6). This integrin receptor binds to the E8 fragment of laminin-1 and mediates cell adhesion to this ligand (6). In melanoma, the expression of the \( \alpha7 \) integrin receptor is elevated in the non-metastatic phenotype but is lacking in metastatic cells, suggesting that absence of this laminin adhesion receptor can play a role in melanoma tumor cell dissemination.\(^1\)

Expression of \( \alpha7 \), as detected by reverse transcriptase-PCR,\(^2\) has also been reported to be present in a number of tissues including for example stomach and uterus (7). However, it has not yet been determined if any of the \( \alpha7 \) protein isoforms are expressed in these tissues. In myoblasts and cardiomyocytes, \( \alpha7 \) appears to be the predominant laminin-binding integrin expressed (8, 9). In vitro, basal levels of \( \alpha7 \) expression are seen in replicating rat and mouse myoblasts (7, 10, 11). Expression of \( \alpha7 \) is increased upon differentiation of myoblasts to myotubes. This up-regulation is paralleled by increased expression of myogenin (11), a member of the muscle-specific basic helix-loop-helix (bHLH) transcription factor family, suggesting that these factors play a regulatory role in \( \alpha7 \) expression during muscle development.

At embryonic day 7.5, \( \alpha7 \) can be detected in the ectoplacental cone (differentiating murine trophoblast), where it is believed to play a role in trophoblast adhesion and/or differentiation. The use of laminin fragments suggested that \( \alpha7 \beta1 \) may be one of the major trophoblast laminin-binding integrins involved in embryo implantation (12). Recently, the cDNAs for rat and mouse \( \alpha7 \) have been isolated and shown to be alternatively spliced in both the extracellular (giving rise to two isoforms, X1 and X2) and cytoplasmic (producing three isoforms, A, B, and C) domains. Subsequent work has shown that these alternatively spliced forms are also developmentally regulated in muscle (7, 10, 13).

Taken together, these results indicate that expression of \( \alpha7 \) plays a role in embryo implantation and muscle development. Furthermore, the developmental and restricted tissue expression (in skeletal muscle and cardiac muscle) of the \( \alpha7 \) subunit suggests that the elements governing its expression are complex. As an approach to understanding the mechanisms regulating cell type- and differentiation-specific expression of the \( \alpha7 \) subunit, we isolated and characterized the promoter region for the murine \( \alpha7 \) integrin gene.

**EXPERIMENTAL PROCEDURES**

**Isolation of a Mouse \( \alpha7 \) Genomic Clone, Restriction Enzyme Analysis, and Sequencing**—Approximately 1 \( \times \) 10\(^6\) plaque-forming units of a mouse spleen genomic library constructed in a \( \phi \)Fix II phage vector (Stratagene Cloning Systems, La Jolla, CA) were screened as described previously (10). An \( \sim 17-kb \) positive clone, C9, was identified and subcloned into pBluescript (Stratagene Cloning Systems). A partial restriction enzyme map was determined by standard techniques using single

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\(^2\) The abbreviations used are: PCR, polymerase chain reaction; bHLH, basic helix loop helix; CAT, chloramphenicol acetyltransferase; bp, base pair(s); kb, kilobase pair(s); Pipes, 1,4-piperazinediethanesulfonic acid; Inr, initiator sequence.
and double restriction enzyme digestion and Southern blot analysis (14). Primer extension was performed on the 5'-flanking region of the CAT reporter gene construct and the 3'-end (underlined) was used in the PCR reactions to create all deletion constructs. The oligonucleotides used for PCR reactions each contained a HindIII restriction enzyme site (underlined) and are as follows: -1.22 kb, 5'(-dAAGTTGAAGTCGCTCAAGGC)-3', -400 bp, 5'-dAAAGCTTGGCGGTTGATTCTTACGGC)-3', -200 bp, 5'-dAAAGCTTGGCGGTTGATTCTTACGGC)-3'. All PCR reactions were performed in 1 × Vent buffer (New England Bio-Labs, Beverly, MA) with 200 μM each of deoxynucleotide, 40 pmol of primer, and 2 units of Vent DNA polymerase (New England Bio-Labs) with proofreading activity. Typically, the thermocycling program consisted of an initial denaturation step at 94°C followed by 20–25 cycles consisting of 1 min denaturation at 94°C, 30 s annealing at 55°C, and 30 s elongation at 72°C. A final 10-min elongation at 72°C followed. The PCR fragments were digested with HindIII and SalI and ligated into the corresponding sites in pCAT-Basic. Each construct was verified by sequencing.

**Cell Culture**—The mouse C2C12 myoblast cell line was provided by Dr. H. Blau (Stanford University). C2C12 cells were maintained in Dulbecco's minimal essential medium (DME-H-21, Cell Culture Facility, New England Bio-Labs) supplemented with 20% fetal bovine serum. For differentiation into myotubes, myoblasts were switched to differentiation medium (DMM) containing DME-H-16 supplemented with 10% fetal bovine serum. The mouse melanoma cell line K1735 c19 was maintained as described previously (6).

**Transfection Assays**—Mouse C2C12 cells were maintained in DME-H-21 containing 20% fetal bovine serum. Cells were plated at 1.35 × 10^5 cells per 35-mm dish and incubated for 18–24 h. Two μg of either CAT reporter gene construct and 0.5 μg of a reference plasmid luciferase plasmid (pGL2, Promega) were combined with 6 μg of a reference plasmid containing [α-32P]dCTP using a Multiprime DNA labelling kit (Amersham Corp.). Hybridized nylon filters (Nytran +, Schleicher & Schuell) were stringently washed and exposed to x-ray film at −80°C as described previously (10). A subclone of G9 designated as G2 that contains the α7 promoter region was sequenced and analyzed further.

All nucleotide sequences were determined by the dideoxy chain termination method for double-stranded DNA as described previously (10). Primers for sequencing and polymerase chain reaction (PCR) were synthesized using the 391 DNA Synthesizer PCR-Mate from Applied Biosystems (Foster City, CA).

**Plasmid Constructs**—To create the ~2.8-kb α7 chloramphenicol acetyltransferase (CAT) construct, the α7 genomic clone G2 was digested with NcoI and SalI and blunt-ended with Klenow and ligated into the ATG start site (14). A second digest with SalI released the ~2.8-kb fragment. The XbaI site of pCAT-Basic (Promega, Madison, WI) was digested and filled in with Klenow, and pCAT-Basic was then digested with SalI and ligated to the blunt-ended SalI fragment to create the ~2.8-kb α7 promoter fragment. The orientation of the insert was confirmed by sequencing. pCAT-Basic plasmids containing deletions of the α7 gene 5'-flanking region were constructed by digestion with each pair of HindIII and SalI restriction enzyme sites (underlined) and are as follows: -1.22 kb, 5'(-dAAGTTGAAGTCGCTCAAGGAAGAATGGAGTGCACAGG)-3', -400 bp, 5' (-dAAAGCTTGGCGGTTGATTCTTACGGC)-3', -200 bp, 5'-dAAAGCTTGGCGGTTGATTCTTACGGC)-3'. All PCR reactions were performed in 1 × Vent buffer (New England Bio-Labs) with proofreading activity. Typically, the thermocycling program consisted of an initial denaturation step at 94°C followed by 20–25 cycles consisting of 1 min denaturation at 94°C, 30 s annealing at 55°C, and 30 s elongation at 72°C. A final 10-min elongation at 72°C followed. The PCR fragments were digested with HindIII and SalI and ligated into the corresponding sites in pCAT-Basic. Each construct was verified by sequencing.

**CAT Assays**—Cells were rinsed twice in phosphate-buffered saline and manually harvested using 1 ml of phosphate-buffered saline. Cell pellets were resuspended in 100 μl of phosphate-buffered saline containing 1 mM phenylmethylsulfonil fluoride. Fifty μl of this cell suspension was subjected to three freeze-thaw cycles, spun at 500 × g for 5 min to remove cell debris, and assayed for CAT activity. CAT activity was detected and quantitated by assaying 20 μl of either the C2C12 or the HLM2 cell lysate, using an enzyme-linked immunosorbent assay (ELISA). This colorimetric CAT-ELISA (5 Prime-3 Prime, Boulder, CO) detection system was fully automated, enzymatically inactive (monomeric) form of the CAT protein. Each ELISA was performed according to the manufacturer’s instructions, and CAT activity in cell lysates was determined from a standard curve run for each experiment.

To normalize the CAT activity between transfections, we assayed the luciferase activity transcribed by cotransfecting plasmid pGL2. The remaining 50 μl of the cell suspension (see above) was lysed using 1 × Reporter lysis buffer (Promega) and spun briefly to pellet cell debris. Three μl of the cell extract was mixed with 100 μl of luciferase assay reagent (Promega), and luciferase activity was measured using a Turner luminometer (Promega). The luciferase values were used to normalize CAT activity readings for transfection efficiency.

**Isolation of a 5' α7 Genomic Clone**—The 5'-flanking sequences of genes, which contain promoter and enhancer elements, regulate developmental and tissue-specific gene transcription. In order to study the elements governing the tissue-specific and developmental regulation of the α7 integrin subunit in more detail, we screened a mouse genomic library in

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Fig. 1. Partial restriction enzyme map of the 5′-flanking region, exon 1, intron 1, and exon 2 of the α7 integrin gene. The top line demonstrates the partial restriction map of the mouse α7 genomic subclone G2. The solid line represents the 5′-flanking sequences and the first intron, whereas exons 1 and 2 are depicted by rectangles. This subclone is comprised of ~7 kilobases, containing 2.8 kb of the α7 5′-flanking region, exon 1, intron 1, and exon 2. The position of exon 1 and exon 2 was assigned by sequencing. The arrow at +1 denotes the transcription start site.

Fig. 2. S1 nuclease protection analysis to identify the transcription initiation site. A single-stranded ~290-bp 5′ genomic fragment of α7 genomic clone G2, extending from the initiation methionine to −115, was used as the probe for S1 analysis. This probe contained 175 bp of the untranscribed region and 115 bp of the 5′-flanking sequences. The probe was end-labeled with T4 polynucleotide kinase and hybridized with 40 μg of total RNA from K1735 cells (lane 3) and from C2C12 myoblasts (lane 1) and myotubes (lane 2). The RNA-DNA hybrid was subjected to S1 digestion, and the protected products were analyzed on a denaturing 6% polyacrylamide gel. Digested probe only was run in lane 4. The sequence (order of sequencing reactions is TCAG) of the two transcription start sites is denoted by ** and *. Oligonucleotide PE1 was used as the primer for sequencing analysis.

αFix II for the 5′-untranscribed and 5′-flanking region of the α7 gene. Using a cDNA fragment that contained sequences near the start of translation, we isolated one positive clone and plaque-purified it. A subclone (designated clone G2) that contained the 5′-untranscribed region and 5′-flanking region of the α7 gene was examined further.

Mapping the Site of Transcription Initiation—Partial restriction enzyme mapping and Southern blotting indicated that clone G2 contains ~7 kb (Fig. 1) of sequence. To map the site of transcription initiation, we used a combination of primer extension and S1 nuclease analysis. S1 nuclease analysis was performed by hybridizing a 5′ end-labeled 290-bp genomic fragment (extending from −115 to +175) with total mRNA from mouse melanoma K1735 cells, C2C12 myoblasts, and C2C12 myotubes (Fig. 2). Two protected fragments were identified in all cell lines indicating that there are two major transcriptional start sites, located 170 and 176 bp, respectively, upstream of the translation initiation methionine (Figs. 2 and 4). This result also indicates that there is no change in the transcription start positions in myoblast versus myotubes. To confirm the S1 analysis result, we performed primer extension with antisense oligonucleotide PE1 (Figs. 3 and 4). Primer extension identified two major extension products whose sizes matched the two transcriptional initiation sites determined by S1 nuclease analysis. Previously, we reported the isolation and characterization of the mouse α7 cDNA (10). The start of the 5′-untranslated region, as determined by 5′ rapid amplification of cDNA ends, of the full-length α7 cDNA terminated at the same position as the 170-bp fragment identified in S1 nuclease and primer extension analysis. Thus, by S1 analysis and primer extension, we have confirmed the presence of two primary transcription initiation sites at 176 and 170 bp upstream of the initiation methionine. Multiple transcriptional start sites are not uncommon for promoter regions lacking a TATA box.

Sequence analysis indicated that the genomic clone G2 contains ~2.8 kb of the 5′-flanking sequence, the first exon, all of intron 1, and exon 2. Designating the −176 initiation site as the start of transcription and performing sequence analysis indicated that exon 1 is comprised of 383 bp, of which 212 bp encodes the signal sequence and translation start site (Figs. 1 and 2). Exon 1 is followed by −3.5 kb that makes up the first intron. Adjacent to this intron is an open reading frame of −124 bp that encodes exon 2. Similar genomic organization is seen for other integrin genes (21, 22). Each of the intron/exon boundaries analyzed (Fig. 2) conformed to the consensus 5′ and 3′ splice sequences, G/T(A)G (23).

The 5′-Flanking Region of the α7 Gene—The promoter regions for a number of the integrin receptor genes (αIIβ, α2, α4, α5, α6, αv, β1, β2, β3, β7, CD11a, CD11b, CD11c and CD18) have recently been identified and characterized (24–37). The 5′-flanking sequences near the transcriptional start site of the α7 gene, like the other identified integrin promoters (except the

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human a4 promoter) (26), lacks both a TATA and a CCAAT box (Fig. 4). Transcription from promoters without TATA and CCAAT boxes can initiate at a consensus sequence defined as the initiator sequence (Inr) (38). The original consensus Inr sequences, as designated by the terminal deoxynucleotide transferase gene (38), closely resemble the initiator sequences found in the aIIb, a5, CD11a, and CD11b 5'-flanking regions (24, 27, 32, 34). The sequence at the initiation site for a7, like that for the a2 integrin gene, diverges somewhat from the consensus Inr sequence (consensus, CTCANTCT; a7, CTGGGTCC) (Fig. 2; 25). In addition, like the promoters for a5 and a6, the transcription start site for the a7 gene appears to start not in the Inr-like sequences but in close proximity to it (27). In fact, the start site at 170 bp 5' of the translational start site is only 2 bp upstream of the Inr-like sequence (27). Typically, the Inr, in combination with nearby Sp1/GC boxes (38, 39), directs correct transcription initiation. Located within 90 bp of the transcription start sites of the a7 gene are three tandem sequences for Sp1 (Fig. 4). Further upstream, at positions -216 and -194, are two additional Sp1 sites. All five Sp1 sites are located in the 5'-flanking region between +1 and +238. This region is composed of 70% guanine (G) and cytosine (C) residues with three PspAI restriction enzyme sites (CCGG). Similar G/C rich regions, termed GpC islands, have been described for the a2, a5, and a63 promoters (25, 27). When methylated, GC islands can affect chromatin structure and thus influence the regulation and tissue specificity of gene expression (40, 41).

In the Inr sequence and the five putative Sp1 binding sites, the ~2.8-kb sequence of the a7 5'-flanking region contains numerous binding sites for ubiquitous, developmental, and cell type-specific transcription factors. Binding sites for ubiquitous transcription factors include three sites for AP-1 at -2210, -2838, and -2109 and four AP-2/AP-2-like sites at -2955, -2147, -2127, and -120 (Fig. 4). Transcription is mediated from AP-1 and AP-2 sites via the phorbol ester/diacylglycerol-activated protein kinase C pathway, whereas the cAMP pathway induces gene transcription from AP-2 (25, 42). Whether these two pathways play a role in a7 transcription remains to be determined. AP-1 and AP-2 sites are present in a number of the integrin genes including a2, a5, and CD11c and a number of muscle-specific genes such as muscle creatine kinase and desmin (25–27, 36). In addition to sequence recognition sites for AP-1 and AP-2, two GATA sites are present, at position -327 (GATA-like) and -756 (consensus site) (Fig. 4). GATA sites bind the heart progenitor cell-specific transcription factor, GATA-4, which has recently been shown to be necessary for cardiac muscle cell development (43). This factor may be important in regulating expression of a7 in late fetal and adult heart. A second putative developmental binding site, for the GATA DNA-binding domain was shown to be required for high level b'-enolase promoter activity in myoblasts and to be important for repression of expression in fibroblasts (44). Further analysis of the 5'-flanking region of the a7 gene revealed a number of potential cell-specific and differentiation-related regulatory sequence elements. There are eight consensus E-boxes (CANNNTG), located at -23, -2201, -2146,
−2127, −2059, −2032, −1449, and −571 (Fig. 4). E-boxes are the DNA-binding sites for a family of muscle-specific bHLH proteins (45, 46). The members of this family, comprised of MyoD, myf5, MRF4, and myogenin, are able to convert non-muscle cells into myogenic lineages, can direct expression of many muscle-specific genes including desmin, troponin I, M-creatine kinase, are expressed only in skeletal (not cardiac) muscle, and are believed to play a major role in muscle differentiation (47–49). Previously, it has been shown that the α7 mRNA level in rat L8 myoblast is increased 3–4-fold upon conversion of myoblasts to myotubes (11). This increase in α7 mRNA is paralleled by increases in the bHLH transcription factor myogenin. In addition, α7 expression is seen in the developing embryo during the time the MRFs, MyoD and myogenin are expressed (50–52). These results suggest that the E-boxes in the promoter region of α7 may play an important role in α7 expression during muscle development and differentiation. Finally, it is interesting to note that the E-box at α7 −2127 contains the symmetrical core consensus sequences, CACGCTG, that have been shown by in vitro studies to be the preferred binding sites for MyoD (53).

A/T-rich sites, termed the MADS box, CARG box, and the serum response element, have recently been identified in a number of muscle-specific genes (54–56). Several factors that are important in embryogenesis and skeletal and heart muscle development (Mhox, Oct 1) and are required for transcriptional activity in skeletal and cardiac myocytes (MEFS, Mefa2-a, serum response factor) bind to these sequence elements (56, 57). There are five A/T-rich sites in the α7 promoter (Fig. 4) that may function as binding sites for these factors and possibly regulate α7 expression in skeletal and/or cardiac tissue. Finally, at position −585 is an MCAT-like sequence. The MCAT motif is the binding site for transcriptional enhancer factor-1 (TEF-1) or a TEF-1-like protein (58, 59) and is believed to be critical in directing cardiac-specific expression (60, 61).

The 5′-Flanking Region of the α7 Gene Acts as a Promoter and Directs Cell-specific Transcription in Transfection Assays—To determine whether the 5′-flanking region of the mouse α7 gene could act as a promoter and direct transcription in a cell-specific and differentiation-specific manner, we ligated the −2.8-kb genomic fragment upstream of the CAT gene in pCAT-Basic (see Fig. 6 and “Experimental Procedures”). The CAT-promoting activity of this fragment was determined by transiently transfecting the −2.8-kb/CAT (p2.8kb-CAT) construct into HtLM2 mouse breast carcinoma cells and C2C12 myoblasts. Replicating C2C12 myoblasts express moderate levels of α7 mRNA (Fig. 5). In contrast, the HtLM2 mouse breast carcinoma cell line is of epithelial origin and does not express α7, as determined by Western blotting (not shown). The α7 gene 5′-flanking region directs CAT enzymatic activity in C2C12 myoblasts. When normalized for transfection efficiency, the −2.8-kb/CAT construct generated 14-fold greater activity than the CAT-structural sequences alone in C2C12 myoblasts (Fig. 6). In contrast, the same construct showed no activity in HtLM2 mouse breast carcinoma cells. Thus, the −2.8-kb 5′ flanking sequence of the α7 gene can function as a promoter and demonstrates cell-specific activity.

To characterize the 5′-flanking region required for either myoblast- or myotube-specific promoter activity, 5′ deletion mutants of p2.8kb-CAT (−2669 to +175), p1.22kb-CAT (−982 to +175), p600bp-CAT (−439 to +175), p400bp-CAT (−225 to +175), p300bp-CAT (−114 to +175), and p200bp-CAT (−23 to +175) were constructed (Fig. 6). We assayed the promoter deletion constructs in C2C12 myoblasts since they display basal levels of α7 expression as myoblasts and an increasing level of α7 mRNA when induced to form myotubes (Fig. 5). All deletion constructs directed CAT expression in both C2C12 myoblasts and myotubes. The p200bp-CAT construct, which contains only 23 bp 5′ of the transcription start site, displayed the lowest promoter activity in both myoblast and myotubes. In myotubes this construct directed 2-fold higher enzymatic activity than in myoblasts. In contrast, the same construct was unable to direct CAT activity in HtLM2 mouse breast carcinoma cells. The addition of −96 bp further 5′ in the p300bp-CAT construct resulted in a −14-fold increase in enzymatic activity in both myoblasts and myotubes. This construct directed the greatest CAT activity of all promoter deletion constructs. The p300bp-CAT construct displayed the highest activity in the HtLM2 mouse breast carcinoma cells as well. Comparison of the activity of p300bp-CAT in myoblasts and myotubes indicated that it was capable of directing −2-fold higher CAT expression in myotubes than in myoblasts. A number of possible factors may be important for the increase in promoter activity displayed by this construct. For example, located within p300bp-CAT are three tandem Sp1/GC boxes, an AP-1 site, and an ets-like site (Fig. 2). These sites, either alone or in combination, may play a role in this increased activity.

The p400bp-CAT construct appears to contain a negative element, as indicated by the 3.5- and 12-fold reduction in CAT activity in myoblasts and myotubes, respectively (Fig. 6). No consensus sequences for known negative regulatory elements or silencers are located in this region. Either an unidentified element is present in this construct or other regulatory elements located further upstream are required for efficient expression of these sequences. In HtLM2 cells, this construct also directed decreased enzymatic activity. In contrast, the addition of 200 bp 5′ in p600bp-CAT only slightly elevated the CAT activity in myoblasts but increased the enzyme level 5-fold in C2C12 myotubes. Comparison of the CAT activity stimulated by this construct in myoblasts and myotubes showed only a modest increase in myotubes. In the mouse breast epithelial cell line, little to no activity was elicited by this construct.

Both the p1.22kb-CAT and p2.8kb-CAT deletion constructs were able to efficiently direct transcription in both myoblasts and myotubes (Fig. 6). The p1.22kb-CAT was able to promote −13- and −10-fold enzymatic activity in myoblasts and myotubes, respectively, in comparison to p200bp-CAT. p1.22kb-CAT showed only slightly less activity than the highest-promoting deletion construct, p300bp-CAT. p1.22kb-CAT contains an E-box and an A/T-rich region, which may explain the higher CAT activity of this construct compared with p600bp-CAT.
p2.8kb-CAT was able to induce −10- and −7-fold higher CAT activity in myoblasts and myotubes, respectively, as compared with p200bp-CAT. The increased expression elicited by both constructs suggests that a positive regulatory element or an enhancer-like element may be located 5' of the p400bp-CAT construct. Neither construct was able to direct enzymatic activity in the murine breast carcinoma cell line, indicating that both constructs contain sequence elements required for tissue-specific expression. p1.22-CAT could direct nearly 2-fold more transcription in myotubes than in myoblasts, which may be due to the E-box. The p2.8kb-CAT that contains all E-boxes was only able to promote −1.3-fold more activity in myotubes than myoblast. The 2- and 1.3-fold induction elicited by these constructs is of borderline relevance given the complexity of the
CAT reporter assay system, suggesting other factors may account for the large increase in α7 mRNA during myoblast differentiation. It is possible that this increase may be due to a combination of factors, for example elevated promoter activity and mRNA stability. Taken together, these results indicate that the 5′-flanking region of the α7 gene functions as a promoter and shows the expected tissue-specific and developmentally regulated activity. Studies are ongoing to determine which sequence elements and post-transcriptional events are involved in this regulation.

As a start to determine the elements involved in the regulation of α7 in myoblasts and myotubes, we undertook trans-activation assays (see “Experimental Procedures”). Trans-Activation assays have been routinely used to determine if the family of muscle-specific bHLH factors can activate transcription from muscle-specific gene promoters (47, 62–65). Vectors expressing MyoD, myogenin, MRF4, and myf5 were transiently cotransfected into 10T1/2 cells along with p2.8kb-CAT. The data are expressed as fold activation of the cotransfection of the muscle-specific transcription factors over the p2.8kb-CAT cotransfected with pGEM-11Zf (+) alone. CAT activity in cell extracts was determined by a CAT-ELISA assay. The results presented are the average of two independent co-transfections.

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REFERENCES
1. Hynes, R. O. (1992) Cell 69, 11–25
2. Ruoslahti, E., and Reed, J. C. (1994) Cell 77, 477–478
3. Rosales, C., O’Brien, V., Kornberg, L., and Juliano, R. (1995) Biochim. Biophys. Acta 1242, 77–96
4. Bosman, F. T. (1993) Histocherm. J. 25, 469–477
5. Kramer, R. H., McDonald, K. A., and Vu, M. P. (1989) J. Biol. Chem. 264, 15642–15649
6. Kramer, R. H., Vu, M. P., Cheng, Y. P., Ramos, D. M., Timpl, R., and Waleh, N. (1991) Cell Regul. 2, 805–817
7. Collo, G., Starr, L., and Quaranta, V. (1995) J. Biol. Chem. 268, 19019–19024
8. Kaufman, S. J., Foster, R. F., Haye, K. R., and Faiman, L. E. (1986) J. Cell Sci. 82, 1–9

FIG. 7. Trans-Activation of the α7 promoter by bHLH transcription factors. Expression plasmids (pEMSV) encoding myogenin (myoG), myf5, MyoD, or MRF4 were cotransfected into 10T1/2 cells along with p2.8kb-CAT. The data are expressed as fold activation of the cotransfection of the muscle-specific transcription factors over the p2.8kb-CAT cotransfected with pGEM-11Zf (+) alone. CAT activity in cell extracts was determined by a CAT-ELISA assay. The results presented are the average of two independent co-transfections.

FIG. 8. Induction of α7 mRNA expression by MyoD. Northern blot analysis. 10T1/2 fibroblasts untransfected (lane 1) or transfected with pEMSV-MyoD (lane 2) and plated into differentiated medium. After 72 h, total RNA was prepared and analyzed by Northern blot analysis using an α7 cDNA probe. The line indicates the position of the ~4.8-kb α7 mRNA transcript. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
