Multiple Promoter Elements Interact to Control the Transcription of the Potassium Channel Gene, KCNJ2*

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Potassium channels play important roles in shaping the electrical properties of excitable cells. Toward understanding the transcriptional regulation of a member of the inwardly rectifying potassium channel family, we have characterized the genomic structure and 5′-proximal promoter of the murine Kcnj2 gene (also referred to as IRK1 and Kir2.1). The Kcnj2 transcription unit is composed of two exons separated by a 5.5-kilobase pair intron. Deletion analysis of 5′-flanking sequences identified a promiscuously active 172-base pair minimal promoter, whereas expression from a construct containing additional upstream sequences was cell type-restricted.

The minimal promoter contained an E box, a Y box, and three GC box consensus elements but lacked both TATA and CCAAT box elements. The activity of the minimal promoter was found to be controlled by a combination of the activities of the transcription factors Sp1, Sp3, and NF-Y. The interplay between Sp1, Sp3, and NF-Y within the architecture of the Kcnj2 promoter, the ubiquitous nature of these transcription factors, and the action of tissue-selective repressor element(s) may combine to enable a wide variety of cell types to differentially regulate Kcnj2 expression through transcriptional control.

The classical inward rectifier subfamily is represented by at least three members, Kir2.1–2.3. These channels play important roles in setting and maintaining the resting membrane potential, buffering the extracellular K⁺ concentration, controlling excitability and action potential shape, and allowing the formation of prolonged action potentials (e.g., cardiac myocytes) without excessive K⁺ depletion (4). Classical inward rectifiers have been electrophysiologically identified in a wide variety of tissues and cell types, ranging from central and peripheral nervous system neurons to glia, muscle, and immune system cells (3). As expected from the electrophysiologic data, the first member of the classical inward rectifiers to be cloned, Kcnj2 (also called IRK1 and Kir2.1) (5), has been shown to be expressed in a variety of cells, including central nervous system neurons (6), glia (7), Schwann cells (8), macrophages (5), osteoclasts (9), and skeletal and cardiac muscle (5, 10).

Despite playing a critical role in determining the electrical properties of a wide variety of cell types, there has been little investigation of the promoter elements that control the expression of Kir channels. Indeed, until recently, there has been only limited characterization of the promoters controlling the expression of any type of ion channel gene. However, of the ion channel gene promoters that have been studied, some common features are apparent. Comparisons of the 5′-promoters of several channel gene promoters revealed general structural features common to the housekeeping-type promoters. The promoters of the voltage-gated potassium (Kv) channels Kv1.5 (11), Kv1.4 (12), and Kv3.1 (13) lack consensus TATA and CCAAT boxes, are GC-rich with multiple putative Sp1 consensus elements, direct transcriptional initiation from multiple sites, and contain a relatively large number of putative regulatory elements. Initial characterizations of the N-type α₁B (14) and L-type α₁D (15) calcium channel promoters, and the skeletal muscle (16) and brain type II (17) sodium channel promoters, also revealed the presence of these same general features. In addition, many of the promoters contained one or more strong silencer elements. In the case of the brain type II sodium channel promoter, it has been shown that binding of the protein REST to an upstream silencer element is critical for directing the highly neuron-specific expression pattern of this channel (18). Similarly, a dinucleotide repeat silencer element (19) and a glucocorticoid response element, found in the 5′-untranslated region (UTR) (20), may regulate Kv1.5 expression in a tissue-selective manner.

An initial characterization of the Kir3.1 promoter, a member of the G-protein-regulated inwardly rectifying potassium channel subfamily, revealed many similarities to the previously described voltage-gated potassium, calcium, and sodium channel gene promoters. The Kir3.1 promoter was found to be GC-rich, lacked a TATA box element, and contained at least two silencer elements in addition to a multitude of potential positive regulatory elements (21). In contrast to the housekeep-
ing-type structures of Kir3.1 and the voltage-gated ion channel promoters, the Kir1.1 gene was found to possess a unique multi-promoter structure that utilizes at least three different CCAAT and TATA box-containing promoters to drive the expression of distinct alternatively spliced transcripts (22).

In the present study, we determined the genomic structure of the mouse Kcnj2 (Kir2.1) gene and provided a critical analysis of the cis-elements and corresponding trans-acting factors acting at the minimal promoter. We characterized the Kcnj2 promoter in the murine C2C12 skeletal muscle cell line because (i) Kcnj2 mRNA is most highly expressed in adult mouse skeletal muscle, and preliminary experiments indicated a robust expression in C2C12 myoblasts as well, and (ii) the C2C12 cell line is a well characterized model system for muscle differentiation, thus allowing the future potential to investigate Kcnj2 promoter regulation during differentiation of myoblasts to myotubes. In addition, we also utilized a non-expressing neuroblastoma cell line, Neuro 2A, to assess the tissue selectivity of various Kcnj2 promoter constructs. Our data suggests that the Kcnj2 promoter is organized such that the level of mRNA expression in a wide variety of cell types may be controlled by blending the activities of many ubiquitous trans-acting factors acting within the context of a generalized minimal promoter. Determination of expressing versus non-expressing cell types may be accomplished through the activity-specific silencer element(s) located distal to the non-selective minimal promoter.

**Experimental Procedures**

**Library Screening and Subcloning**—Titering and screening of a 129Svd mouse genomic library (AFXIIX, Stratagene) was carried out as described in the manufacturer’s protocol. The library was screened using a 1.7-kb Kcnj2 genomic fragment (see Materials and Methods) containing the 5‘-UTR and open reading frame (ORF) of the Kcnj2 cDNA (5). The probe was labeled to high specific activity with [32P]dCTP (3000 Ci/mmol, New England Nuclear) by random primer extension and hybridized overnight at 65°C in buffer containing 1 × 106 cpm/ml labeled probe, 250 µg/ml herring sperm DNA, 2× SSC, 5× Denhardt’s, 1% SDS, and 0.5% Na2PO4. The filters were washed at 65°C in 0.5× SSC, 1% SDS and 0.1× SSC, respectively. After hybridization, filters were washed 1× SSC, 0.1% SDS and exposed to film. Initially, 18 positive clones were identified by autoradiography. Directional ligation was carried out on two overlapping clones that were determined to carry the entire Kcnj2 ORF and extended the furthest 5‘ and 3‘ of the coding region.

The genomic DNA fragments generated by SacI or SacII/SalI digests of phage DNA were subcloned into pBS SK‘ (Stratagene) using standard subcloning techniques (23, 24). This generated the subclones 1.16pBS 5kb-2, 1.16pBS 7.5kb-2, and 1.16pBS 7kb-2 (Fig. 1). To analyze 5‘- and 3‘-flanking regions not represented by subclones obtained using the above strategy, two additional plasmids were generated by LA-PCR (25). To generate plasmid 1.8pBS 2.2kb-2, a 3.7-kb PCR product was amplified from 1.8A1 phage DNA using oligos T3 (5‘-GAAATTACCCCTCACTAGG-3‘) and S90 (5‘-GOTTTCACTCTCCAGCTTGGC-3‘), digested with SacI, and the resulting 2.2-kb fragment was subcloned into pBS SK‘. Plasmid 1.8pBS 5‘-proximal was generated by amplification of a 6.5-kb PCR product from 1.8A1 with oligos T7 (5‘-GTAATACGACTCACTATAGGG-3‘) and S62 (5‘-AATTC-CCAGACACTTGCTG-3‘), digested with XbaI/KspI to yield a 3.1-kb fragment, and directionally ligated into pBS SK‘—Amplification reactions were performed using the buffers described (25). The amplification protocol consisted of 10 cycles of 15 s at 94°C, 30 s at 65°C, and 3 min at 68°C, followed by 20 cycles of 15 s at 94°C, 30 s at 65°C, and 3 min +10 s/cycle at 68°C, with a final extension step of 7 min at 68°C.

**Luciferase Reporter Construction**—The luciferase reporter vector pGL2 Basic (Promega) was used to generate all of the reporter constructs. The parental construct pGL2 SacRI was generated by directional ligation of the ~800-bp SacI/EcoRI (blunted) fragment digested from pGL2-SacRI (Promega) with 1.16pBS 5kb-2 and pGL2 Basic (Promega) (blunted) using T4 DNA ligase. This placed the Kcnj2 promoter fragment (~787/+62) immediately upstream of the luciferase gene in the 5′ to 3′ orientation. Sequential 5′ deletion constructs were generated by digestion and intramolecular ligation using the following enzymes: SacI (blunted)/NheI (blunted) to form pGL2N2B NheRI (~629/+62), SacI (blunted)/AvaiI (blunted) to form pGL2B ApaRI (~418/+62), XmaI to form pGL2B XmaRI (~109/+62), S6aI (blunted)/BbvClI to form pGL2B BbvClI/R (~85/+62), and SacI (blunted)/BglII (partial digest, blunted) to form pGL2B BglII/R (~41/+62). To extend constructs beyond ~787, plasmid 1.6pBS 5‘-proximal was digested with KpnI/KspI, and the resulting 3.1-kb fragment was directionally ligated into pGL2B SacRI restricted at KspI to generate the promoter construct pGL2B ~900 bp. A reverse orientation construct, pGL2B RI/Sall (~627/~787), was generated by subcloning the ~850-bp Sall (blunted)/EcoRI (blunted) fragment from 1.16pBS 5kb-2 into pGL2 Basic linearized with HindIII (blunted). Reverse orientation was confirmed by sequencing. Blunting the 5‘ or 3‘ overhangs was accomplished by incubation for 15 min at 37°C with 1 U of T4 DNA polymerase in the presence or absence, respectively, of 100 µM dNTPs. Construct insertion and deletion junctions were confirmed by sequencing using TaqDyeDecoy™ Terminator Cycle Sequencing (ABI), or TaqTrack Sequencing (Promega), or by restriction digests.

**Site-directed Mutagenesis**—Complementary mutant oligonucleotides (Fig. 4) were synthesized (Life Technologies, Inc.) and used in a PCR-based strategy to generate luciferase reporter constructs with point mutations disrupting selected promoter elements. The initial step consisted of two separate PCR reactions that paired oligo 572 (5‘-GGGTTT-TCCACAGCTCTAGGGC-3‘) with a specific mutant reverse primer and the complementary mutant forward primer paired with oligo 586 (5‘-GCAATTGTTCCAGAAACCCAGG-3‘). Amplifications were carried out using 20 ng of pGL2B SacRI DNA in a 50-µl reaction containing 20 µM Taq DNA polymerase (Promega), 1× PCR buffer (Promega), 1× High Fidelity Buffer (Stratagene), 125 µM 32P-labeled GTP (3000 Ci/mmol, Amer sham), 10 µM dNTPs, 1 mM MgCl2, 0.1% Triton X-100, 100 µg/ml bovine serum albumin, 400 µM dNTPs, 2 µM each primer, and 2.5 units of Pfu polymerase. The reactions were amplified for 35 cycles of 1 min at 94°C, 1 min at 65°C, 1 min at 72°C, with 2.5°C/s ramps. The PCR products were purified and combined for a second PCR reaction containing oligo 572 and oligo 614 (5‘-CCCTTTCTTTATTTTCTTGGGCTCTTCC-3‘), located internal to oligo 586, using the same protocol substituting 55°C for annealing. The final PCR product was purged, digested with KspI/BsmII to generate a 173-bp transfer cassette containing the desired point mutation, and ligated in place of the wild-type KspI/BsmII cassette in pGL2B SacRI using standard subcloning techniques.

To generate multiple point mutations, the strategy outlined above was repeated using the second set of mutant oligos. In this instance, the wild-type pGL2B SacRI DNA used in the initial amplification was replaced with the pGL2B SacRI construct containing the desired point mutant background. The transfer cassettes of each of the resulting point mutant constructs was sequenced by the dyeoxy terminator method to confirm the point mutations and to check for extraneous mutations that may have resulted during the course of the PCR amplifications.

**Cell Culture**—C2C12 and Neuro 2A cells were purchased from the American Type Culture Collection (Rockville, MD). Myoblast stage C2C12 cells were plated at 1000–2000/cm2 and maintained at ~70% confluence in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 µg/ml streptomycin sulfate in a 37°C 5% CO2 incubator. Neuro 2A cells were plated at 4 x 104 cells/cm2 in 10% fetal bovine serum, 400 units/ml penicillin G, and 100 µg/ml streptomycin sulfate in a 37°C 5% CO2 incubator.

**Transfections**—Plasmid DNA was prepared using Qiagen or Quantum (Bio-Rad) DNA purification columns as per manufacturer’s instructions. C2C12 myoblasts were transfectionally transduced in triplicate using the DEAE-dextran method as outlined (23). Kcnj2 reporter constructs, in combination with a secreted embryonic alkaline phosphatase (SEAP) reporter vector (pBC12/CMV/SEAP, kindly provided by Dr. M. Katze, University of Washington) used as an internal control, were transfected at a ratio of 4:1. C2C12 myoblasts were seeded at 1500 cells/cm2 in 100-mm tissue culture-treated plates and grown for 2 days to approximate 40–60% confluence. The plates were rinsed, drained, and incubated 4 h at 37°C, 5% CO2 with 4 ml of Dulbecco’s modified Eagle’s medium, containing 10% diazylated fetal bovine serum, 12 µg of the luciferase construct, 3 µg of pBC12/CMV/SEAP, and 200 µg/ml DEAE-dextran. The cells were shocked 1 min with 10% MeSO4, rinsed, and incubated an additional 3 days with 8 ml of fresh media at 37°C, 5% CO2 to allow for expression of the reporter proteins. Neuro 2A neuroblastoma cells were plated at 10,000 cells/cm2 in 100-mm tissue culture-treated plates and grown 2 days, and transfected using the calcium phosphate method as outlined (23). For Neuro 2A transfections, the reporter construct to pBC12/CMV/ SEAP ratio was 8:1. Cells were incubated in calcium phosphate 6 h, shocked 3 min with 10% MeSO4, rinsed in phosphate-buffered saline, and incubated 24 h with 3 ml of complete media at 37°C, 5% CO2 to allow for expression of the reporter proteins.
One milliliter of the conditioned media was reserved for the secreted alkaline phosphatase assay described previously (26), the results of which were later used to correct for transfection efficiency. To measure luciferase activity, the transfected plates were rinsed and lysed in 500 or 200 μL of buffer containing 1% Triton X-100, 25 mM glycglycine, pH 7.8, 15 mM MgCl₂, 1 mM EDTA, and 2 mg/mL BSA. After processing, the protected fragments were separated by electrophoresis on a 10% polyacrylamide, 7.5M urea, 1× TBE gel, fixed, dried, and exposed to film overnight with an enhancing screen.

RESULTS

Genomic Structure of Kcnj2—To determine the structure of the Kcnj2 gene, we screened a 129SvJ mouse genomic library with a 1.7-kb Kcnj2 cDNA probe containing the 5’-UTR and ORF. Nine positive phage clones were plaque-purified to homo- geneity and classified by restriction digest into five overlapping groups of clones. Each group was determined by Southern blot analysis to contain the entire Kcnj2 ORF. Clones 1.8A1 and 1.16A1, spanning a total of 25-kb genomic DNA, were found to extend the furthest 5’ and 3’, respectively, of the ORF. Extensive characterization of these clones by Southern blot, subcloning, and sequencing confirmed that the Kcnj2 ORF is intronless (32). Our analysis extends this finding to the 3’-UTR, which was found to be contiguous with the ORF and also intronless. The 5’-UTR, however, was found to contain a large, approximately 5.5-kb, intron (Fig. 1A).

Sequencing of subclones 1.16BS5kb-2 and 1.16BS-17 identified consensus 5’ donor and 3’ acceptor splice sites in the genomic sequence that places the exon 1/exon 2 boundary at position +168/+169 in the 5’-UTR of the Kcnj2 mRNA (Fig. 1C). Sequence comparison of the distal portion of the cDNA clone 3’-UTR with sequence from the genomic clones 1.16BS-3 and 1.8BS 7kb-2 identified two putative polyadenylation consensus sequences located approximately 3.8-kb downstream of the stop codon (Fig. 1D). Immediately 3’ of the polyadenylation consensus sequences in the genomic sequence are a number of GT-rich sequence motifs similar to sequences often found associated with functional polyadenylation sites (33).

Sequence analysis upstream of the identified intron revealed a region of high GC content suggestive of the GC-rich islands often associated with promoter regions (34, 35). To determine if transcription of the Kcnj2 gene was initiated in this region, we performed RNase protection assays using antisense RNA probes that spanned, or were just upstream of, the 5’ end of the previously published cDNA sequence (5). As shown in Fig. 2, a cluster of at least four strong transcriptional start sites was found situated 63 bp upstream of the EcoRI site that marked the 3’ boundary of the RNase probe. Thus, the mature Kcnj2 mRNA would be predicted to possess a 5’-UTR of 403 bp. Surrounding the strong start sites were multiple poorly protected bands that were not reproduced consistently in all assays and may reflect incomplete or over-digestion products. In addition, in some assays we noted a very weak band corresponding to a protected full-length probe (not shown). Although this indicated the possible presence of additional upstream start sites, they were not pursued at this time because of their extremely low abundance. In support of our assignment of the strong proximal sites as being the primary start sites, the entire 5.5-kb size of the Kcnj2 mRNA detected by Northern blot (5) can be fully accounted for using the transcriptional start sites and poly(A)’s sites depicted in Figs. 1 and 2. We found no evidence for the utilization of the strong transcriptional start sites in brain, ventricle, skeletal muscle, or C2C12 myoblasts.

Sequence Analysis of the 5’-Flanking Region—As a preliminary step in analyzing the Kcnj2 promoter, we sequenced approximately 800 bases of the 5’-flanking region upstream of exon 1 in the plasmid 1.16BS5kb-2 (Fig. 1B). These sequences were searched for known transcription factor consensus ele-
FIG. 1. *Kcnj2* genomic structure and flanking nucleotide sequence. A, representation of the *Kcnj2* gene showing a partial restriction enzyme map of the genomic phage clones 1.8A1 and 1.16A1. Restriction enzyme sites are as follows: \( X \), \( XbaI \); \( S \), \( SacI \); \( SL \), \( SalI \); \( B \), \( BstX1 \); \( P \), \( PstI \). Shown above the phage clones are the positions of the subclones described under “Experimental Procedures”: 1, 1.8pBS 5'9-proximal; 2, 1.16pBS 5'kb-2; 3, 1.16pBS-17; 4, 1.8pBS7kb-2; 5, 1.16pBS-3; 6, 1.8pBS2.2kb-2; and 7, 1.16pBS2kb-2. The boldface lines marked \( B \), \( C \), and \( D \) correspond to the sequenced regions shown in \( B - D \), respectively. \( B \), 5'9-flanking sequence. 887 bases of the 5'9-flanking region and 90 bases of 5'9-UTR of the *Kcnj2* gene are shown. Flanking sequences are represented by lowercase letters, and UTR sequences are represented by uppercase letters. Transcription initiation sites are indicated by asterisks. The open box, shaded box, broken-line box, bold underlined, dashed underlined, and double overlined sequences correspond to published cDNA sequence. Restriction enzyme sites relevant to 5' deletion constructs and EMSA probes are also indicated. C, the 5' donor and 3' acceptor splice sites at the intron/exon junctions. Consensus splice site sequences are depicted above the corresponding *Kcnj2* genomic sequences. The hatched box represents the 5.5-kb intron separating exons 1 and 2. The intron was not sequenced completely. D, putative
Transcriptional Regulation of the Kcnj2 Promoter

To identify the upstream promoter elements controlling the transcriptional activity of the Kcnj2 gene, we generated a series of 5‘ deletion constructs and transiently expressed them in either C2C12 myoblast or Neuro 2A neuroblastoma cells. As controls for spurious promoter activity, we included transfections of the reporter plasmid alone (pGL2B) and a reverse orientation construct (pGL2B RI/Sal, Fig. 3). The reporter vector alone possessed essentially no transcriptional activity in either cell line when compared with the pGL2B Sac/RI construct. We attribute the minimal amount of transcriptional activity observed in the reverse orientation construct to the repositioning of the distal GC box at −776 into close proximity with the luciferase gene.

Deletion of the upstream sequences from −3300 to −109 had no significant effect on the transcriptional activity of the Kcnj2 promoter in C2C12 myoblasts (p > 0.1, Student’s t test, Fig. 3). However, deletion of sequences downstream of −109 dramatically reduced Kcnj2 promoter activity. Removal of the upstream promoter sequences to −85 resulted in an approximately 50% decrease in transcriptional activity when compared with the pGL2B Xma/RI construct (p < 0.005, Student’s t test). Further deletion of the 5’-flanking sequence to −41 resulted in more than a 90% decrease in activity (p < 0.001, Student’s t test). The relative promoter activity of the Kcnj2 constructs obtained in the Kcnj2 non-expressing Neuro 2A cell line paralleled the results obtained from C2C12 myoblasts, with the notable exception of the −3300/+62 construct. In Neuro 2A cells, inclusion of the sequences from −3300 to −787 resulted in an approximately 65% decrease in promoter activity relative to the activity expressed in C2C12 myoblasts (p < 0.001, Student’s t test). This result is indicative of the presence of at least one active tissue selective repressor element within this region.

These results defined a promiscuous minimal promoter, active in both C2C12 myoblast and Neuro 2A neuroblastoma cells, that spanned the sequences from −109 to +62. This region of the Kcnj2 upstream promoter contains an E box (−88), Y box (−78), and three GC box (−105, −54, and −19) consensus sequence motifs. We subsequently focused on these motifs as the promoter elements most likely supporting Kcnj2 transcription.

Both Sp1 and Sp3 Bind to the Minimal Promoter—To determine which consensus elements might be transcriptionally active, we generated double-stranded oligo probes corresponding to each of the minimal promoter consensus sequence elements (see Fig. 4) for use in EMSAs. The probe designations Sp1(M) and Sp1(H) were derived from the predicted affinities of the GC box elements at −105 and −54 (medium and high, see below), respectively, whereas Sp1(N) was used to designate the inverted orientation of the GC box element at −19. As demonstrated in Fig. 5A, the end-labeled oligo probes Sp1(H), Sp1(N), and Sp1(M) each shifted three distinct DNA-protein complexes. Probes containing mutations within the core GC box, however, were unable to shift these complexes. These shifted complexes were specific since they were efficiently competed by unlabeled self oligo but not by unlabeled nonspecific oligos present at up to 500-fold molar excess or by oligos containing point mutations within the core consensus sequence (Fig. 5B).

Because several DNA-protein complexes were shifted using the GC box oligo probes, we considered the likelihood that multiple members of the Sp family of transcription factors might be involved. It has been reported that Sp2 does not bind to the GC box consensus sequence with high affinity (57). Therefore, we concentrated on Sp1, Sp3, and Sp4 as being the most likely candidates responsible for the observed shifted

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complexes. By using αSp1-specific antibodies, we were able to specifically supershift the low mobility DNA-protein complex produced by EMSA with the Sp1(H) oligo probe. Likewise, αSp3-specific antibodies effectively disrupted the formation of the remaining two fast mobility complexes (Fig. 5B). Incubation with an αNF-I antibody produced no changes in the pattern of shifted complexes, indicating that an interaction between IgG and the oligo probe was not responsible for these results (data not shown). Similar supershift results were obtained using the Sp1(M), E box, Y box, and Sp1(N) used in EMSA are indicated. Point mutations are shown in bold.

After determining that the Sp1(M), Sp1(H), and Sp1(N) promoter elements could each bind both Sp1 and Sp3, we were interested in determining their rank order of binding affinities. In approaching this problem, we first calculated a theoretical affinity value for each of the core GC box elements using the weight matrix devised by Bucher (59). In this analysis, values closer to zero correspond to higher predicted binding affinities. Using the matrix, the calculated values for the Sp1(M), Sp1(H), and Sp1(N) elements are 2.618, 2.207, and 3.91, respectively, indicating a predicted rank order of affinity of Sp1(H) → Sp1(N) → Sp1(M).

To test this prediction, we utilized an end-labeled exogenous Sp1 consensus oligo probe (60) with a calculated value of 3.03 in competition EMSAs with excess unlabeled Kcnj2 element oligos. As shown in Fig. 5C, the competition assays confirmed the weight matrix predictions. The Sp1(H) oligo probe was able to efficiently compete the labeled probe at a 10-fold molar excess concentration, whereas the Sp1(N) and Sp1(M) oligos required at least 100- and 1000-fold excess concentrations, respectively, for efficient competition. These data indicate the relative affinities of the Kcnj2 GC box elements as determined by EMSA, Sp1(H) > Sp1(N) > Sp1(M), are as predicted by the weight matrix analysis.}

**USF Interacts at the Minimal Promoter E Box Element—** E box motifs are relatively promiscuous promoter elements that can often be found in the promoters of muscle-specific genes. In
many cases, the E box elements in muscle-specific genes interact with members of the myogenic basic helix-loop-helix family of transcription factors such as MyoD, myogenin, and Myf-5 (61, 62). However, E boxes have also been shown to interact with a number of non-myogenic transcription factors, including members of the Myc, TFE3, and USF families (63–65).

We have determined that the double-stranded Kcnj2 E box oligo probe will specifically shift a major DNA-protein complex, in addition to a weaker complex, that is efficiently competed by 100-fold excess cold self oligo but not by an unrelated oligo or an oligo containing point mutations that disrupt the core E box sequence (Fig. 6A). Nuclear extracts from a non-muscle source (HeLa cells) were able to shift a DNA-protein complex of the same apparent size, suggesting that this complex is not the result of an interaction with one of the myogenic transcription factors (Fig. 6A). Similar to that of the C2C12 extract, the DNA-protein complex generated with the HeLa extract was also competed by excess unlabeled Kcnj2 oligo but not by nonspecific or mutation-containing (mutE box) oligos (data not shown).

Since this E box element contains the more specific consensus sequence CACGTG, we considered the possibility that this element could interact with a member of the Myc family of transcription factors (64, 66). This does not appear to be the case, however, since aMyc and aMax antibodies failed to appreciably supershift the DNA-protein complex (data not shown). While testing the thermostability of this binding activity, we found that significant activity is retained even after preincubation of the nuclear extract for 10 min at 100 °C (Fig. 6A). This degree of thermostability is a characteristic of the ubiquitous transcription factor USF (67, 68), which is also known to bind to the core CACGTG sequence (69). To confirm the presence of USF in the shifted complexes, aUSF-1 and aUSF-2 antibodies were used in supershift assays. Both antibodies were able to supershift the DNA-protein complexes to similar degrees (Fig. 6A). These data suggest that binding of USF-1/USF-2 hetero- and/or homodimers to the E box element is responsible for the major DNA-protein complex seen by EMSA using C2C12 myoblast extracts.

**NF-Y Binds the Y Box Element of the Minimal Promoter**—An oligo spanning the Y box element specifically shifted a major DNA-protein complex, in addition to a weaker complex, that is efficiently competed by 100-fold excess cold self oligo but not by an unrelated oligo or an oligo containing point mutations that disrupt the core E box sequence (Fig. 6A). Nuclear extracts from a non-muscle source (HeLa cells) were able to shift a DNA-protein complex of the same apparent size, suggesting that this complex is not the result of an interaction with one of the myogenic transcription factors (Fig. 6A). Similar to that of the C2C12 extract, the DNA-protein complex generated with the HeLa extract was also competed by excess unlabeled E box oligo but not by nonspecific or mutation-containing (mutE box) oligos (data not shown).

Since this E box element contains the more specific consensus sequence CACGTG, we considered the possibility that this element could interact with a member of the Myc family of transcription factors (64, 66). This does not appear to be the case, however, since aMyc and aMax antibodies failed to appreciably supershift the DNA-protein complex (data not shown). While testing the thermostability of this binding activity, we found that significant activity is retained even after preincubation of the nuclear extract for 10 min at 100 °C (Fig. 6A). This degree of thermostability is a characteristic of the ubiquitous transcription factor USF (67, 68), which is also known to bind to the core CACGTG sequence (69). To confirm the presence of USF in the shifted complexes, aUSF-1 and aUSF-2 antibodies were used in supershift assays. Both antibodies were able to supershift the DNA-protein complexes to similar degrees (Fig. 6A). These data suggest that binding of USF-1/USF-2 hetero- and/or homodimers to the E box element is responsible for the major DNA-protein complex seen by EMSA using C2C12 myoblast extracts.

**Transcriptional Regulation of the Kcnj2 Promoter**

**Fig. 5.** EMSA analysis of the minimal promoter Sp1 elements. **A**, binding activity of wild-type and point mutant GC box oligos. 20 fmol of labeled double-stranded wild-type (wt) and mutant (mut) oligos were incubated with 2 μg of C2C12 myoblast nuclear extracts and analyzed for binding activity by gel-shift assay as described under “Experimental Procedures.” **B**, identification of Sp1 and Sp3 binding activities. 20 fmol of end-labeled Sp1(H) oligo probe was incubated in the absence (control lane) or presence of 500-fold molar excess of the indicated competitor oligonucleotides and analyzed by gel-shift assay. Where indicated, polyclonal αSp1 or αSp3 antibodies (0.2 μg IgG) were added 30 min prior to the addition of the labeled probe. The asterisk indicates the supershifted Sp1 product. **C**, relative binding affinities of the Kcnj2 GC box elements. 20 fmol of an end-labeled exogenous Sp1 consensus oligo was used in competition studies with the Kcnj2 Sp1(M), (H), and (N) element oligos. 1-, 10-, 100-, and 1000-fold molar excess unlabeled Kcnj2 oligo were preincubated 30 min in reactions containing 2 μg of C2C12 myoblast nuclear extracts prior to the addition of the labeled probe to determine relative binding affinities.
purified antibodies to the NF-Ya subunit as shown in Fig. 6C.

Although NF-Y appeared to be the dominant trans-acting factor responsible for the observed Y box-shifted complex in C2C12 myoblasts by supershift assay, we also investigated the possibility that YB-1, a member of the Y box family of transcription factors that has been previously shown to interact with Y box elements (44), might also interact with the Kcnj2 Y box element. The possibility of promoter regulation through competitive binding at the same Y box element has previously been demonstrated for the major histocompatibility complex class II DRA promoter, in which the positive effect of NF-Y binding could be negatively regulated by competitive binding of YB-1 at the same element (70, 71). An unusual characteristic of many of the Y box proteins that have been cloned is an ability to bind single-stranded DNA (43, 71, 72). We found that both the top and bottom single-stranded Y box element oligos could be specifically shifted, although weakly, by C2C12 nuclear extracts, with a slight preference shown for the top strand (data not shown). In addition, antibodies to the human YB-1 protein (71) were able to weakly supershift a small portion of the Y box-protein complex, indicating that a protein antigenically similar to YB-1 binds, albeit poorly (Fig. 6C), to the Kcnj2 Y box element.

Competitive Binding within the Minimal Promoter—Having identified the specific transcription factors capable of interacting at the Kcnj2 minimal promoter E box, Y box, and GC box elements, we were interested in determining if the relatively close proximity of these promoter elements to each other might effect binding within a larger context. To accomplish this, we isolated a promoter fragment probe containing the E box, Y box, Sp1(H), and Sp1(N) elements (SmaI/BssHII 2107/20), from wild-type and point mutant-containing promoter constructs. To ensure that the introduced point mutations ablated specific binding, we used the point mutant oligos as probes in EMSAs and determined that the mutations effectively blocked specific binding to the targeted promoter elements (see Figs. 5A and 6, A and C).

Using the wild-type SmaI/BssHII probe, we identified the transcription factor(s) responsible for generating the specifically shifted complexes produced in EMSA by a combination of
specific antibodies and/or excess competitor oligos (not shown). The Smal/BssHII probe robustly shifted complexes corresponding to both Sp1 and Sp3 binding at a single GC box element, in addition to multiple slowly migrating complexes resulting from binding simultaneously to both GC box elements (Sp1(H) and Sp1(N)) present within the probe. In addition, a single weak band corresponding to NF-Y binding activity was also identified (Fig. 7). We were unable to identify a specifically shifted complex that could be attributed to USF binding to the E box element.

Point mutations within the inverted CCAAT sequence of the Y box (µ Y box) resulted in the loss of the NF-Y complex (Fig. 7). As expected, mutations within either the GC box at −54 or at −19 (µSp1(H) and µSp1(N), respectively) resulted in the specific loss of the Sp1 and/or Sp3 multimer bands, whereas the Sp1 and Sp3 complexes formed by interactions at the remaining GC box element were unaffected. When both GC box elements were disrupted (µSp1(H/N)), only the complex resulting from NF-Y binding at the Y box element remained. In addition, we observed a marked increase in the relative binding of NF-Y to the µSp1(H) probe, whereas no corresponding increase of NF-Y binding was observed with the µSp1(N) probe. Conversely, mutation of the Y box resulted in an increase in both Sp1 and Sp3 binding (Fig. 7). These results indicated that binding at the Y box and Sp1(H) elements is competitive, possibly due to simple steric interference between the closely positioned elements.

Mutation of the E box element (µE box) had no discernible effect on the pattern of shifted complexes when compared with the wild-type probe. This finding was consistent with our inability to detect any binding activity attributable to USF in the wild-type Smal/BssHII probe, and the results obtained using the µSp1(H/N) probe, which possessed no other binding activity except NF-Y. Given the proximity of the E box to the Y box element, the competitive nature of binding between the Y box and Sp1(H) elements, and the divergence of the Kcnj2 E box-flanking sequence from an optimal USF consensus sequence (69), the lack of detectable USF binding to the fragment probe is not surprising.

**Effect of Point Mutations on Kcnj2 Promoter Activity**—In order to assess the contribution of individual promoter elements toward the overall transcriptional activity of the Kcnj2 promoter, we transiently transfected the point mutation-containing reporter constructs into C2C12 myoblasts and assayed for luciferase activity. Transfection efficiencies were adjusted using a co-transfected plasmid expressing the secreted embryonic alkaline phosphatase gene (pBC12/CMV/SEAP) as an internal control. The Student's *t* test was used to assess statistically significant changes in reporter construct activity.

We found that mutation of the E box element at −88 resulted in a small, but not statistically significant, effect on the Kcnj2 promoter activity (Fig. 8, *p* > 0.1, Student's *t* test). This finding was consistent with the previous EMSA results using the µE box SmaI/BssHII probe (Fig. 7), which found no change in binding activity when compared with the wild-type probe. The small decrease in promoter activity noted with the µE box construct may be attributable to effects on the adjacent Sp1(M) or Y box elements. Similar to the µE box results, mutation of the Sp1(M) element resulted in a small, but not statistically significant, decrease in promoter activity. Although the µSp1(M) mutation failed to produce a statistically significant effect in and of itself, its introduction consistently resulted in an approximately 20% decrease in activity (compare wild-type versus µSp1(M), µSp1(H) versus µSp1(M/H), and µSp1(N) versus µSp1(M/N) in Fig. 8). The low level transcriptional activity attributable to the Sp1(M) element is consistent with the contribution that might be expected of a relatively low affinity element in comparison to the Sp1(H) and Sp1(N) elements. In contrast, mutation of the high affinity Sp1(H) element at −54 produced a robust decrease in luciferase activity (∼40%, *p* < 0.005, Student's *t* test). This measured effect from disrupting the Sp1(H) element may underestimate its actual contribution.
toward transcriptional activity of the Kcnj2 promoter because of compensation through increased NF-Y binding at the Y box element (see Fig. 7).

In contrast to the reduced promoter activity of the $\mu$Sp1(M) and $\mu$Sp1(H) constructs, mutations of either the Sp1(N) or Y box elements each resulted in approximately 60% increases in luciferase activity ($p < 0.002$, Student's $t$ test). Because of the competitive binding observed between the Y box and Sp1(H) elements (Fig. 7), we attributed the increase in promoter activity seen in the Y box construct to increased binding at the Sp1(H) element. Despite this apparent repressor effect, the Y box element also seems to function as an transcriptional activator, although a weak one. Evidence of this can be seen with the triple mutant construct $\mu$Sp1(M/H/N), which retained only NF-Y binding activity by EMSA. Rather than being inactive, as would be expected if the Y box functioned solely as a repressor element, this construct retained approximately 30% of the wild-type transcriptional activity. The simplest explanation for these results is that the Y box mutation removed a weak activator (NF-Y), allowing a strong activator (Sp1) to bind at the nearby Sp1(H) element. The increase in luciferase activity seen with the $\mu$Sp1(N) mutation is less easily explained. It is possible that the Sp1(N) element preferentially binds the inhibitory isoforms of Sp3 better than Sp1, thereby leading to the observed increase in luciferase activity when this element is mutated. Alternatively, the reverse orientation of this element, or its close proximity to the transcription initiation sites, may cause it to normally function as a negative regulator. Like the Y box element, however, the Sp1(N) element may also function as a transcriptional activator in some circumstances. Evidence of this may be inferred by comparison of the activities of the $\mu$Sp1(M/H/N) and $\mu$Sp1(M/H) constructs (Fig. 8). In this instance, mutation of the Sp1(N) element resulted in an additional loss of activity, rather than the increase in activity associated with the $\mu$Sp1(N) mutation observed in other contexts. Both the Y box and Sp1(N) elements may therefore serve as bifunctional promoter elements, acting as either positive or negative regulatory elements depending upon the complement of trans-acting factors interacting at nearby elements. These context-dependent elements may be important in integrating the combined inputs of the multiple elements controlling the activity of the Kcnj2 minimal promoter in different cell types.

**DISCUSSION**

The Kcnj2 gene encodes an inwardly rectifying K$^+$ channel (variously named IRK1, Kir2.1, and Kcnj2) that is found in a wide variety of tissue and cell types. In an effort to understand the elements involved in regulating the expression of this channel, we determined the genomic structure of Kcnj2 and characterized its minimal promoter in C2C12 myoblasts. The murine Kcnj2 gene isolated from 129SvJ genomic DNA is comprised of 2 exons separated by a single 5.5-kb intron. Exon 1 encodes 168 bases of the 5'-UTR, whereas exon 2 encodes the remaining 235 bases of the 5'-UTR and the entire ORF and 3'-UTR. The intronless ORF structure of the Kcnj2 gene is similar to that of several Shaker-like Kv channels genes (73) and the rat Kir3.4 gene (74) but different from the human Kir1.1 (22), Kir3.1 (21), Kir3.2 (75, 76), Kir3.4 (74), and mouse Kir3.4 (77) inward rectifier genes, whose ORFs are all encoded by multiple exons.

Comparison of the Kcnj2 129SvJ genomic sequence with the cDNA sequence derived from the J774.1 macrophage cell line (5) revealed only minor differences attributable to normal sequence variations between the different sources (data not shown). Immediately upstream of the 3'-polyadenylation consensus sequences found in the genomic sequence are two motifs similar to the “UUUUUAU” sequences found to be important for the regulation of expression of specific mRNAs via poly (A)$^+$ addition during early oocyte maturation and development (78). Interestingly, an inwardly rectifying K$^+$ current has been seen to undergo changes during maturation of starfish oocytes (79). The presence of these motifs in the Kcnj2 transcript suggests the possibility that, in addition to traditional transcriptional mechanisms, Kcnj2 expression may be regulated during the very early stages of maturation and development through a non-transcriptional mechanism.

Analysis of the Kcnj2 promoter 5'-deletion constructs determined that only the full-length reporter construct (-3300/+62) displayed a tissue-specific expression pattern, seemingly due to a repressor element(s) located within the sequences from -3300 to -787 that significantly reduced expression in the Neuro 2A but not the C2C12 cell line. The presence of this repressor activity in the neuronal, but not muscle-derived, cell line may explain the relatively low level of Kcnj2 mRNA expression observed in whole brain (5, Fig. 2). Similar tissue-specific silencing activities have been previously described for the Kv1.5 (19) and type II sodium channel genes (18). The dinucleotide repeat element in the Kv1.5 promoter seemingly acts to repress expression in non-GH3 cell lines by interaction with nonhistone high mobility group 1 protein. The RE1 silencer element found in the type II sodium channel gene promoter, as well as other nervous system-restricted genes, binds the zinc finger protein REST. REST expression is inversely correlated with type II gene expression, with high REST levels found in non-neuronal and differentiated peripheral nervous tissues. In both these cases, the repressor element and associated protein have been well characterized; further localization of the Kcnj2 promoter repressor element(s) may help to identify the associated binding protein(s).

The housekeeping characteristics of the Kcnj2 tissue nonspecific minimal promoter (−109 to +62), and the ubiquitous nature of its multiple controlling trans-acting factors, may be important to allow Kcnj2 expression in many different tissue and cell types. Recent studies have demonstrated that genes possessing TATA-less promoters are often the subject of active transcriptional regulation (80). There is also mounting evidence indicating that Sp1-driven promoters may be acutely regulated by multiple signal transduction pathways that can influence the activity of Sp1 (81–83). These types of transcriptional regulation of the generalized Kcnj2 promoter, which would be expected to allow expression in a variety of cell types, may be critical in allowing the different cell types to fine tune their level of Kcnj2 expression in order to maintain or adjust their electrical properties as needed.

Competitive binding between different trans-factors at the same or nearby promoter elements, often having opposing effects, is a mechanism of transcriptional regulation seen in many promoters. This type of regulation may be a particularly important mode for the Kcnj2 promoter. Sp1 and Sp3 have been shown to compete with similar affinities for binding at the same GC box elements (57, 84, 85). Although Sp1 has long been known to function as an activating transcription factor, in many instances overexpression of Sp3 has indicated that it may serve as an inhibitory member of the Sp family, causing repression of Sp1-mediated promoter activation (85–88). Therefore, the relative levels of Sp1 and Sp3 proteins in a given cell are likely to be important determinants of the transcriptional activity of a target promoter (89, 90). However, regulation by Sp1 and Sp3 is more complex than a simple Sp1/activator and Sp3/repressor model. Recent work has demonstrated that Sp3, like Sp1, can also mediate the transcriptional activation of at least some promoters (58, 91, 92). It now appears that the full-length Sp3 protein can act as a transcriptional activator in
some instances, while two internally initiated Sp3 protein species lacking part of the N-terminal activation domain are likely responsible for the inhibition of Sp1-mediated activation (58). In addition, a recently described repressor domain located just 5’ of the zinc finger domain in Sp3 may be an additional determinant for whether or not it can activate a specific promoter (93, 94). Given the multiple Sp factors found within the Kcnj2 minimal promoter and the complexity of the interactions between Sp1 and the various Sp3 isoforms, it seems likely that these elements may serve as important regulatory targets in setting the mRNA expression levels in various cell types.

The observed competitive binding between NF-Y and Sp1/Sp3 at the Y box and Sp1(H) elements, respectively, offers another potential regulatory point within the Kcnj2 promoter. In previously characterized promoters containing closely positioned NF-Y- and Sp1-binding sites, investigators have noted cooperative interactions that stabilized binding between the transcription factors (52, 95, 96). NF-Y has also been shown to be important for the recruitment of additional upstream transcription factors (52, 95, 96). NF-Y has also been shown to cooperate with Sp3 at the Y box and Sp1(H) elements, respectively, offering some instances, while two internally initiated Sp3 protein species lacking part of the N-terminal activation domain are likely responsible for the inhibition of Sp1-mediated activation (58). However, much lower levels of competitor oligo (50–100-fold) were sufficient to completely compete the fast mobility Sp3 band (data not shown), which correlates with the inhibitory Sp3 species described in Kennett et al. (58).

In conclusion, we find that the Kcnj2 promoter possesses a complex structure, allowing interactions with several different ubiquitous transcription factors. This structure, in conjunction with the relative abundance and state of activity of the transcription factors, may provide the basis for selective cell- and tissue-specific transcriptional regulation of the widely distributed Kcnj2 mRNA.

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