Two E-Boxes Are the Focal Point of Muscle-specific Skeletal Muscle Type 1 Na\(^+\) Channel Gene Expression*

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We have characterized a group of cis-regulatory elements that control muscle-specific expression of the rat skeletal muscle type 1 sodium channel (SkM1) gene. These elements are located within a 3.1-kilobase fragment that encompasses the 5′-flanking region, first exon, and part of the first intron of SkM1. We sequenced the region between −1062 and +311 and determined the start sites of transcription; multiple sites were identified between +1 and +30. The basal promoter (−65/+11) lacks cell-type specificity, while an upstream repressor (−174/−65) confers muscle-specific expression. A positive element (+49/+254) increases muscle-specific expression. Within these broad elements, two E boxes play a pivotal role. One E box at −31/−26 within the promoter, acting in part through its ability to bind the myogenic basic helix-loop-helix proteins, recruits additional factor(s) that bind elsewhere within the SkM1 sequence to control positive expression of the gene. A second E box at −90/−85 within the repressor controls negative regulation of the gene and acts through a different complex of proteins. Several of these cis-regulatory elements share both sequence and functional similarities with cis-regulatory elements of the acetylcholine receptor δ-subunit; the different arrangement of these elements may contribute to unique expression patterns for the two genes.

Sodium channels comprise a multigene family, the members of which are expressed in a tissue-specific and developmentally-regulated manner. The electrophysiological properties of the different channel isoforms differ subtly, and presumably reflect the underlying need for slight variations in electrical conduction in the different tissues. Several members of the sodium channel family have been implicated in various inherited disorders in muscle, heart, and neuronal tissues (1–3). Although significant progress has been made in understanding the structural defects of mutant channel proteins, the mechanisms that regulate transcriptional control of these genes have been examined in detail only for the rat brain II (RBII)\(^1\) and skeletal muscle type 2 (SkM2) isoforms (4–7). In this report, we characterize several of the cis-regulatory elements that control transcription of the skeletal muscle type 1 sodium channel (SkM1).

The two isoforms of skeletal muscle sodium channel, SkM1 and SkM2, are regulated differentially during development. SkM2 is expressed in embryonic muscle but is down-regulated postnatally; denervation of adult muscle leads to the reactivation of SkM2 transcription (8, 9). SkM1 comprises most of the sodium channel expressed in adult skeletal muscle, with the mRNA levels up-regulating 10-fold postnatally (8–10), and it is expressed in the same temporal manner as the ancillary β\(_1\) subunit (11).

Since the SkM1 isoform is expressed in skeletal muscle, it seemed likely that it is regulated in part by muscle-specific transcription factors from the MyoD and MEF2 families (see Refs. 12–14, for reviews). Unlike most skeletal muscle genes, which are expressed at highest levels before birth, SkM1 reaches peak expression in adult muscle. Only a few other muscle genes, such as myoglobin, exhibit this late rise in expression levels (15), and novel factors may be involved in the regulation of such genes (16, 17). In addition, like the acetylcholine receptor (AChR), the sodium channel is expressed at higher levels beneath the neuromuscular junction than in the surrounding sarcolemma (18, 19); comparison of the SkM1 regulatory sequences to those for the AChR subunits may reveal common regulatory pathways.

We anticipate that the expression of the SkM1 gene is regulated by a complex combination of tissue- and development-specific factors working in conjunction with the general transcription apparatus. As a first step toward elucidating the regulation of this gene, we have carried out a detailed analysis of the cis-regulatory elements that control its expression, elucidated similarities between some of these elements and those of the AChR δ-subunit, and studied in more detail the critical role played by two E boxes. One E box, located within the promoter of the gene, plays a critical role in positive, tissue-specific gene expression. We demonstrate that members of the MyoD family of transcription factors contribute to this positive regulation, although they are not wholly responsible for it. The second E box plays a critical role in the repression of SkM1 expression in non-muscle cells through binding a complex of proteins.

**EXPERIMENTAL PROCEDURES**

**Isolation of Genomic Clones**—A rat spleen genomic library in λDASH was screened using probes derived from the 5′-untranslated region of the SkM1 gene (20), and five overlapping clones were obtained. Clones λ14.2, λ29.1, and λ221A were positive in a Southern blot to a PstI fragment encoding sequences −310 to +119 relative to the published translation start site of the SkM1 gene (20). Sequence analysis of λ14.2, reported previously (21), encodes the −401 to −226 segment but then deviates from that of the cDNA clone (20), revealing the presence of an intron between −402 and −401. The library was screened using se-
sequences −451 to −420 as a probe, and two clones, designated X2633 and λ2A.1.1, were obtained. A restriction site map was derived using the enzymes HindIII, EcoRI, BamHI, and KpnI, indicating that the five clones encompass almost 40 kb of DNA surrounding the transcription initiation site of the SkM1 gene. A 3.1-kb HindIII fragment obtained from λ2A.1.1 that was positive in a Southern blot with the −457 to −420 probe was cloned into pBlueScript (KS) (SkM1-3.1) and used for all subsequent analysis.

DNA Sequence Analysis—DNA sequencing (Sequenase; U. S. Biochemical Corp.) was carried out on the SkM1-3.1 clone from the 3′ edge using T7 and a series of primers to the SkM1 sequence. Using the resulting sequence, additional primers were synthesized and annealing performed on the complementary strand. Three clones containing sequence between −1062 (BglII) and −438 (SphI), −438 (SphI) and +11 (SacI), and +11 (SacI), and +254 (XbaI) were cloned into pAlter (Promega) and single strand DNA generated. This DNA was both sequenced and used for generation of site-directed mutations.

RNAse Protection and 5′-RACE—RNAse protections were carried out as described (4), using a [32P]UTP-labeled antisense RNA probe containing sequences −640 to +127 hybridized to 50 μg of total RNA obtained from rat skeletal muscle or liver.

For 5′-RACE, poly(A)+ mRNA was purified from rat skeletal muscle, and cDNA synthesized using a primer containing the −325 to −349 segment relative to the translation start site. A poly(G) tail was added to the 3′ end by incubating in 25 units of terminal transferase, 40 μM dGTP, 0.72 mM CoCl2, and the buffer supplied by the manufacturer (Boehringer Mannheim) for 15 min at 37 °C. PCR was performed using this template, an oligo(C) anchor primer (CAGTCCAGAAGCTTG-135 to 135), and single strand DNA generated. This DNA was both sequenced and used for generation of site-directed mutations.

Generation of Reporter Gene Constructs—The SkM1-3.1 clone was digested with HindIII (−2.8) and XbaI (+49) and the resulting fragment was ligated into pCAT-Basic (Promega) digested with HindIII and XbaI using a linker (XbaI-XhoI-XbaI). This clone was designated SkM1CAT (−2800 to +94). Five deletion mutants −1062 and +11 were created from SkM1CAT using naturally occurring restriction sites at −1062 (BglII), −78 (SacI) and −11 (SacI), −273, −174, −135, −95, −85, and −65 were created by PCR. The 5′ primers contained 20 bp of SkM1 sequence starting at the designated point, and a restriction site for enzyme indicated below for cloning purposes; the 3′ primer was complementary to +56 to +78. The PCR products were digested with PstI and XbaI (−273 and −174), SalI and XbaI (+65), or HindIII and XhoI (−135, −95, −85, and −65), and cloned into pCAT-Basic digested with the same enzymes. All mutants generated by PCR were sequenced. To generate the constructs XmaS and XmaG, the XmaI fragment encoding sequences +50 to +254 was cloned into the XmaI site of the SkM1CAT vector and clones corresponding to the native (XmaS) and reverse (XmaG) orientation were selected.

The repressor constructs on the heterologous rat brain type II sodium channel promoter, containing sequences between −150 and +177 (RBII), were generated by introducing HindIII-BglII fragments generated by PCR (for −174−50 and −174−65) or double-stranded synthetic oligonucleotides (−135−95, −135−82, and −93−82) into the gel-purified pSDK7 vector (5), with HindIII and BglII. The wild-type SkM1 promoter E box (wt −34−23) and mutants (c/g, g/a, u/a, g/t, and t/c) were generated by introducing double-stranded synthetic oligonucleotides containing flanking sequences into the HindIII site of pCAT-Basic. All constructs were sequenced.

RESULTS

Isolation and Sequencing of Genomic Clones and Identification of Transcriptional Start Sites—Five SkM1 genomic clones were obtained from a rat spleen genomic library, and the overlaps determined by restriction mapping (Fig. 1). A 3.1-kb HindIII fragment derived from λ2A.1.1 was positive in a Southern blot using a probe to sequences −451 to −420 relative to the translational start site (20). This fragment was cloned into Bluescript KSII, and used as the starting material for all subsequent analysis. Sequence analysis between −1062 and +511 revealed that this fragment contains 225 bp of the first intron, the first exon (87 bp), and approximately 2.8 kb of 5′-flanking sequence (Fig. 2A).

Transcriptional start site analysis was carried out by both nuclease protection and 5′-RACE. RNAse protection assays were performed with an [32P]UTP-labeled antisense RNA probe containing sequences −640 to +127 hybridized to total RNA prepared from both adult rat skeletal muscle and liver. Protected fragments ranged in size from 87 to 70 bp (Fig. 3), indicating multiple transcriptional start sites.

5′-RACE was used to confirm the transcriptional start sites.
Fig. 2. A, SkM1 genomic sequence. The 3.1-kb clone was sequenced from −1062 to +311 and found to encode approximately 2.8 kb of flanking sequence, the first exon (87 bp), and part of the first intron, designated by lowercase letters.

The SkM1 sequence contains two E boxes between the 5′ and 3′ flanking sequences (26). There is a CG-rich sequence just upstream of the transcription initiation sites that includes a consensus binding site for the AP-2 transcription factor (GCCNCGCC) as well as an E box (CANNTG), the consensus binding site for the AP-2 transcription factor (GCCNCGCC). These sequences indicate functionally important E boxes. The double underlines indicate motifs conserved between the SkM1 −85/−60 positive element and an enhancer region of the AChR δ-subunit.

B, the 5′-flanking sequence for the AChR δ-subunit (27) and SkM1 genes are aligned at the first transcription initiation site, as indicated. The single underline indicates similarities in the promoter regions and the boxed sequences indicate functionally important E boxes.

Of 21 clones sequenced, 16 encoded the SkM1 5′-untranslated region and 11 of these initiated between +1 and +29, in good agreement with the distribution pattern observed by the RNase protection assay. The five remaining clones initiated between +40 and +78, and may represent transcripts that terminated prematurely. The sequence of 5 clones obtained with 5′-RACE is shown (Fig. 3). We numbered the first transcription initiation site observed by 5′-RACE as +1 and all other sites are numbered relative to it, as is shown in the sequence (Fig. 2A).

Inspection of the sequence just upstream of the transcriptional start sites indicates that the SkM1 promoter region lacks canonical TATA boxes, CAAT boxes, or binding sites for SP-1, and also lacks conserved initiator sequence surrounding the transcription start sites (26). There is a CG-rich sequence just upstream of the transcription initiation sites that includes a consensus binding site for the AP-2 transcription factor (GCCNCGCC) as well as an E box (CANNTG), the consensus binding site for the AP-2 transcription factor (GCCNCGCC). These sequences indicate functionally important E boxes. The double underlines indicate motifs conserved between the SkM1 −85/−60 positive element and an enhancer region of the AChR δ-subunit.

Deletion Analysis of the SkM1 Genomic Sequence Reveals Multiple cis-Regulatory Elements That Control Expression of the Gene—We created a series of deletion mutants of the SkM1 genomic sequence linked to the reporter gene for chloramphenicol acetyltransferase (CAT) and transfected these mutants into either rat primary muscle cultures, which express the SkM1 gene, or NIH 3T3 cells, which do not express the gene. The series of 5′ deletion mutants shown in Fig. 4A terminate at +49. The longest construction of this group, beginning approximately −2800 bp upstream of the transcription start sites, and 5′ deletion mutants to −174, produced approximately 10-fold higher CAT levels in rat primary muscle cells than in NIH 3T3 cells, demonstrating the ability of the 5′-flanking region to drive muscle-specific expression. Deletion of sequences from −174 to −65 resulted in a 10-fold up-regulation in the 3T3 cell line, with only a 2-fold increase in primary muscle cells. Further deletion to +11, which falls within the cluster of transcription start sites, reduced expression in both lines to levels only 2–3-fold that of the pCAT-Basic vector itself. A 3′ deletion mutant that includes sequence between −2800 to +11 exhibited expression levels in primary muscle cells similar to that of the mutation that ends at +49, whereas further 3′ deletion to −438 abolished expression in primary muscle cells. Thus, the rough boundaries of the SkM1 “core” promoter fall between nucleotides −65 and +11, although for convenience, our core promoter constructs extend from −65 to +49. This core promoter is not muscle-specific; the repressor element located downstream of the SkM1 promoter.

The addition of nucleotides between +50 and +254 to the −2800/+49 sequence increased expression in primary muscle.
cells, 10-fold, to achieve a final level 50-fold higher than in 3T3 cells (Fig. 4B). This increase in gene expression is partly dependent on orientation, since placement of the +50/+254 fragment in a reverse orientation reduced the effect by 50%. This fragment did not function in an enhancer trap assay with either the heterologous SV-40 promoter or its native SkM1 promoter (data not shown) and is, therefore, a position-dependent positive element.

We refer to the genomic sequences spanning nucleotides −2800 to +254 as the full-length SkM1 regulatory sequence. Within this region, we have initially defined three broad elements important for the expression of the SkM1 gene: 1) the core promoter, which lies between −65 and +11 and lacks muscle-specificity; 2) a repressor, which lies between −174 and −65 and confers muscle-specificity on the promoter; and 3) a muscle-specific positive element which lies between +50 and +254.

Analysis of the −174/−65 Repressor Element by Gel Shift and DNase Footprint Assays—Because the repressor located between −174 and −65 confers muscle-specific gene expression, we investigated this element in greater detail. A 32P-labeled −174/−65 probe was analyzed in a gel shift assay using nuclear extracts prepared either from primary muscle cells at various stages of development or from NIH 3T3 cells (Fig. 5A). As an independent control for the quality of the nuclear extracts, a 32P-labeled SP-1 recognition site synthetic oligonucleotide was tested with the same extracts. A prominent shift, designated as the upper band (UB), was detected at the same mobility in primary muscle cells at all stages of development and in 3T3 cells. Although the intensity of the upper band was greater in 3T3 cells than primary muscle cells, the presence of a shift in the primary muscle cultures once myotubes had fully formed (day 7) seemed at odds with the activity profile in the primary muscle cells (Fig. 4B). This increase in gene expression is partly dependent on orientation, since placement of the +50/+254 fragment in a reverse orientation reduced the effect by 50%. This fragment did not function in an enhancer trap assay with either the heterologous SV-40 promoter or its native SkM1 promoter (data not shown) and is, therefore, a position-dependent positive element.

To further delineate the boundaries of the protein binding region, we performed DNase footprint assays using extracts from both day 7 primary muscle and 3T3 cells (Fig. 5B). The footprints in the two cell types had in common a series of hypersensitive sites and a protected region extending from −135 to −82 that may indicate the binding of a factor common to both cell types. In the primary muscle cells, there was also additional protection both within the −135 to −82 region and upstream to −65, indicating either that an additional factor binds the DNA in the primary muscle cells or that a different factor binds. The combination of the gel shift and footprint analysis suggested that repressor-binding protein(s) are present in both cell types, but that the identity of the protein or protein complex may not be the same.

The footprinted −135/−82 region produced the same gel shift pattern as observed for the −174/−65 repressor, although two lower bands (labeled LB) are more easily visualized when using the smaller probe (Fig. 5C). These shifts were also observed with the −174/−65 probe, but were partly obscured by the unbound probe. A −93/−82 fragment encompassing only the −90/−85 E box failed to produce a gel shift (data not shown), but when present at micromolar concentrations, this E box displaced the probe from the upper band in a competition assay, demonstrating that the relevant factor binds to the E box, although with a lower affinity than for the larger fragment.

An E Box at −90/−85 Plays a Crucial Role in the Function of the Repressor—Extending the results of the footprint analysis, we constructed a series of mutations in which the −174/−65 repressor element or various permutations of this sequence were transferred to a heterologous promoter. We chose a minimal rat brain type II sodium channel promoter contain...
of the footprint. The region of the probe extending from the lowest hypersensitive site to the highest protected part in the 3T3 lane indicates protected regions. A Maxam and Gilbert A + G sequencing reaction was carried out on the same probe to mark the position of the footprint. The region of the probe extending from the lowest hypersensitive site to the highest protected part in the 3T3 lane corresponds to sequences −135 to −82. C, gel shift competition with repressor E box. The gel shift performed with the −135/−82 probe forms a series of complexes, as does the −174/−65 probe, although with the larger probe, the lower bands (LB) are partially obscured by the free probe. Increasing concentrations of a competitor DNA encoding the repressor E box at −90/−85 disrupt the formation of the upper band (UB) in the gel shift assay performed with a −135/−82 probe.

**Smaller Deletions between −174 and −65 on the Native Promoter Reveal the Presence of a Muscle-specific cis-Regulatory Element**—Deletions on the native promoter confirmed the importance of the E box between −95 and −85 in the function of the SkM1 repressor (Fig. 6B). While deletions to −135 and −95 had only small effects, deletions that included the E box produced a much higher expression level in primary muscle than 3T3 cells. Further deletion to −65 greatly reduced this muscle-specific expression, indicating the presence of a positive element adjacent to the repressor. As noted above, there are two motifs within this region, AGATTG at −83/−78 and GTTTC at −64/−60, that are conserved exactly between the SkM1 gene and a region of the AChR δ-subunit gene that acts as an enhancer (25, 27, 28).

**An E Box at −31/−26 Coordinates a Positive Muscle-specific Interaction of the SkM1 Promoter with Factors That Bind Elsewhere in the Full-length SkM1 Regulatory Sequence**—We evaluated the SkM1 promoter by characterizing a series of linker-scanning mutations through the region homologous to the δ-subunit promoter. All mutations were created within two different “backgrounds” to assess their impact both on the promoter itself and on the larger, full-length SkM1 regulatory sequence. Within the background of the core promoter, all mutations reduced expression to 20–80% of wild-type levels in both the positive and negative cell types, and mutations within the E box itself did not have special significance (Fig. 7A). The basal promoter activity arose from a distributed sequence of nucleotides, such that all mutations diminished promoter activity while none abolished it.

In contrast, expression in the background of the full-length SkM1 regulatory sequence clearly indicates the unique importance of the region between −31 to −23 that encompasses the promoter E box. Although mutations upstream of −31 and downstream of −23 reduced expression in primary muscle cells to 40–60% of control levels, the mutations between −31 and −23 reduced expression to 5–20% of control levels (Fig. 7A). All mutations within the −31 to −23 region were not equivalent; the most severe disruptions involved the conserved consensus sites within the E box, while less severe effects resulted from mutation of adjacent nucleotides. The difference between the effect of these mutations in the full-length SkM1 regulatory sequence background as compared with the core promoter suggests that a factor bound to the E box orchestrates strong positive muscle-specific SkM1 expression in part through interaction with another factor(s) bound elsewhere in the full-length SkM1 regulatory sequence.

Although the expression level in primary muscle cells was
severely reduced by mutations in the promoter E box, the expression level in 3T3 cells was not increased by any of the mutations within the context of the full-length SkM1 regulatory sequence, in contrast to previously reported results for the E box of the δ-subunit promoter (25).

MyoD and Myogenin Form Part of the Complex of Proteins That Bind to the SkM1 Promoter E Box—A 32P-labeled probe encompassing the E box (−34/−23) gave rise to a prominent group of shifted bands in primary muscle cells, while fewer bands were seen in 3T3 cells (Figs. 7B and 8). In primary muscle cells, this pattern consisted of an upper band (UB), two closely spaced middle bands (UMB and LMB), and a lower band (LB). Competition with both the wild-type sequence and the mutants revealed a close correlation between the functional assay and the degree to which each mutant disrupted the gel shift (Fig. 7D). In 3T3 cells, there was a band of similar size and intensity to the UMB and two lower bands of different size from those in primary muscle cells.

Using antibodies to MyoD and myogenin, supershift assays were carried out to determine whether these factors are part of the complex of proteins that give rise to the gel shift with the promoter E box probe. As expected, assays performed with 3T3 nuclear extracts did not yield supershifted bands, confirming that MyoD and myogenin are not bound to this E box in the non-muscle cell line. Both antibodies produced supershifted complexes with primary muscle nuclear extracts. The UB was shifted upward by the MyoD antibody, indicating that MyoD is present in the largest complex, while the intensity of the LB signal was greatly diminished by the myogenin antibody, suggesting that the LB is composed of two independent complexes, one of which includes myogenin. Because the myogenin supershift fell on top of the UB, it was not possible to discern whether or not the myogenin antibody shifted the upper band. Since not all bands shifted, there must be factors other than MyoD and myogenin that bind this region.

**DISCUSSION**

We have identified multiple cis-regulatory elements that control expression of the SkM1 gene in a primary muscle culture system. The combination of 5′ and 3′ deletion analysis suggests that the core promoter of the SkM1 gene is located between nucleotides −65 and +11. Within this sequence lie consensus binding sites for two families of transcription factors. The CG-rich sequence (−20 to −9) is characteristic of housekeeping genes and includes a binding site for the AP-2 factor, while the E box at −31/−26 is a binding site for the basic helix-loop-helix (bHLH) proteins that include members of the muscle-specific MyoD family. Linker-scanning mutations of nucleotides −40 to −11 within the background of a basal promoter sequence of −65 to +49 indicates that both families of factors are involved in basal expression of the gene, since no single mutation eliminates promoter function, although all reduce function. This core promoter drives transcription in both primary muscle cells and NIH 3T3 cells, indicating that it lacks muscle specificity.

The linker-scanning analysis of the nucleotides −40 to −11 within the larger background of the full-length SkM1 regulatory sequence reveals that the −31/−26 promoter E box plays a role in coordination of positive muscle-specific expression of the SkM1 gene. Because this property is seen only in the background of the full-length SkM1 regulatory sequence, factors that bind to this E box must work in concert with factors that bind elsewhere in the full-length SkM1 sequence. The gel shift competition and supershift assays implicate the myogenic bHLH factors in this function.

An element located between +50 and +254 exerts a powerful positive effect on the overall expression of the SkM1 gene. This region encodes the 3′ end of the first exon and the first 167 nucleotides of the first intron. Because this element exerts at least 50% of its effect in the reverse orientation, it likely acts by binding a transcription factor rather than by stabilizing the RNA structure. Since this element is included in the full-length SkM1 regulatory sequence, it is a candidate for interacting with the promoter E box.

A repressor element located between −174 and −65 confers muscle-specific expression on the native SkM1 promoter, although this specificity is not seen with a heterologous promoter. Interactions between the native promoter and the repressor must produce the muscle specificity observed on the native promoter, although we have yet to discover the nature of this interaction. The footprint of the −174/−65 probe localizes the binding of the repressor complex of proteins to −135/−82, and this fragment confers the same degree of repressor activity as the larger fragment. Although the E box at −90/−85 exhibits only low-affinity binding restricted to the upper band, it is required for repressor activity, implicating the factor which gives rise to this gel shift in the activity of the repressor. This factor is present in both muscle and non-muscle cell types, which lack the MyoD family of proteins.

Immediately downstream of the repressor E box, there is an additional muscle-specific positive element at −85/−60, within which occur two sequence motifs, AGATTG at −83/−78 and GTTTC at −64/−60, also found in an AChR δ-subunit enhancer (25). In our primary muscle culture model system, the −90/−85
E box masks the activity of this element. The overall effect of the repressor is therefore to counteract the activity of the −85/−60 element and to confer muscle specificity on the native SkM1 promoter. The possibility that the −85/−60 positive element mediates the muscle-specific aspect of the interaction between the repressor and promoter was tested by using a construction extending from −174 to −50 on the heterologous promoter, but inclusion of the −85/−60 element fails to supply muscle specificity. Clearly, the interaction between the repressor, promoter, and −85/−60 positive element is complex, and it is unclear that their activity in vitro reflects their activity in vivo. In the δ-subunit, the region of the second motif (GTTC/CC) was shown to be important in the subsynaptic expression of that gene (28). Since sodium channels, like AChR, are clustered preferentially at the neuromuscular junction, it will be important to determine if these elements play a role in localized gene expression in vivo.

One candidate for the factor that binds the repressor E box is the transrepressor ZEB, which binds to a subset of E boxes that encode CACCTG (29–31). Like our factor, ZEB is a direct repressor, and is found in both non-muscle cells and in adult skeletal muscle (29, 31). Others have shown that there is competition between the bHLH proteins and ZEB for binding to E boxes and that this competition reduces ZEB’s activity in muscle (29, 31).

Based on this competitive mechanism, we suggest a model that could explain the difference in expression patterns of SkM1 and AChR δ-subunit. Following denervation, SkM1 mRNA levels remain relatively constant (9), while the δ-subunit mRNA levels increase 30-fold (32). Although there is considerable similarity between the 5′-flanking regions of the two genes, the layout of the cis-regulatory elements is different. The δ-subunit contains one E box that controls both positive and negative regulation (25), while the SkM1 contains two E boxes that control these functions separately. For the SkM1 gene, we propose that the repressor E box is always bound by a repressor factor, and the promoter E box is always bound by a bHLH protein. The 40-fold increase in myogenin levels and 15-fold increase in MyoD levels following denervation (33) have minimal impact since both E boxes are already occupied. In contrast, the bHLH proteins could displace repressor factor(s) bound at the sole E box of the δ-subunit, resulting in the dramatic increase in mRNA levels post-denervation.

In the course of this work, we have identified several cis-regulatory elements that control expression of the SkM1 gene and have begun to address the question of how they work together. These elements include: 1) a core promoter, which lies between −65 and +11 and lacks muscle specificity; 2) a repressor, which lies between −135 and −92 and confers muscle specificity on the promoter; 3) a muscle-specific positive element at −85 to −60, which partially overlaps the repressor and the action of which is masked by the repressor; and 4) a muscle-specific positive element that lies between +50 and +254 and confers a 10-fold up-regulation of expression. The organization of these four elements, and others yet to be identified, contribute to aspects of SkM1 gene regulation shared with other muscle genes, as well as those unique to itself.

Within the these larger regions, two E boxes play a pivotal role in the regulation of the gene, with an E box at −90/−85
controlling negative regulation and an E box at −31/−26 controlling positive regulation. Although some of the transcription factors binding these elements are the same factors that control expression of other genes, the arrangement of the cis-regulatory elements in SkM1 may lead to interactions between those factors that give rise to the unique regulation of this gene.

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