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Basic helix-loop-helix factors recruit nuclear factor I to enhance expression of the Na_v 1.4 Na⁺ channel gene

Sadie L. Hebert^{1,*}, Christine Simmons^{1,*}, Amy L. Thompson¹, Catherine S. Zorc², Eric M. Blalock¹, and Susan D. Kraner¹

¹ Department of Molecular and Biomedical Pharmacology, University of Kentucky Medical Center, Lexington, KY 40536

² Biological Basis of Behavior, University of Pennsylvania, Philadelphia, PA 19104

Summary

We have previously shown that the basic helix-loop-helix (bHLH) transcription factors coordinate Na_v 1.4 Na⁺ channel gene expression in skeletal muscle, but the identity of the co-factors they direct is unknown. Using C2C12 muscle cells as a model system, we test the hypothesis that the bHLH factors counteract negative regulation exerted through a repressor E box (−90/−85) by recruiting positive-acting transcription factors to the nucleotides (−135/−57) surrounding the repressor E box. We used electrophoretic mobility shift assays to identify candidate factors that bound the repressor E box or these adjacent regions. Repressor E box-binding factors included the known transcription factor, ZEB/AREB6, and a novel repressor E box-binding factor designated REB. Mutations of the repressor E box that interfere with the binding of these factors prevented repression. The transcription factor, nuclear factor I (NFI), bound immediately upstream and downstream of the repressor E box. Mutation of the NFI binding sites diminished the ability of myogenin and MRF4 to counteract repression. Based on these observations we suggest that bHLH factors recruit NFI to enhance skeletal muscle Na⁺ channel expression.

Keywords

GABP; MRF4; myogenin; neuromuscular junction; NFI; skeletal muscle; synaptogenesis

1. Introduction

Voltage-gated Na⁺ channels are responsible for propagating the action potential in skeletal muscle. Inherited human mutations in the Na⁺ channel protein result in conduction abnormalities that cause periodic paralyses or other channelopathies (reviewed in [1]). A second type of human syndrome, critical illness myopathy, also arises from a loss of skeletal muscle Na⁺ channel function, although the molecular mechanisms underlying this disease are not well understood [2,3]. Critical illness myopathy may belong to a new category of channelopathies that are transcriptional in nature [4], emphasizing the need for understanding the molecular basis of channel gene regulation as it relates to control of electrical signaling.

Correspondence to: Sadie Hebert, UKMC MS-306, 800 Rose Street, Lexington, KY 40536-0298, Phone: 859-323-4875, FAX: 859-323-1981, Email: slhebe2@email.uky.edu.

*These authors contributed equally to this project

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To fulfill its functional role, the adult skeletal muscle Na⁺ channel, Na_v 1.4, must be expressed in a precise developmental and spatial pattern. The channel protein is expressed at highest levels at neuromuscular junctions (NMJs), but also at lower levels throughout the surface membrane and in the T-tubular membranes [5,6]. Although protein-protein interactions likely “fine-tune” channel spatial distribution [7,8,9,10,11], we and others have shown that transcriptional mechanisms are very important in sculpting the development of the surface membrane and synapse [12,13,14,15].

Our previous work demonstrated that most of the transcription factors that regulate the Na_v 1.4 Na⁺ channel gene are not cell-type specific but rather that muscle specificity is conferred by the binding of the myogenic basic helix-loop-helix (bHLH) transcription factors at a promoter E box [16,17]. Utilizing the C2C12 muscle cell line as a convenient model system, we demonstrated that Na⁺ channel expression is initiated by myogenin and maintained at the highest levels by MRF4 [15,17]. Although the bHLH factors counteract the activity of an upstream repressor located between –135 and –57 [17], the precise mechanism involved is poorly understood.

In this study, we present evidence that the bHLH factors recruit nuclear factor I (NFI) to this upstream region to counteract a dominant repression exerted by ZEB/AREB6 and REB through the repressor E box. Although NFI is known to regulate other genes expressed in skeletal muscle, notably the GLUT4 glucose transporter [18], the mechanism presented in this paper is novel. Myogenin and especially MRF4 recruit NFI to enhance Na⁺ channel expression, consistent with our previous observation that expression of the Na_v 1.4 Na⁺ channel is decreased in MRF4-null mice suggesting this mechanism is important not only in cell culture but also in vivo [15].

2. Materials and methods

2.1. Construction of Na_v 1.4 reporter gene mutants

All Na_v 1.4 reporter genes were inserted into the pCAT-Basic vector, which encodes the reporter gene for chloramphenicol acetyl-transferase. The wt and c/g promoter E box mutant –2800/+254 Na_v 1.4 reporter genes were made previously [16]. The –2800/+254 a, b, c, and d mutations were created by PCR. One PCR fragment was generated with a –852/–832 forward primer coupled with –155/–136, –147/–128, –139/–120, or –131/–112 reverse primers for the a, b, c, and d mutations, respectively, with a Pac I extension on the 3' end. A second PCR fragment was generated using a +194/+213 reverse primer coupled with –127/–106, –119/–100, –111/–92, or –103/–84 forward primers for the a, b, c, and d mutations, respectively with a Pac I site extension on the 5' end. The first set of PCR products was restricted with Sph I and Pac I and the second set of PCR products was restricted with Sac I and Pac I, and three-way ligations of the gel-purified products were carried out into the Sph I (–438) and Sac I (+11) sites of the –2800/+254 Na_v 1.4 reporter gene. The final Na_v 1.4 reporter gene mutants were sequenced between the Sph I and Sac I sites to confirm that only the desired scanning mutations, an 8 bp Pac I site, were present in each of the a, b, c, and d scanning mutations (ACGT, Inc.).

All other mutations were created using the Altered Sites II kit (Promega) according to manufacturer's directions. Briefly, the Sph I to Sac I portion of the Na_v 1.4 5' flanking region was cloned into the corresponding sites of the pAlter-1 vector and single-strand DNA was prepared. Mutagenesis was carried out by annealing a primer bearing a mismatch of the desired Na_v 1.4 region and an Amp^R primer to confer resistance to the antibiotic ampicillin. Following plating, colonies were selected and DNA prepared and restricted to verify production of the appropriate mutation. For creating double mutations (ce, and eM1), a second mutation was laid down on the previously mutated template, and for the triple ceM1 mutant, a third mutation was laid down on the previously mutated eM1 mutant. The following scanning mutations were

created by this technique, mREB (primer, CCTGAGGACTGGGCCAATCTTCTTAATTAAAGCCTCAGCCACACTTCCCTC), e (primer, GGCCAATCTTCAGGTGGGTGCTTAATTAACTTCCTCTTGGCATGTTCC), M1 (primer, GAGTGAAACCTGAGGACTGGGCGCTAGCTCAGGTGGGTGCCTCAGCCACAC), eM1 (primer, GAGGACTGGGCGCTAGCTCAGGTGGGTGCTTAATTAACTTCCTCTTGGC) ce (primer, CTTTCAGGTGGGTGCTTAATTAACTTCCCTTAATTAAATGTTCCAGGCTTA CCCTGCG) and ceM1 (primer, GCTCAGGTGGGTGCTTAATTAACTTCCCTTAATTAAATGTTCCAGGCTTACCCTGCG). All primers introduced the indicated restriction site differences between the mutant and the wt Na_v 1.4 and were used in initial screening. Selected mutants were then confirmed by sequencing the entire region between the Sph I and Sac I to assure that only the desired mutation (s) was introduced (ACGT, Inc.). The Sph I to Sac I region was then sub-cloned back into the corresponding sites of the -2800/+254 Na_v 1.4 reporter gene.

2.2. Cell culture, transient expression assays, and adenoviral-treatments

C2C12 cells were cultured in 6-well dishes using growth medium containing 10% fetal bovine serum in DMEM supplemented with 100 I.U. of penicillin and 100 µg/ml of streptomycin. Cells were transfected the following day when 80–90% confluent using Lipofectamine 2000 according to manufacturer's directions. Briefly, all cells were transfected as myoblasts using 2.4 µg of Na_v 1.4 reporter gene, 2.4 µg pCAT-Basic as a negative control, or 0.3 µg of pCAT-Control/2.1 µg pCAT-Basic as a positive control. Following the 4 hour transfection, cells were either maintained in the growth medium for 48 hrs and harvested as myoblasts or switched to a differentiation medium containing 2% horse serum rather than fetal bovine serum and harvested as either day 2 myotubes or day 7 myotubes. CAT (chloramphenicol acetyltransferase) reporter gene assays and quantification were carried out as described previously [16,17]. Expression of all Na_v 1.4 reporter genes is shown relative to the positive pCAT-Control.

For studies on the effects of exogenously added myogenin and MRF4, 1000 MOI of myogenin or MRF4 adenoviruses were used, with a virus expressing β-galactosidase serving as a negative control. Viruses were created and titers determined as reported previously [15,17].

2.3. Electrophoretic Mobility Shift Assays

Probes used for the electrophoretic mobility shift assays (EMSA) were created by annealing primers and ligating them into a cloning vector using Hind 3 and Bgl 2 restriction sites on the 5' and 3' ends, respectively. The probes created in this manner include the -135/-95 probe, the -93/-82 repressor E box probe, and the -103/-66 ZEB probe, the -99/-80 Sp1 probe, and the -33/-23 promoter E box probe. To make radiolabeled EMSA probes, the plasmids were cut with either Hind III and Bgl 2 or Hind III and Acc I, which cuts 8 bp downstream of the Bgl 2 site. The restricted fragments were incubated with 100 µCi of α-P³² dATP and 10 units of Klenow fragment, run through a desalting column, and the desired probe purified on a non-denaturing 5% acrylamide gel. Following excision of the desired band, the probe was eluted using a buffer containing 170 mM Na⁺ acetate and 0.1 mM EDTA. Probes were stored at -80 until used.

The -85/-57 and NFI probes were radiolabeled with 100 µCi of γ-P³² ATP and 25 units of T4 polynucleotide kinase. The probes and competitors (100-fold excess) used in the EMSA assays are indicated in each figure. The NFI and C/EBP consensus probes were obtained commercially (Santa Cruz) and the -85/-57 probe was created previously [17]. To detect only the NFI complex bound to the -85/-57 probe, EMSAs were carried out in the presence of the -85/-57 M1 competitor.

Nuclear extracts were prepared as previously described [19], except phosphatase inhibitors (Calbiochem #524625) were used in addition to the protease inhibitors 0.1 mM PMSF and 2 µg/ml each of leupeptin, aprotinin, and pepstatin A. To carry out EMSA assays, 20 µg of nuclear extract protein was incubated with 200,000 CPM (1 nmole) of radiolabeled probe. For the -135/-95 and -85/-57 probes, a low ionic buffer (4 mM HEPES, pH, 7.9, 1% glycerol, 1% Ficoll, 20 mM KCl, 50 µM EDTA, and 0.1 mM DTT) was used. For the other probes, an E box-binding buffer (25 mM HEPES, pH 7.5, 50 mM KCl, 12.5 µM ZnCl₂, 5% glycerol, 0.1% Nonidet P-40, 0.5 mM DTT) was used. For all probes, 2 µg of poly dIC and 5 µg BSA were also included. The low ionic samples were run on a gel in a low ionic strength buffer (6.4 mM Tris, pH 7.5, 3.3 mM sodium acetate, and 1 mM EDTA) at 4°C, and the other samples were run on a gel in ½ X TBE buffer at 4°C. Where indicated, competitors were used at a 100-fold excess, except for the short e, wt, and mutant -93/-82 REB competitors, which required a higher 1000-fold excess for complete competition. Antibodies for supershifts used in this study were obtained from the following companies: MRF4 (sc-784, Santa Cruz), myogenin (556358, BD Biosciences Pharmingen), Sp1 (07-124, Upstate), Sp3 (07-107, Upstate), ZEB (sc-10572, Santa Cruz), GABPα (sc-28312, Santa Cruz), NFI (sc-5567, Santa Cruz). Typically 1–5 µl of antibody was needed for supershift assays and the amount was determined empirically by titration.

Acid phosphatase treatments were carried out on some of the nuclear extracts by incubating 0.5 ml of nuclear extract containing 1.0 mg of protein with 5 units of acid phosphatase (Sigma) overnight in buffer D (20 mM HEPES, pH 6.0, 40 mM NaCl, 5mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 20% glycerol). As a control, corresponding nuclear extracts were also incubated overnight in pH 6.0 buffer D (vehicle) alone. The appearance of the bands in the EMSA were similar in pH 6.0 buffer to those observed in the normal buffer D.

2.5 Statistical Analysis

In figure 1 statistical analyses were carried out using Two way Analysis of Variance (2-ANOVA) followed by a posthoc Tukey's comparison. Asterisks indicate a statistical significance of $p < 0.05$.

Statistical analyses for figure 3 were carried by doing Student's t-test comparing the wild type and mREB under each condition. Asterisks indicate a statistical significance of $p < 0.05$.

In figure 7 data were analyzed by 2-way ANOVA using SigmaStat (v. 3.01a, Systat Inc.) followed by four post hoc planned linear contrasts of the general form:

$$LC = \frac{\left| \left(\sum_{1-x} 1/7\bar{x} \right) - \left(\sum_{1-y} 1/4\bar{y} \right) \right| n_i}{\sqrt{MSE} * \sqrt{\sum_{1-x} 1/7^2 + \sum_{1-y} 1/4^2}}$$

where LC = linear contrast t statistic; n_i = geometric mean of the number of observations in each individual group; MSE = mean squared residual error from the 2-ANOVA. The four hypotheses tested were that mutations found in plasmids M1, ce, ceM1, and eM1 did not alter expression levels: 1-overall; 2- in LacZ treatment; 3- in the MGN treatment; and 4- in the MRF4 treatment. $x = 7$ plasmids without and $y = 4$ plasmids with, mutations of interest. Asterisks indicate a statistical significance of $p < 0.05$, while double asterisks indicate a statistical significance of $p < 2^{-9}$.

3. Results

3.1. The repressor E box at -90/-85 controls negative regulation of the Nav 1.4 Na⁺ channel gene

Several Nav 1.4 Na⁺ channel reporter genes were analyzed following transient expression in C2C12 muscle cells at the indicated stage of development (Fig. 1B). Expression of the wild-type -2800/+254 reporter gene increased with developmental progression from myoblasts (MB) to mature myotubes (D7), consistent with previous results [17]. Mutation of the promoter E box abolished expression, consistent with the previously reported role of bHLH factors in coordinating overall positive regulation of the Nav 1.4 gene [16,17]. The bHLH factors MRF4 and MGN bound the promoter E box in electrophoretic mobility shift assays (EMSAs), as demonstrated by supershifts (Fig. 1C). In contrast to the promoter E box, mutation of the repressor E box increased expression approximately 5-fold at all stages of development (Fig. 1B). Taken together, these data indicate that the promoter E box is the focal point of positive regulation and the repressor E box is the focal point of negative regulation, as indicated schematically above the graph (Fig. 1A).

3.2. The transcription factors ZEB and REB are candidates for acting at the repressor E box

To identify factors that control negative regulation exerted through the repressor E box, we carried out EMSA analyses. Three different probes were designed based on potential cognate binding sites using transcription factor database analysis (<http://motif.genome.jp/>). These probes potentially bind the transcription factor ZEB/AREB6 (-103/-66), the Sp1 family of transcription factors (-99/-80), or to the core REB itself (-93/-82) (Fig. 2A). To correlate factor binding with the functional analysis, competitions were carried out with either the wild-type repressor E box or the same mutation of the repressor E box used for the functional analysis.

EMSAs with the ZEB probe gave rise to a prominent band that was supershifted with addition of the ZEB antibody (Fig. 2B, white asterisks). This supershifted band was displaced by inclusion of the wild-type but not the mutant repressor E box competitor. Taken together, these data indicate that the well known transrepressor ZEB is capable of binding to the repressor E box in a manner consistent with it exerting a functional effect.

EMSAs were also carried out with cognate binding sites for the Sp1 family, but because the antibodies displaced the factors from the probes rather than supershifting them, competitions were analyzed separately (Fig. 2C). Sp1 and Sp3 both bound at all stages of development, although Sp1 decreased by day 7 and thus allowed Sp3 to be seen more clearly. Addition of the Sp1 antibody displaced a high band at all stages of development leaving the residual Sp3 factor bound to its probe. Conversely the Sp3 antibody displaced a lower band at all stages of development leaving the prominent Sp1 factor bound to its probe. However in competition assays, the wild-type REB competitor did not displace binding indicating that the Sp1 family could bind this general region but did not primarily utilize the repressor E box for binding. Taken together these data indicate that the Sp1 family does not exert repression through the repressor E box.

Additional EMSAs were carried out with a small probe encompassing the repressor E box and three nucleotides on either side. This probe gave rise to a series of bands in the EMSA all of which were displaced by the wild-type REB competitor (Fig. 2D). However, only the highest band was restored with the mREB competitor, indicating that this was the primary band involved in repressor function. None of these bands were shifted by addition of ZEB, Sp1, or Sp3 antibodies (Fig. 2E). Collectively our data indicate that ZEB and a unique repressor E box

binding factor designated REB bind the repressor E box and are likely responsible for its function.

3.3. The bHLH factors MGN and MRF4 counteract negative regulation exerted through the repressor E box

C2C12 cells were transiently transfected with either the wild-type or repressor E box mutant Nav 1.4 reporter genes, followed by infection with control, myogenin or MRF4 adenoviruses. The cells were then allowed to develop to day 7 myotubes (D7). Both myogenin and MRF4 counteracted repression through the repressor E box (Fig. 3). To compare the extent to which the bHLH factors counteracted repression, the fold difference between the wild type and repressor E box mutant under each condition was compared. With the control virus, this difference was 4.6-fold; with the bHLH factors, this difference diminished to 2.3-fold with myogenin and finally to 1.6-fold with MRF4 (Fig. 3). EMSA analysis showed that addition of these viruses did not alter levels of the negative factors ZEB or REB expression (data not shown). These data suggest that the bHLH factors recruit a positive factor to counteract negative regulation exerted through the repressor E box.

3.4. A transcription factor complex binds immediately upstream and downstream of the repressor E box

We postulated that the positive factor recruited by myogenin or MRF4 might lie adjacent to the repressor E box and aimed to identify it through EMSA analyses. The sequence of the -135/-57 region and the probes and wild type and mutant competitors used in these assays is schematically depicted (Fig. 4A). We chose to use probes that were immediately downstream (-85/-57) or upstream (-135/-95) of the repressor E box. The wt 85/57 probe bound two complexes, the 64/59 and the transcription factor complex (TFC) (Fig. 4B, left panel). Use of the 85/57 M1 competitor interfered with binding of the 64/59 complex, while use of the 85/57 M2 competitor interfered with binding of the TFC. The wt 135/95 competitor displaced the diffuse complex, suggesting that this TFC bound common sequences in the upstream and downstream probes.

To localize these common sequences within the two probes, a series of scanning mutations of the wt 135/95 probe were screened, as indicated in the right panel (Fig. 4B). This analysis identified two binding sites, c and e. The TFC was displaced by the 85/57 M2 competitor, again indicating that this complex bound both the upstream and downstream probes.

To identify the cognate binding site(s) for the TFC, we surveyed these regions for common elements (Fig. 4A). We were able to identify a sequence, TGGC or TGGCNNAG, common to the sites that bind the TFC. To determine which nucleotides within the TGGCNNAG sequence were most important, competitions using mutants that altered only two nucleotides at a time were carried out (Fig. 4C). The results of this competition indicated that the TGGC nucleotides were most important.

To identify potential transcription factors that bind this core sequence, we submitted the entire -135/-57 region to TESS (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>) and also surveyed the transcription factor binding database (<http://motif.genome.jp/>). These results indicated that the transcription factor NFI has core consensus sequence that includes TGGC. In addition, there are binding sites for the ets transcription factor, GA-binding protein (GABP) in this sequence immediately upstream of each TGGC site. The arrangement of these binding sites on either side of the repressor E box is indicated schematically (Fig. 4A).

3.5. Nuclear factor I is the primary component of the TFC

Using nuclear extracts from C2C12 cells at different stages of development, EMSAs with the 135/95, 85/57, and NFI probes were carried out (Fig. 5A). For these and all subsequent EMSAs with the 85/57 probe, the 85/57 M1 competitor was used so that all other factors that bind the -85/-57 sequence were removed, allowing us to discern only the factor that bound the TFC site. All probes revealed EMSAs that had a similar change in appearance with development (Fig. 5A). Competition with the 85/57 and NFI competitors displaced these complexes, while that of another transcription factor that potentially binds in this region, C/EBP, did not.

To confirm that NFI is part of the TFC, supershifts with NFI and GABP α antibodies were carried out (Fig. 5B). For both the 135/95 probe and the 85/57 probe, the NFI antibody disrupted the complex formation (Fig 5B, black asterisks). For the 135/95 probe only, the GABP α antibody induced a slight supershift only in myoblasts (Fig. 5B, white asterisk). Taken together, these data confirm that the primary protein component of the TFC is NFI, although GABP also seems to bind the upstream region to some degree.

3.6. NFI is phosphorylated

The diffuse appearance of the NFI complex suggested that the proteins in the complex may be phosphorylated. EMSAs were carried out with the 135/95 and 85/57 probes at all stages of development and in the presence or absence of acid phosphatase, which removes phosphates. Again, both probes showed a similar change in the appearance of the EMSA as the C2C12 cells developed (Fig. 6A). De-phosphorylation of the factor by addition of acid phosphatase reduced the migration to one tight band at all stages of development for both the 135/95 and 85/57 probes, although binding of the de-phosphorylated band to the 85/57 probe was very faint. To determine the identity of the de-phosphorylated factor bound to the 135/95 probe, antibodies to NFI or GABP α were used in EMSA analysis. An antibody to NFI, but not GABP, interfered with binding, confirming that the de-phosphorylated factor is NFI itself. Collectively these data suggest that activity of NFI during development may be regulated by phosphorylation.

3.7. Both MGN and MRF4 recruit NFI to drive Nav 1.4 reporter gene expression

Having established the identity of key transcription factors, it was necessary to determine their functional contributions. Since expression is highest in day 7 C2C12 myotubes, we introduced wild type and mutant Nav 1.4 reporter genes in the presence of control, MGN, or MRF4 adenoviruses at this developmental stage. The mutants used in these analyses corresponded to the same mutations used in EMSA analyses (Fig. 4A), although all mutations were in the context of the -2800/+254 Nav 1.4 reporter gene. We anticipated mutations in the NFI sites would diminish Nav 1.4 reporter gene expression, if MGN- and MRF4-driven recruitment of NFI is important to counteract the action of ZEB/REB at the repressor E box. Mutations at single sites upstream of the repressor E box did not reduce gene expression, while mutation of the downstream NFI site did reduce gene expression (Fig. 7). Double mutation of the upstream NFI sites also diminished expression, although triple mutation of the NFI sites did not reduce expression to a greater extent.

Although overall Nav 1.4 reporter gene expression is higher in the presence of MRF4 than MGN, much of this difference was lost once the NFI sites were mutated, suggesting that part of the difference in activity of these two bHLH factors is due to their differential ability to recruit NFI. Taken together, our data indicate that bHLH-driven recruitment of NFI counteracts repression exerted through the repressor E box.

4. Discussion

Work from several laboratories suggests that development of the skeletal muscle surface membrane and synapse is an extended process with both initiation and maturation phases, which are represented by aggregation of acetylcholine receptors (AChRs) and Na_v 1.4 Na⁺ channels, respectively [6,7,21]. We and others suggest that transcriptional control is important for both phases and, additionally, that muscle-intrinsic factors are important for synapse formation [12,14,22,23,24]. One group of transcription factors that regulate the AChRs, Na⁺ channels, and many other muscle genes are the basic helix-loop-helix (bHLH) transcription factors, of which four members are expressed in skeletal muscle—myf-5, MyoD, myogenin, and MRF4 (reviewed in [25]). E boxes, the cognate binding site for the bHLH factors, regulate AChR subunit genes and Na_v 1.4, but there are both similarities and differences. For example, the promoter region of the AChR δ subunit has a great deal of similarity to that of the Na_v 1.4 gene, but the E box of the AChR δ subunit controls both positive and negative regulation of that gene, while these two functions are divided into a positive-acting promoter E box and a negative-acting repressor E box in the Na_v 1.4 gene [16,26].

Previous work from our lab indicates that bHLH factors bound at the promoter E box influence the behavior of transcription factors bound at other sites in the 5' flanking region of the Na_v 1.4 gene, but these other factors were not identified [15,16,17]. In this work, we now demonstrate that the repressor E box binds ZEB/AREB6 and a factor we designate REB, and that mutation of this site interferes with binding of these proteins. The transcription factor ZEB/AREB6 is known to exert negative regulation in skeletal muscle through a subset of E boxes [27,28,29]. Mutational analysis of the repressor E box indicates that ZEB/AREB6 and REB play a similar role in negative regulation in the Na_v 1.4 Na⁺ channel.

Although Sp1 family members also bind this general region, the repressor E box mutation we used did not alter binding of these factors. This does not rule out a role for these factors, but they are not implicated in direct regulation through the repressor E box. Based on the known function of these factors (reviewed in [30]), a possible role for them is bending the DNA to bring the upstream region in contact with the promoter region, but this will have to be resolved in future work.

The repressor E box is closely adjacent to binding sites for NFI and GABP. The most recently identified consensus site for NFI is TTGGC (N5)GCCAA, but NFI can also bind to the half sites TTGGC or GCCAA[31]. In the Na_v 1.4 gene there are two half sites upstream of the repressor E box and one downstream (Fig. 4C). There are also three GABP consensus sites with the core motif of GGA (Fig. 4C). Experimental results with direct EMSA binding assays, competitions, and supershift analyses indicate that NFI is bound at both the upstream and downstream sites. Although there are two upstream NFI half sites, only one of them appears to bind NFI at a time on this probe, since the mobility of the complex is the same for both the upstream and downstream probes, as shown in Fig. 5B. While there are sites for GABP, the supershift analysis indicates that GABP binds to a small degree in the upstream region and not at all in the downstream region. Since GABP is stimulated by motorneuron-derived factors such as neuregulins [32,33], GABP might be recruited to this region under different conditions, but our results suggest that the primary protein binding to the regions flanking the repressor E box under these conditions is NFI.

NFI is known to bind the GLUT4 glucose transporter gene, which is expressed in skeletal muscle and adipocytes [18]. Most gene regulation studies with this promoter have focused on expression in 3T3-L1 adipocytes [34,35,36,37] and suggest that NFI is a negative regulator in these cells. Many transcription factors act as both positive and negative regulators under different conditions and this is true for NFI [31]. NFI is known to be a phosphoprotein [34],

consistent with the results of our acid phosphatase-treatment experiment. Changes in NFI migration in the EMSA during development indicate phosphorylation or other post-translational modification of the protein may change, possibly allowing it to transition from being a negative to a positive regulator in the Na_v 1.4 gene.

Previous CASTing experiments have shown that myogenin interacts with NFI [38], consistent with our observations. Functional assays indicate that NFI is required for myogenin- and MRF4-driven expression in day 7 C2C12 cells. However, the NFI binding sites are not equivalent. Mutation of the downstream site by itself diminishes expression, while mutation of both upstream sites simultaneously were required to diminish expression. Mutation of all three sites did not have a greater effect. Taken together, these results suggest that NFI must be recruited to either the downstream site or both of the upstream sites to counteract negative regulation.

In summary, we suggest that NFI is a major regulator of Na_v 1.4 expression and that it works in concert with the bHLH factors myogenin and especially MRF4. This mechanism has not been reported for other genes expressed at neuromuscular junctions, which are widely reported to be regulated primarily through GABP [13,14,22,23,39,40,41]. As noted above, other muscle synaptic proteins such as the AChR are expressed uniquely at the synapse and earlier in development, whereas Na_v 1.4 Na⁺ channels are expressed in the extrajunctional surface membrane as well as the synapse with this pattern forming later. Thus it is not entirely surprising that different transcriptional mechanisms regulate the Na_v 1.4 Na⁺ channel and these other muscle synaptic genes. Future work will be directed at analyzing expression of Na⁺ channels in NFI-null mice to confirm the role of NFI in Na⁺ channel regulation in vivo.

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Abbreviations used

AChR	acetylcholine receptor
bHLH	basic helix-loop-helix
CAT	chloramphenicol acetyltransferase
DMEM	Dulbecco's modified Eagle's medium
dithiothreitol	DTT
EMSA	electrophoretic mobility shift assay
GABP	GA-Binding Protein
Nav 1.4	adult skeletal muscle Na ⁺ channel α subunit
Nav 1.5	embryonic skeletal muscle Na ⁺ channel α subunit
NMJ	neuromuscular junction

NFI	nuclear factor I, PEB, promoter E box
REB	repressor E box
Sp1 and Sp3	stimulating protein 1 and 3
TFC	transcription factor complex
ZEB	zinc-finger E box-binding protein

Fig. 1. The repressor E box controls negative regulation of the skeletal muscle $\text{Na}_v 1.4$ Na^+ channel gene. (A) A schematic layout of several elements that regulate expression of the $\text{Na}_v 1.4$ Na^+ channel is shown, including the repressor E box (REB) and promoter E box (PEB). (B) The wild type -2800/+254 $\text{Na}_v 1.4$ regulatory region is expressed in C2C12 muscle cells at increased levels as development proceeds from myoblasts (MB) to nascent day 2 myotubes (D2) to fully mature day 7 myotubes (D7). Mutation of the promoter E box site (mPEB) abolishes expression of the reporter gene. In contrast, mutation of the repressor E box (mREB) increases expression at all stages of development. Asterisks indicate statistical significance (p

< 0.05). (C) The PEB binds both myogenin (MGN) and MRF4, as indicated by the supershift induced with antibodies to these factors in the EMSA (white asterisks indicate supershifts).

Fig. 2.

The transcription factors ZEB and REB are candidates for exerting negative regulation through the repressor E box. All EMSAs were carried out using nuclear extracts from myoblasts (MB), day 2 myotubes (D2 MT), or day 7 myotubes (D7 MT). Antibodies and competitors were added as indicated above each lane. (A) A schematic depiction of probes and competitors used in the EMSA assays is shown. (B) EMSAs were carried out with the ZEB probe. The ZEB antibody induced a supershift, as indicated by the white asterisks. The supershifted band was diminished by the wild type but not the mutant REB competitor. (C) EMSAs were carried out with the Sp1, Sp3 probe. Addition of Sp1 or Sp3 antibodies prevented formation of complexes, as indicated by the white asterisks. Competition with the wild type REB and mutant REB had little effect on the Sp1 and Sp3 complexes. (D) Using the REB probe in EMSAs, a number of complexes form. The highest complex is displaced by the wt REB but not the mREB competitors. (E) This REB factor is not supershifted by antibodies to ZEB or the Sp1 family, as shown in panel E.

Fig. 3.

The bHLH factors myogenin and MRF4 counteract negative regulation exerted through the repressor E box. Although there was a significant difference between the wild type and mREB reporter genes under all conditions, in the presence of myogenin and especially MRF4, expression of the wt $\text{Na}_v 1.4$ reporter gene increased relative to the mREB, as indicated by the change in the fold difference. The asterisks indicate that the two were significantly different ($p < 0.05$) under each set of conditions.

Fig. 4.

A Transcription Factor Complex (TFC) binds sites upstream and downstream of the repressor E box. (A) Probes and competitors used in B are shown schematically above the -135 to -57 portion of the Na⁺ channel sequence. This portion of the sequence contains potential binding sites for nuclear factor I (NFI) and GA-Binding Protein (GABP) which are shown below the -135 to -57 sequence with their consensus sequences. (B) EMSAs with the wt 85/57 probe gave rise to two distinct bands—a high tight band indicated by the small arrow labeled 64/59, and a lower, more diffuse complex indicated by the large arrow labeled TFC (transcription factor complex). Binding sites for both the 64/59 factor and TFC are shown in A. Addition of the wt 135/95 competitor is able to displace the TFC, indicating that a common factor binds

the 85/57 and 135/95 probes. To determine what sites within the 135/95 probe bound the TFC, competitions with scanning mutants indicated that competitors 135/95 c and 135/95 e were unable to displace the TFC. The TFC is also displaced by the short e and the 85/57 M2 competitors. (C) To determine the precise nucleotide involved in TFC binding scanning mutants of the short e site were used as competitors in another EMSA using the short e probe. Sequences are indicated below the EMSA. The m1 and m2 competitors were unable to displace the TFC. The “core” TFC sequences are shown to the left.

Fig. 5.

NFI is the primary component of the TFC. (A) EMSAs were carried out using the indicated probes and competitors. EMSAs with the 85/57 probe were done in the presence of the 85/57 M1 competitor (see Fig. 4A) to displace binding of all proteins except the TFC. Using nuclear extracts from the indicated stage of development, a similar developmental alteration in the mobility of the EMSAs were observed for all probes, including the NFI probe. The NFI consensus competitor, but not the C/EBP competitor, displaced the TFC from the 135/95 and 85/57 probes. (B) EMSAs were carried out with the indicated probes in the presence of the indicated antibodies. NFI antibody dissociated NFI from both the 135/95 and 85/57 probes using nuclear extracts from both myoblasts and day 7 myotubes (indicated by black asterisks).

GABP α antibody induced a small supershift only in myoblasts with the 135/95 probe (indicated by the white asterisk).

Fig. 6.

NFI is a phosphoprotein that changes appearance with development in C2C12 cells. (A) Nuclear extracts were prepared from cells at the indicated stage of development and treated with vehicle (control) or acid phosphatase (AP-treated). The appearance of NFI is diffuse and changes with development. For both probes, de-phosphorylation yielded a single “tight” band at all stages of development, although the binding to the 85/57 probe was greatly reduced. (B) Using only acid phosphatase treated nuclear extracts, EMSAs were carried out as indicated in the presence of NFI and GABP α antibodies, using the 135/95 probe. The NFI antibody interfered with binding to the 135/95 probe, as indicated by the black asterisks.

Fig. 7.

MGN and MRF4 recruit NFI to counteract negative regulation through the repressor E box. (A) A schematic diagram of the scanning mutations used in B are shown in the context of the full length sequence (-2800/+254). These scanning mutations correspond to those used in the EMSA analyses (Fig. 4A). (B) As shown previously in Fig. 3, expression of the wt -2800/+254 reporter gene approaches that of the mREB in the presence of the bHLH factors, especially MRF4. Individual mutations in the upstream NFI sites did not diminish expression, while the individual mutation of the downstream GABP and NFI site, M1, significantly reduced expression. Double mutation of the upstream NFI sites, c and e, significantly reduced expression, but the triple NFI mutation did not reduce expression further. Asterisks indicate a statistical significance of $p < 0.05$, while double asterisks indicate a statistical significance of $p < 2^{-9}$.