Interaction between the Skeletal Muscle Type 1 Na\(^+\) Channel Promoter E-box and an Upstream Repressor Element

RELEASE OF REPRESSION BY MYOGENIN

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We have defined how four elements that regulate expression of the rat skeletal muscle type 1 sodium channel (SkM1) gene cooperate to yield specific expression in differentiated muscle. A basal promoter region containing within it a promoter E-box (−31/−26) is broadly expressed in many cells, including myoblasts and myotubes; mutations within the promoter E-box that disrupt binding of the myogenic basic helix-loop-helix (bHLH) factors reduce expression in all cell types only slightly. Sequential addition of upstream elements to the wild-type promoter confer increasing specificity of expression in differentiated cells, even though all three upstream elements, including a positive E-box (−85/−57), a repressor E-box (−90/−85), and upstream repressor sequences (−135/−95), bind ubiquitously expressed transcription factors. Mutations in the promoter E-box that disrupt the binding of the bHLH factors counteract the specificity conferred by addition of the upstream elements, with the greatest interaction observed between the upstream repressor sequences and the promoter E-box. Forced expression of myogenin in myoblasts releases repression exerted by the upstream repressor sequences in conjunction with the wild-type, but not mutant, promoter E-box, and also initiates expression of the endogenous SkM1 protein. Our data suggest that particular myogenic bHLH proteins bound at the promoter E-box control expression of SkM1 by releasing repression exerted by upstream repressor sequences in differentiated muscle cells.

Expression of the rat SkM1 sodium channel isoform is restricted almost exclusively to skeletal muscle; following a rapid post-natal increase in mRNA and protein levels, SkM1 becomes the predominant voltage-dependent sodium channel expressed in adult skeletal muscle (1, 2). The spatial distribution of the channel within a myofiber is also tightly regulated, with the highest density of channel protein found within the folds of the neuromuscular junction, and lower levels throughout the sarcolemma and T-tubular membrane (3–5). Given the multiple levels at which channel expression is regulated, complex interactions of transcription factors probably govern SkM1 transcription.

We have previously characterized several cis-regulatory elements that control expression of this gene in a primary muscle culture system (6). We found that both positive and negative mechanisms combine to modulate expression, and that two E-boxes play pivotal roles in this process. One E-box, located at −31/−26 within the promoter, works with other elements to orchestrate positive regulation of the gene, while a second E-box, located at −90/−85 within a larger upstream repressor region, confers muscle-specific expression on the basal promoter that otherwise lacks cell-type specific function.

One of the unresolved issues from our earlier work was the mechanism by which the upstream repressor region achieved muscle-specific function. Transcription factors that bind to this region are present in all cell types examined, and transfer of either the entire repressor or its various sub-components to a heterologous rat brain type II sodium channel (RBII) promoter repressed expression in muscle cells as well as non-muscle cells. We postulated that the native SkM1 promoter influenced the ability of the upstream repressor region to act selectively in non-muscle cells, perhaps through the E-box within the SkM1 promoter.

Several muscle-specific genes, including those for troponin I, desmin, and the acetylcholine receptor (AChR) α, β, γ, δ, and ε subunits, contain E-boxes within their promoter regions that are involved in regulating positive gene expression. These E-boxes function in part through their interaction with myogenic basic helix-loop-helix (bHLH) proteins (7–14). Although positive regulation through the E-box is common to all these genes, the interplay between the bHLH factors and other transcription factors is more variable, and in some cases has not been completely resolved. The E-box within the promoter of the desmin gene coordinates positive regulation through interaction with a distal enhancer that contains a second E-box and an MEF2 binding site (7), while the E-boxes of the β AChR subunit promoter interact with an M-CAT sequence adjacent to it (10). The AChR δ subunit and SkM1 5′-flanking sequences have substantial sequence and functional similarities (6, 12, 13). However, the E-box within the δ subunit promoter controls both positive and negative regulation of that gene, while these functions are split between two E-boxes within the SkM1 sequence (6, 13).

In this report we demonstrate that the SkM1 promoter E-box influences the ability of the upstream repressor region to function, and that the binding of bHLH factors to the promoter E-box releases repression exerted by this element in the muscle...
lineage. Furthermore, comparison of repressor function in different muscle cell types and at different developmental stages reveals specificity in the ability of particular myogenic bHLH factors to effect this release of repression.

**EXPERIMENTAL PROCEDURES**

*Generation of Reporter Gene Constructs*—The −174, −135, −95, and −85 promoter E-box mutations were created from previously characterized full-length (−2800/+249) promoter E-box mutations contained in pCAT-Basic (Promega; 6). Briefly, PCR was performed using either the cgg or tcc/gaa mutant −2800/+254 as a template. The 5′-primer contained 20 base pairs of SkM1 sequence starting at the designated point and a restriction site (either HindIII or PstI) for cloning purposes. The 3′-primer was complementary to +56 to +78 of the SkM1 sequence. The PCR products were digested with HindIII and SacI (−135, −95, −85) or PstI and SacI (−174) and cloned into the same sites of the corresponding wild-type 5′-deletion mutant. Mutants generated by PCR were sequenced (Sequenase; U. S. Biochemical Corp.). All other mutations used in this report were created and characterized previously (6).

*Cell Culture and Transient Expression Assays*—Culture and transfection of primary muscle cells was carried out as reported previously (2, 6). The C2C12 cell line was maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.). To initiate and maintain differentiated C2C12 cells, 2% horse serum replaced the fetal bovine serum. LipofectAMINE and OptiMEM (Life Technologies, Inc.) were used to transfect C2C12 cells, according to manufacturer directions. To allow comparison between calcium phosphate-transfected primary cultures and LipofectAMINE-transfected C2C12 cells, a constant molecular ratio of test DNA and pCI polylinker in the pCI expression vector (Promega) vector expressing the gene for chloramphenicol acetyltransferase driven by the SV-40 promoter and enhancer as a positive control, and the pCAT-Basic vector as a negative control (6).

Cells that were both transfected with reporter gene constructs and infected with the CMV-myogenin IRES-β-galactosidase adenovirus were assayed for β-galactosidase activity using X-gal as previously described (11). The CMV-myogenin IRES-β-galactosidase adenovirus was obtained from the Vector Core of the University of Pennsylvania (18, 19). Cells were either switched to differentiation medium or maintained in growth medium overnight (16–18 h). Control cells were fed medium without adenovirus. Cells were either switched to differentiation medium or maintained in medium containing 10% fetal bovine serum for an additional 28–30 h prior to harvest. Cells that were not transfected with reporter gene constructs were infected with the CMV-myogenin IRES-β-galactosidase adenovirus according to the same paradigm and harvested for nuclear extracts or membrane proteins.

*Gel-shift Assays*—Gel-shift assays and supershift assays were carried out as previously reported (6) with the following modifications and additions. Gel-shift assays for the repressor probes were carried out at 4 °C rather than room temperature. The antibodies used to supershift the various βHLH factors were obtained from Santa Cruz (E2A and myf-6), PharMingen (myogenin), and Novocastra (MyoD).

*Preparation of Protein Fractions and Western Blotting*—Membrane fractions containing sodium channel protein or nuclear extracts containing transcription factors were prepared as reported previously (6, 15). Gel electrophoresis and Western blotting were carried out as reported using the Western Star kit (Tropix; Ref. 15). The primary antibodies used to detect the βHLH factors were the same as those used in the supershifts. To remove particulate matter and reduced background staining, the myf-6 antibody was treated as follows. The antibody was diluted 1:50 in 10% heat-inactivated horse serum in phosphate-buffered saline and incubated with 20 μg/ml porcine liver extract (Sigma) for 1 h at 4 °C. The extract and particulate matter were removed by centrifugation at 100,000 × g for 2 h. The final antibody solution was diluted 1:250 in 10% horse serum, 0.4% 1-block (Tropix), and 0.1% Tween in phosphate-buffered saline. Final dilutions for the MyoD antibody was 1:250, and the myogenin antibody 1:500. The monoclonal antibody (anti-β-actin) used to detect the sodium channel has been extensively characterized and is specific for the SkM1 isoform of sodium channel (16, 17).

**Generation of Replication-deficient CMV-myogenin IRES β-galactosidase Adenovirus**—The dl327 adenovirus and pAd-Link vector used to create the CMV-myogenin IRES β-galactosidase adenovirus were obtained from the Vector Core of the University of Pennsylvania (18, 19). 293 cells (American Type Cell Culture) were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

The polynucleotide in the pCI expression vector (Promega) was altered to contain restriction sites for the enzymes EcoRI, StyI, NotI, SnaBI, and BclI, and an EcoRI to StyI fragment containing myogenin was inserted. A NotI to BamHI fragment containing an IRES in frame with the β-galactosidase gene, obtained from pLIGns (20), was inserted between the EcoRI and BclI site of CMV-myogenin to yield the CMV-myogenin IRES-β-galactosidase. This construct was digested to completion with BglII, and a partial digest carried out with ClaI to yield a fragment extending from the CMV promoter to the SV40 poly(A) site. This fragment was cloned into the BglII and ClaI sites of pAd-Link and prepared for recombination by linearizing with NheI. The dl327 adenovirus was grown in 293 cells and prepared for recombination by digestion with ClaI as reported (19). The initial transfection was carried out as reported previously (19), but the standard agar overlay procedure was replaced by plaque-purification using 96-well plates. Serial dilutions of the transfected cells were combined with 1 × 10⁶ 293 cells in a 20-ml total volume and dispensed into 96-well plates using 100 μl well. The plates were maintained for 6 days, then fed with 100 μl of medium. After another 5–6 days, plaques were observed by eye. Dilutions resulting in more than 20 plaques/plate were discarded. A total of 30 plaques were screened by Southern blot, and of these, 9 were positive.

Plaque purification was carried out in the same manner, using serial dilutions ranging between 10⁻⁸ and 10⁻¹⁰. Eleven plaques were screened in a Southern blot, and all were positive. One of these was expanded for large scale production according to published methods (19).

**RESULTS**

*Arrangement and Activity of cis-regulatory Elements*—We previously characterized several cis-regulatory elements that control expression of the SkM1 gene (6, Fig. 1). For most of the experiments reported here, we focused on four major functional elements between −174 and +49. Our previous results are summarized as follows. The promoter E-box at −31/−26 directs
positive modulation of the gene through an interaction with elements elsewhere in the SkM1 genomic sequence. Myogenic bHLH proteins play a role in this interaction. The activity of the −85/−75 positive element is largely muscle-specific and confers 7-fold higher expression levels on the promoter, although its activity is masked in cultured muscle cells by the repressor E-box immediately upstream at −90/−85. DNase footprinting of the transcription factors that bind the upstream repressor region or repressor have shown that this element extends upstream to approximately −135, and functional studies described below substantiate an independent function for these upstream repressor sequences. A 3′-positive element that includes part of the 5′-untranslated region and part of the first intron lies between +50/+254; this element increases expression levels 10-fold in muscle cells.

Fig. 2. Myogenic bHLH proteins bind the wild-type SkM1 promoter E-box but not the promoter E-box mutants. The wild-type SkM1 promoter E-box region (cagCACGCTgta), a c/g mutant promoter E-box region (cagCACGCTgta), and a tcc/gaa mutant promoter E-box region (cagCACGCTgaa) were used in gel-shift assays with nuclear extracts prepared from the following cell types: A, day 4 primary muscle cells; B, day 7 primary muscle cells; C, C2C12 myoblasts; D, C2C12 day 2 myotubes; E, PC12 cells. For all panels, antibodies against the following factors were used for supershift assays: 1, no antibody; 2, MyoD; 3, myogenin; 4, MRF4; 5, E2A. The supershifts are indicated by asterisks. The SkM1 promoter E-box bound multiple proteins in both muscle and non-muscle cells, but the myogenic bHLH proteins were bound only in muscle cells, as indicated by the asterisks. The two mutations in the promoter E-box either abolished (c/g mutant) or markedly reduced (tcc/gaa mutant) the ability of this region to bind myogenic bHLH factors. The c/g mutant also severely reduced the binding of non-bHLH proteins, while the tcc/gaa mutant retained binding for these additional factors. The tcc/gaa mutant also bound an additional factor not observed with the wild-type probe; this new complex co-migrated with the MyoD gel-shift but was not supershifted by the MyoD antibody. In panel F, a direct comparison was made between the supershifts of the MyoD and E2A antibodies in muscle cells at different stages of development. Two alternative supershifted states of MyoD existed, with the higher complex predominating in myoblasts, and the lower in myotubes. An antibody to the MyoD dimerization partner, E2A, demonstrated that E2A was involved only in the higher complex. The E2A antibody did not supershift the complex corresponding to myogenin. The nuclear extracts were prepared from the cell type indicated beneath each set of lanes.

The Transcription Factors That Bind the −85/−75 Positive Element, Repressor E-box, and the Upstream Repressor Se-
The mutant. 1, no competitor; 2, wild-type competitor (GAAGATTGGC-

Other SkM1 elements.

3

The sequences are expressed in all cell types—

components of the upstream repressor region.

sequences are expressed in all cell types. The −85/−57 positive element binds a simple complex in all cell types examined, including PC12 and muscle cells (Fig. 3A). This factor was displaced by the wild-type competitor (Fig. 3A, lane 2) but not a mutant competitor that altered two short motifs at −83/−78 and −64/−59, represented by the connected boxes in Fig. 1 (Fig. 3A, lane 3). Although this factor is present in all cell types, the −85/−57 positive element exhibited greater activity in differentiated muscle cells, as published previously and shown below (6).

The upstream repressor region is comprised of two components, a repressor E-box and the upstream repressor sequences. Previously, we identified a broad gel-shift that exhibited an extensive footprint covering sequences between −135 and −82 (6). Gel-shift assays carried out at low temperatures revealed an additional factor that associates more uniquely with the repressor E-box. Both the −135/−82 probe that included the repressor E-box and the repressor E-box probe alone associated with a sharp gel-shift that runs at the highest complex, while the shorter −135/−95 probe that excluded the repressor E-box did not bind this factor. The broad middle gel-shift was found with the probes that include the upstream repressor sequences, while the lowest gel-shift band appeared with all three probes. The longest probe, containing both components of the upstream repressor region, generated the most intense gel-shift, suggesting that these factors stabilize each other on the DNA. The repressor-binding transcription factors were found in all cell types.

The promoter determines the cell type in which the SkM1 repressor functions. The −174/+49 SkM1 sequence with the wild-type promoter E-box or the −174/+49 sequence with either the cgg or tcc/gaa mutant promoter E-box were inserted into a vector containing the reporter gene for CAT. These constructions were assayed in transient expression assays in primary muscle cells, which express the SkM1 gene, or PC12 cells, which express the RBII gene. Both cell types were transfected with the pCAT-Control (pCAT-C) plasmid as a positive control. In conjunction with its native promoter, the repressor functioned in the non-muscle PC12 cell line, while it allowed expression in muscle cells. Mutations that disrupt the ability of the promoter E-box to bind the myogenic factors caused the repressor to function in primary muscle cells, but did not further reduce gene expression in the negative PC12 cell line. In the lower panel, the −174/−50 sequence containing the entire upstream repressor region was transferred onto the heterologous RBII promoter, which also contains an E-box, and analyzed in both cell types. This switch in promoters caused the repressor to function in primary muscle cells rather than PC12 cells. The activity of the RBII promoter without added sequences is also shown in both cell types.

expression in PC12 cells. These data suggest that the "default" setting of the upstream repressor region is to function except when the promoter E-box binds bHLH factors.

Transfer of the upstream repressor region to the heterologous RBII sodium channel promoter reduced expression of that promoter in muscle cells, while permitting expression in PC12 cells (Fig. 4, bottom panel). The repressor binding-proteins were clearly present in PC12 cells (Fig. 3B), and the repressor was able to function in conjunction with the native SkM1 promoter in these cells (Fig. 4, top panel), indicating that the repressor-binding proteins were functionally active in PC12 cells. Together, these data indicate that the promoter, and specifically the promoter E-box, determines the cell type in which the repressor functions.

Expression of SkM1 sodium channel protein and Myogenic factors as a function of development in C2C12 cells—We have previously used primary muscle cultures to study the expression of SkM1 mRNA levels and the function of the SkM1 cis-regulatory elements because this system relates most closely to the in vivo setting (1, 2, 6). However, primary muscle cultures express detectable levels of SkM1 mRNA and protein at the earliest times measured in culture, even before myotubes form (2). We therefore examined C2C12 cells as an alternative to primary muscle cultures since C2C12 cells can main-

S. D. Kraner, unpublished observation.
Addition of the \(-85/-57\) element contributed to positive regulation in differentiated myotubes (Fig. 6, A and B; note difference in scale). Although this positive element increased the level of gene expression in both myoblasts and myotubes, consistent with the presence of a ubiquitous transcription factor, the augmentation produced in differentiated muscle cells was 4–6-fold greater than in myoblasts (Fig. 2B). Both mutations in the promoter E-box significantly reduced this differentiation-specific activity (Fig. 6B), indicating that at least part of the activity was derived through an interaction with the promoter E-box.

Addition of the repressor E-box to the combined promoter and positive element reduced expression in all cells, but to a greater degree in C2C12 myoblasts and myotubes (10-fold) than primary muscle cells (7.5-fold). The level of expression observed in myoblasts approached that reported previously for the negative NIH 3T3 cell line (6), and neither promoter mutation further reduced expression levels in myoblasts. In differentiated cells, the tcc/gaa mutation did not significantly alter repressor activity, while the c/g promoter mutation reduced expression in both C2C12 and primary muscle myotubes to nearly the same level as myoblasts, suggesting that the promoter E-box binding proteins that interact with the repressor E-box are the non-bHLH factors.

The greatest effect of the promoter E-box mutations was observed in constructs that included the upstream repressor sequences (Fig. 6D). Addition of these sequences further reduced expression in both myoblasts and myotubes, but the incremental decrease was much less in myotubes, particularly primary muscle myotubes. Promoter mutations had little effect on the residual expression in myoblasts, but both the c/g and tcc/gaa mutations virtually eliminated expression above background in myotubes, producing a 90% reduction in primary culture myotubes. Our data suggest that the upstream repressor sequences play an important role in the interaction with the promoter E-box, and that mutations in the promoter E-box allow the combined components of the upstream repressor region to function to the same degree in differentiated muscle cells as in non-muscle cells. The combination of all four elements produces the highest degree of developmental specificity of SkM1 expression.

**Myogenin Release Repression of the SkM1 Upstream Repressor Region in C2C12 Myoblasts**—To determine if the interaction between the promoter E-box and the upstream repressor sequences was mediated by particular bHLH proteins, we forced expression of myogenin in C2C12 myoblasts using a recombinant adenovirus, and tested the functional impact of this transcription factor on either the wild-type \(-174/+49\) sequence, or the corresponding c/g or tcc/gaa promoter E-box mutants. The production of myogenin in the infected cells was verified by Western blot (Fig. 7). In the absence of myogenin, myoblasts did not express either the wild-type or mutant \(-174/+49\) sequences at levels above background. Introduction of myogenin resulted in expression of the wild-type sequence in both myoblasts and 1 day myotubes at levels 9-fold higher than background (Fig. 7), but the mutations in the promoter E-box interfered with the ability of myogenin to potentiate this increase. The overall level of myogenin-driven expression attained with the \(-174/+49\) construct in these cells was comparable to that observed in primary muscle cells (Figs. 6D and 7).

**Myogenin Is Sufficient to Initiate Expression of the Endogenous Sodium Channel Gene in C2C12 Myoblasts**—Although our data indicate that myogenin releases repression exerted by the upstream repressor region in the small segment of SkM1 flanking sequence used in our functional assays, there are additional elements that control expression of the endogenous
gene. The ability of myogenin to initiate expression of the endogenous sodium channel gene was therefore assessed by directly measuring sodium channel protein levels in the same experimental paradigm used for the functional assays. In control myoblasts, no SkM1 protein product was detected, while the forced expression of myogenin was sufficient to up-regulate SkM1 protein levels to an extent comparable to control day 1 C2C12 myotubes (Fig. 7, inset).

Later Phases of Sodium Channel Up-regulation Correlate with the Activity of a 3\(^{-}\)Positive Element—Although the initiation of SkM1 transcription correlates with the appearance of myogenin in C2C12 myotubes, both MyoD and myogenin levels are relatively low at later times in culture when the highest levels of SkM1 protein are detected, and only low levels of MRF4 are found in these cells, as determined by Western blot (Fig. 5). These observations suggest that additional factors must act to maintain transcription of the SkM1 gene at later times. Since we previously demonstrated the contribution of a 3\(^{-}\)positive element to positive regulation of the SkM1 gene (6), we compared the activity of SkM1 constructs with and without the 3\(^{-}\)-positive element during late myotube development in C2C12 cells (Fig. 8). Although the enhanced expression produced by this positive element in C2C12 cells was less than in primary muscle cultures, the magnitude of the effect did correlate with developmental up-regulation of SkM1 protein expression in C2C12 cells.

DISCUSSION

We have shown previously that multiple cis-regulatory elements control expression of the SkM1 sodium channel gene in primary muscle cells, and two E-boxes located within several larger elements play a dominant role (6). Although we had demonstrated that the promoter E-box interacts with elements outside the promoter to control positive regulation of the gene, we had not determined with what element(s) it interacted or how this positive regulation was achieved. Our earlier experiments also indicated that the native SkM1 promoter controls the cell type in which the upstream repressor region can function, but the mechanism underlying the promoter/repressor interaction was unclear. In this report, we have focused most of our effort on understanding the interactions between four major elements located between \(-85/-57\) in order to systematically approach these unresolved issues.

The promoter E-box influences the ability of upstream repressor region to act in differentiated muscle. As we have shown previously and again in this report, the promoter itself is expressed broadly in many cell types, even though the promoter E-box is the one element characterized to date that binds...
confer specificity by itself. It is the addition of upstream elements that increase expression in differentiated muscle relative to undifferentiated muscle, even though the upstream elements bind ubiquitously expressed transcription factors. Mutations that disrupt the ability of the promoter E-box to bind either the myogenic bHLH factors or non-bHLH factors interfere with the specificity conferred by these upstream elements, indicating that factors bound at the promoter E-box interact with those bound at upstream elements. The interaction of these factors potentiates higher levels of expression in differentiated muscle relative to myoblasts, especially in primary muscle cells where the amount of the myogenin is highest.

Two different mutations in the promoter E-box were used to help characterize the interaction between the promoter E-box and upstream elements. One mutation (c/g) abolishes the binding of both myogenic bHLH proteins and non-bHLH proteins, while a second (tcc/gaa) severely reduces binding of the bHLH proteins but does not diminish binding of the non-bHLH transcription factors. These mutants distinguish the impact of the bHLH factors from that of the other proteins that bind to the promoter.

The interaction between the promoter E-box and the −85/−57 positive element is a supportive one in that factors bound to the promoter E-box “aid” the action of the transcription factor that binds the positive element. Since both promoter mutations affect this interaction, it appears that the myogenic bHLH proteins are involved.

The upstream repressor region is comprised of two individual components, the repressor E-box and the upstream repressor sequences, that have different relationships with the promoter E-box. For both of these components, factors bound to the promoter E-box inhibit the function of the repressor specifically in differentiated muscle cells, resulting in the retention of positive expression in primary muscle cells, and to a lesser extent in C2C12 myotubes. The factors responsible for the promoter E-box/repressor E-box interaction appear to be the non-bHLH factors, since the c/g mutation, but not the tcc/gaa mutation, affect the interaction. Both promoter E-box mutations allow full repression by the upstream repressor sequences, demonstrating that myogenic bHLH are responsible for this release of repression.

Experiments that we carried out previously, in which various subcomponents of the upstream repressor region were transferred to the heterologous RBII promoter, demonstrated that the repressor E-box was both necessary and sufficient for negative regulation (6). Although the data presented in this paper do not allow us to conclude if the upstream repressor sequences can function without the repressor E-box in the −174/+49 sequence, it is clear that the upstream repressor sequences play a key and distinct role in interacting with the bHLH proteins.

The promoter determines in what cell type gene expression is allowed. Transfer of the SkM1 repressor to the RBII promoter results in repression in primary muscle cells, while expression is allowed in PC12 cells, which express the RBII gene (21, 22). One prediction from these data is that the E-box within the RBII promoter will bind bHLH proteins specific to neuronal cells and that these factors might act in PC12 cells to inhibit the action of the SkM1 repressor much as the myogenic factors do in conjunction with the wild-type SkM1 promoter in myotubes. Indeed, bHLH factors have been found in PC12 cells, and increased levels of specific neuronal bHLH factors have been shown to correlate with increased expression of the RBII gene in this cell type when treated with nerve growth factor, suggesting neuronal bHLH proteins play an important role in the regulation of the RBII gene (21, 23). Other neuronal genes
expressed in PC12 cells are regulated by interactions between E-boxes and separate regulatory sequences, and this interaction is mediated in part through bHLH proteins (24, 25). We anticipate that there may be parallel mechanisms controlling expression of the SkM1 and RBII genes, with regulation by bHLH factors acting as a common theme.

Not all bHLH proteins that bind the promoter E-box are equivalent in their ability to release the repression exerted by the upstream sequences. A complex program initiates development in muscle, with different myogenic factors expressed at different times. C2C12 myoblasts, which do not contain detectable levels of SkM1 protein, express only MyoD partnered with E2A. Upon differentiation, myogenin is rapidly up-regulated. Initiation of SkM1 gene expression takes place at this time. This correlation led us to hypothesize that myogenin plays an important role in the regulation of SkM1 expression.

Direct introduction of myogenin in combination with the entire −174/+49 sequence into C2C12 myoblasts and day 1 myotubes released repression, leading to expression levels comparable to those observed for this same construct in primary muscle cells. Mutations in the promoter E-box either abolish (c/g) or greatly reduce (tcc/gaa) the release of repression that myogenin can confer, confirming that myogenin binding at the promoter E-box directly affects negative regulation. Myogenin also activates expression of the endogenous SkM1 gene. However, unlike its action on the short regulatory sequences, the effect of myogenin on the endogenous gene is potentiated by culture conditions that induce differentiation, suggesting that myogenin may initiate expression of the endogenous SkM1 gene through multiple mechanisms.

Levels of SkM1 increase at later times in C2C12 differentiation, suggesting later action of another factor, particularly since the levels of the bHLH factors themselves decrease. We previously demonstrated that the 3′-positive element plays a major role in tissue-specific SkM1 expression (6), and the activity of this element correlates with the late increase in the endogenous SkM1 protein in C2C12 cells, although it never confers the level of activity in C2C12 myotubes that it does in primary muscle cells. Part of the activity of the 3′-positive element is derived through a specific interaction with MRF4, since forced expression of MRF4 in C2C12 cells increases the activity of the 3′-positive element to the same level observed in primary muscle cells.2 The absence of high levels of SkM1 gene expression in the constructs that lack the 3′-positive element indicates its important role, particularly for the maintenance of expression in later stages of differentiation.

Although our initial analysis presents the relationship between the promoter E-box-binding proteins and other transcription factors as a one-on-one interaction, this is certainly an oversimplification of an association that is probably far more complex, with changes occurring in the entire transcription initiation complex to switch it from an “inactive” to an “active” state. The myogenic bHLH proteins and perhaps factors that are still unknown may independently contribute to the assembly of the transcription initiation complex. Our data suggest that only specific myogenic bHLH proteins can function in conjunction with the other SkM1 factors, and it may be that the bHLH proteins confer the muscle-specific action to the complex. However, the myogenic bHLH factors cannot act alone. Our future work will be directed toward identifying all of the factors involved and understanding the interplay between them.

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