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Expression and transcriptional control of human KCNE genes

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Abstract

Potassium channels are essential for a variety of cellular processes ranging from membrane excitability to cellular proliferation. The KCNE genes (*KCNE1–5*) encode a family of single-transmembrane-domain proteins that modulate the properties of several potassium channels, suggesting a physiologic role for these accessory subunits in many human tissues. To investigate the expression and transcriptional control of KCNE genes we mapped transcription start sites, delineated 5′ genomic structure, and characterized functional promoter elements for each gene. We identified alternatively spliced transcripts for both *KCNE1* and *KCNE3,* including a cardiac-specific *KCNE1* transcript. Analysis of relative expression levels of *KCNE1–5* in a panel of human tissues revealed distinct, but overlapping, expression patterns. The coexpression of multiple functionally distinct KCNE genes in some tissues infers complex accessory subunit modification of potassium channels. Identification of the core promoter elements necessary for transcriptional control of the KCNE genes facilitates future work investigating factors responsible for tissue-specific expression as well as the discovery of promoter variants associated with disease.

Keywords: Potassium channel; Promoter; *KCNE1*; *KCNE2*; *KCNE3*; *KCNE4*; *KCNE5*; *KCNQ1*

Potassium channels comprise one of the oldest and most diverse classes of membrane proteins, with genes encoding more than 75 pore-forming subunits [1]. Voltage-gated potassium (K_V) channels constitute a large portion of this family and have been implicated in a variety of physiological processes, including membrane excitability, synaptic transmission, epithelial transport, and cellular proliferation. The diversity of K_V channel activity is further expanded through associations between pore-forming (α) and accessory (β) subunits. Several distinct families of accessory β subunits can have profound effects on biophysical properties, pharmacological responses, tissue distribution, and trafficking of various K_V channel α subunits [2].

One important family of human potassium channel β subunits is encoded by the five KCNE genes (*KCNE1–KCNE5*) [3,4]. In addition to amino acid sequence homology within their single transmembrane domains, a common feature of KCNE subunits is the ability to modulate KCNQ1 and other voltage-gated channels in vitro. The most well studied example is the interaction of KCNE1 with KCNQ1 to form the slow component of the delayed rectifier current (I_{Ks}) essential for normal cardiac repolarization [5,6]. KCNE2 and KCNE3 alter activation and confer constitutive activity when coexpressed with KCNQ1, with KCNE2 decreasing [7] and KCNE3 increasing [8] current magnitude. By contrast, KCNE4 and KCNE5 inhibit heterologously expressed KCNQ1 channel activity [9–11]. The physiological importance of KCNE genes is illustrated by mutations in KCNE1-3 that have been associated with inherited human diseases [12–17].

In this study, we defined the transcription start sites and 5′ flanking genomic sequences of each KCNE gene. These data enabled discovery of alternatively spliced transcripts for *KCNE1* and *KCNE3* and functional characterization of core promoter regions for all genes except *KCNE2.* We further examined relative mRNA levels of the five KCNE genes by quantitative RT-PCR in multiple human tissues and identified distinct but overlapping expression patterns. This study provides evidence that KCNE genes are widely coexpressed, suggesting that complex accessory subunit modulation of

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potassium channels is plausible in many tissues. We also delineate critical genomic elements necessary for transcriptional control of KCNE genes and thus enable future work to determine factors responsible for tissue-specific expression.

Results

Genomic organization of human KCNE genes

Definition of the 5′ genomic structure for each KCNE gene enabled identification of promoter regions and relative mRNA expression analysis in various human tissues. Transcription start sites were mapped for each KCNE gene by the ribo-oligonucleotide ligation method of rapid amplification of cDNA ends (RLM-RACE) [21], followed by nucleotide sequencing of subcloned products. We identified exons containing the 5′ untranslated region (5′-UTR) of each gene by alignment of RLM-RACE sequences with publicly available human genome sequence. We did not determine the genomic organization of the 3′ untranslated regions.

Two alternative transcription start sites were identified for *KCNE1* in cDNA derived from human ventricle, suggesting the presence of multiple promoter regions. The transcripts originating from these two sites were designated KCNE1a (short 5′-UTR, GenBank Accession No. AY789479) and KCNE1b (long 5′-UTR, GenBank Accession No. AY789480). Each transcript contained identical coding regions, differing only by alternative splicing of exons containing portions of the 5′-UTR (Fig. 1). We identified one entry in GenBank corresponding to KCNE1a (Accession No. BC036452), which extended an additional 49 nucleotides in the 5′ direction, but no KCNE1b sequences were identified in GenBank or databases of expressed sequence tags (ESTs). *KCNE3* also exhibited two alternative transcription start sites resulting in two transcripts (KCNE3a for long 5′-UTR; KCNE3b for short 5′-UTR, GenBank Accession Nos. DQ192291, DQ192292). These transcripts differed only by the presence of an additional exon in the 5′-UTR for KCNE3a compared to KCNE3b. One human EST sequence (CR936625) corresponded to KCNE3a exactly, whereas we found no KCNE3b matches in the nucleotide databases. Single sites of transcription initiation were identified in human ventricular mRNA for *KCNE2, KCNE4,* and *KCNE5* (Fig. 1, GenBank Accession Nos. DQ192290, DQ192293, DQ192294), and the corresponding 5′-UTRs extended 4–49 nucleotides 5′ of existing cDNA or EST sequences except one EST corresponding to *KCNE5,* which originated from an RLM-RACE library (AK223306). All KCNE genes except *KCNE5* have exons containing 5′-UTR sequence separated from the coding exon by variable-length introns (size range 0.5–5.1 kb). Consensus splice site sequences including a 5′ GT dinucleotide and 3′ AG dinucleotide were identified in each intron.

Relative KCNE gene expression in human tissues

To examine relative expression of KCNE genes in a variety of human tissues, we employed real-time, quantitative RT-PCR using 5′-nuclease chemistry and fluorogenic oligonucleotide (TaqMan) probes. To achieve a high level of specificity and to avoid detection of genomic DNA, we designed probes for each KCNE transcript to span exon–exon junctions, except for *KCNE5,* which is intronless. We also assayed for expression of *KCNQ1,* a K_V channel α subunit reported to interact with each member of the KCNE family in vitro. Expression levels for each gene were determined by compar-

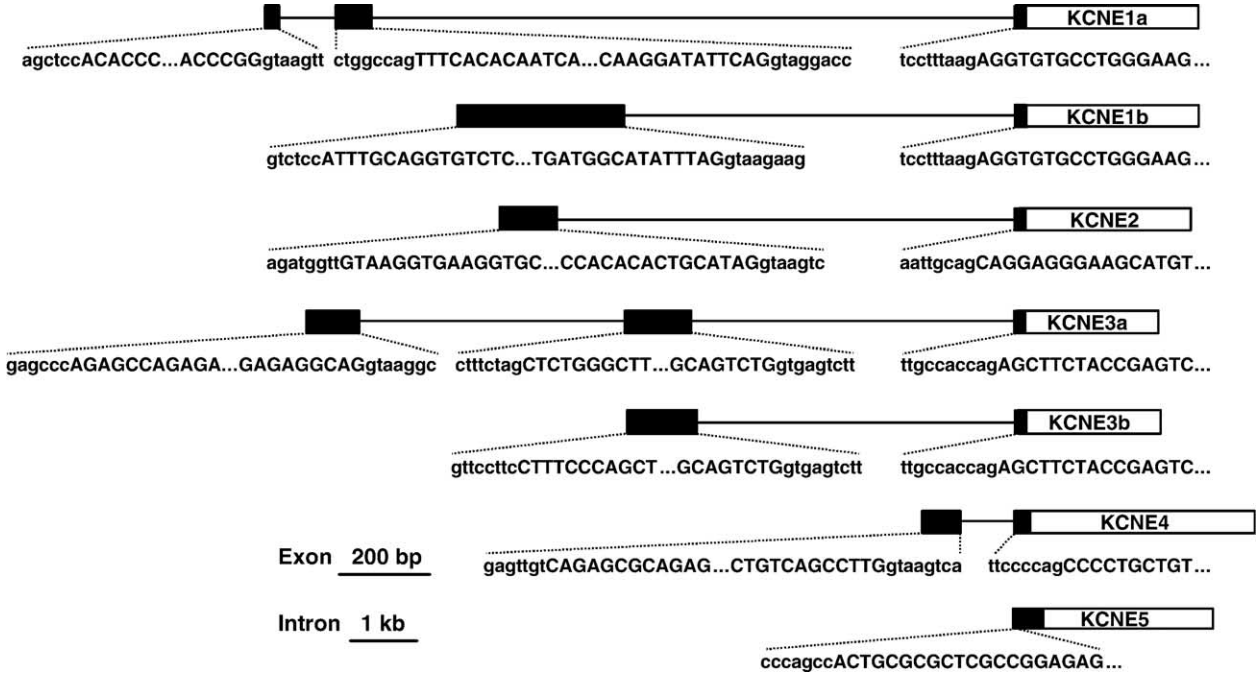

Fig. 1. Genomic organization of human KCNE genes. For each KCNE gene, black boxes denote the 5′ UTR, while white boxes indicate coding regions. Nucleotide sequences correspond to experimentally determined exon boundaries (intronic sequences are in lower case). Intron and exon lengths are scaled differently as indicated by separate scale bars.

ison to a standard curve that was assayed in parallel and the results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

Fig. 2 illustrates the relative levels of all KCNE genes and *KCNQ1* across 15 different human tissues. As shown previously [11], *KCNE1* (KCNE1a + KCNE1b) exhibits the most prominent expression in the heart, but levels of *KCNE4, KCNE3,* and *KCNE5* are also substantial in this tissue (Fig. 2A). Notably, we observed expression of the KCNE1b transcript only in the heart. This suggests that the KCNE1b transcript is cardiac specific, while KCNE1a is expressed in several other tissues, including kidney, testes, and uterus (Figs. 2A and 2B). By contrast, we detected very low levels of cardiac *KCNE2* expression. *KCNE2* expression was low across the majority of tissues compared to the other members of the KCNE family except in colon and small intestine (Fig. 2B).

KCNE3 is the most highly expressed KCNE gene across the various tissues. High relative levels of *KCNE3* and *KCNQ1* were detected in kidney, prostate, and leukocytes, with minimal expression of other KCNE family members in these tissues. However, in other tissues such as the liver, ovary, and placenta, we observed high relative levels of *KCNE3* transcripts in the absence of *KCNQ1* expression. High levels of *KCNE4* and *KCNE5* expression were detected in a variety of tissues. Relative to the other KCNE genes, the greatest expression of *KCNE4* occurred in skeletal muscle, testes, spleen, and ovarian tissue. High levels of *KCNE5* expression were observed in the brain, thymus, testes, spleen, ovaries, and placenta.

Identification of KCNE promoters

Mapping transcription start sites (TSS) for each KCNE gene permitted identification and characterization of their putative promoter regions. To delineate further genomic regions potentially important for transcriptional control, we performed VISTA [19] analysis across human and mouse sequences to identify conserved noncoding elements. We cloned genomic DNA surrounding each identified TSS into the pGL3basic plasmid, which encodes firefly luciferase and lacks a resident promoter. For each KCNE promoter construct, we determined luciferase

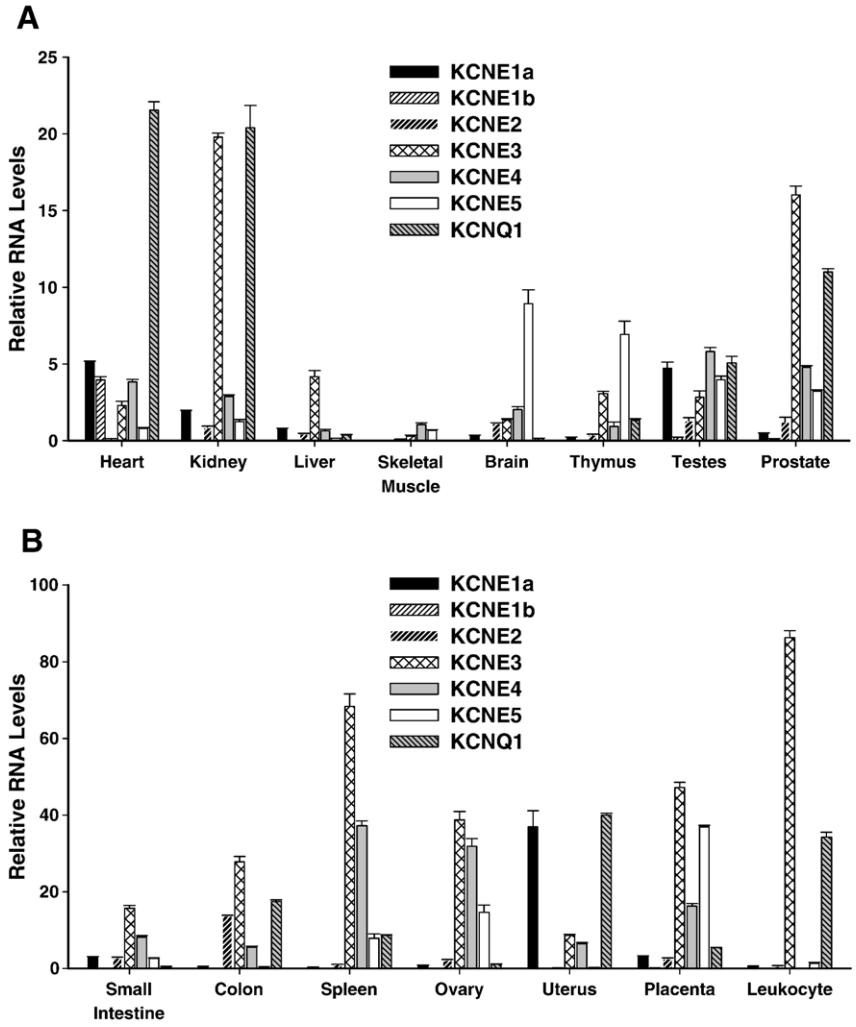

Fig. 2. Relative expression of KCNQ1 and KCNE genes in human tissues. Several human tissues were examined by real-time quantitative RT-PCR. Tissues were grouped by low (A) and high (B) overall expression for display purposes (note differences in the y-axis scale). All data were quantified by gene specific standard curves and results were normalized by GAPDH expression. Data are presented as mean ± SEM for at least 6 replicates from 2 different pooled (2 or more individuals) cDNA samples isolated from healthy individuals.

activity in murine cardiac myocytes (HL-1 cells) and green monkey kidney cells (CosM6). When promoter activity was observed, we tested nested deletions of the full-length cloned promoter constructs. Data were normalized to the activity of a second reporter (*Renilla* luciferase) to control for variation in transfection.

KCNE1 promoter

Two distinct regions were identified corresponding to putative promoters for KCNE1a and KCNE1b. Expression of the putative KCNE1a promoter region (−1278 to +257 relative to the TSS) resulted in high levels of normalized luciferase activity in both CosM6 cells (40-fold) and HL-1 cells (16-fold) compared to nonrecombinant vector, suggesting the identification of a functional promoter region (Figs. 3A and 3B). An unpaired 5′ splice site belonging to the first intron included in this construct elicits mRNA splicing to a cryptic acceptor site 46 bp upstream of the luciferase start codon. However, this splicing event preserves the complete open reading frame of the reporter gene based on sequencing of RT-PCR-generated amplicons from transfected cells (data not shown). Truncation of the genomic region by removal of 1034 bp from the 5′ end did not significantly affect reporter activity, indicating that the core promoter was contained within a 501-bp region (−244 to +257) surrounding the TSS. This hypothesis was confirmed by observing only background luciferase activity when this region was removed (Fig. 3A). A consensus TATA box was not evident in the GC-rich region upstream of the transcription start site, although two conserved transcription factor binding sites (FXR, WHN) were identified (Table 1).

Expression of the region surrounding the KCNE1b transcription start site (−1690 to +70) resulted in reporter activity ninefold greater than the nonrecombinant vector in both CosM6 and HL-1 cells (Fig. 3B). Truncation of the 5′ region led to progressively less activity in the HL-1 cells, while significantly increased activity was observed in CosM6 cells with the removal of the region from −1690 to −921. In both cell types, removal of a 450-bp region surrounding the KCNE1b TSS significantly diminished reporter activity and is likely to contain the core promoter region for this alternatively spliced transcript. There was no identifiable TATA box, although several conserved transcription factor binding sites were predicted, including MYOD, GATA6, SRF, and SF1 (Table 1).

KCNE2 promoter

In a similar fashion, we cloned the genomic region (−1546 to +30) surrounding the transcription start site identified for *KCNE2.* However, expression of this construct, as well as various deletion constructs, did not result in luciferase activity significantly greater than background levels in either HL-1 or CosM6 cells (data not shown). *KCNE2* is not highly expressed in heart or kidney, the two tissue types represented by the cell lines used in our experiments, and the putative promoter may

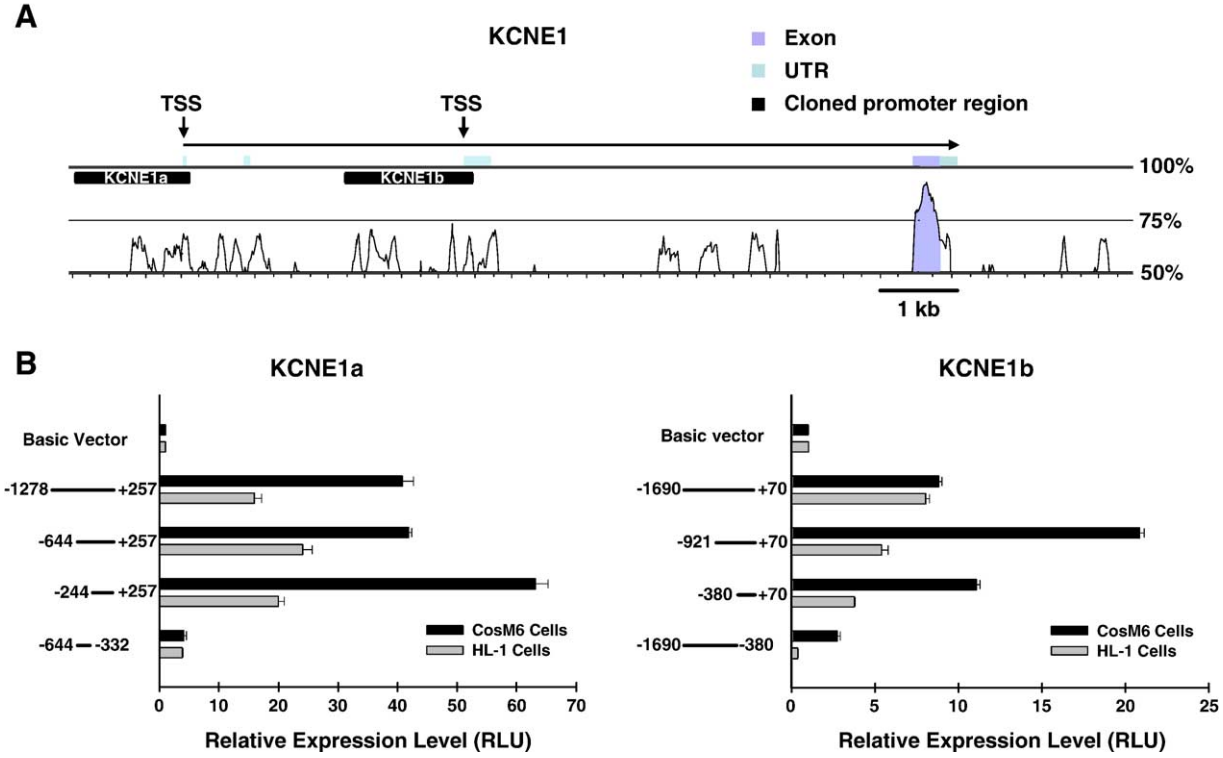

Fig. 3. KCNE1 sequence conservation and promoter analysis. Human and mouse genomic sequence surrounding the KCNE1 locus was compared using VISTA (A). Peaks represent regions of conservation greater than 50%. The locations of untranslated exons (blue) and the coding exon (purple) are depicted above the alignment. The full length cloned promoter regions for both KCNE1a and KCNE1b are represented by black boxes. Full length and truncated promoter regions (described relative to the transcription start site) for both KCNE1a and KCNE1b were subcloned into pGL3basic (Promega) and assayed for activity in CosM6 and HL-1 cells (B). Nonrecombinant vector (promoterless pGL3basic vector) expression was normalized to a value of one. Data were compiled from three independent transfections assayed in triplicate.

Table 1
Predicted transcription factor binding sites in KCNE genes

Gene	Transcription Factor	Position\ Strand (bp relative to TSS)	Conserved Binding Site[a]
KCNE1a	WHN	−751\−	TAGCGTTGCTC
	FXR_IR1	−888\+	GGTTCCTGACCTA
KCNE1b	GATA6	−153\+	CCTATCAGCACTGATAACAG
	SF1	−918\+	GTCCTTGG
	MYOD	−931\+	CCAACTGTCT
	SRF	−977\+	CTATGTCCAAATTTTGGA
KCNE3a	SP1	−61\−	GCCCCGCCCGGC
	SP1	−113\−	CCTCCGCCCAGCT
	ETS	−174\−	CGCCTGCTTCCTGGA
KCNE3b	ETS	−120\−	TCCCCTTCCTGC
	GABP	−120\−	CCCCTTCCTGCA
	NRF2	−120\−	CCTTCCTGCA
	AP4	−132\−	CTGCAGCAGCTGGGTCAG
	NFAT	−976\−	TTTTTCCTCTTC
KCNE4	MYOD	−50\+	CAGAGCACGTGACGATGC
	E4F1	−50\+	TGACGATGCA
	USF	−50\+	GAGCACGTGACGAT
	MYOD	−780\+	AAAACCTGCCCA
KCNE5	STAT	+84\+	GGAAGCGA
	SP1	−54\+	CTCCTCCCCTT
	Muscle INI B	−64\+	CCCTTCCCCACCACGCCCCCT
	SP1	−71\+	ACCACGCCCCCTC
	GC	−71\+	CACCACGCCCCCTC
	OLF1	−226\+	GCGAGGCCCCAGGCAGCAGACG
	E2F	−261\+	TTGGCCCA
	AP2	−276\+	ACCCTGGGCTGCGGAA
	ROAZ	−276\+	GACCCTGGGCTGCG
	YY1	−790\+	GTAAGCCATCTTCCCAGAAG
	STAT	−796\+	CCATCTTCCCAGAAGTGAAAC

[a] Sequence of binding site conserved between mouse and human predicted by rVISTA with a matrix similarity of ≥85%.

be inactive in these cells. Alternatively, it is possible that we did not identify the true promoter region for *KCNE2*.

KCNE3 promoter

Two alternative transcription start sites were identified for *KCNE3* and a significant level of sequence conservation between human and mouse was identified upstream of both sites (Fig. 4A). Expression of a reporter construct encompassing the KCNE3a site (−1677 to +59) resulted in 40- and 20-fold greater reporter activity in CosM6 and HL-1 cells, respectively, compared to the nonrecombinant vector. Removal of nucleotides −736 to −346 resulted in a 2.5-fold increase in reporter activity in both CosM6 and HL-1 lines, suggesting that this region may contain a suppressor element active in kidney and heart cells (Fig. 4B). Deletion of 405 bp (−346 to +59) surrounding the KCNE3a transcription start site reduced reporter activity to background levels, implicating this segment as the location of the core promoter. The region upstream of the TSS was GC rich, lacked a TATA box, and exhibited predicted transcription factor binding sites (two SP1 and one ETS) that were conserved between mouse and human (Table 1).

Expression of the region surrounding the KCNE3b start site (−1182 to +173) resulted in 10- and 6-fold increases in reporter activity in CosM6 and HL-1 cells, respectively, although this activity was significantly less than that of KCNE3a. Removal of the region from −1182 to −655 resulted in increased reporter activity in both cell types, consistent with elimination of suppressor element (Fig. 4B). This putative suppressor coincides with a noncoding region of substantial conservation ~750 bp upstream of the TSS for KCNE3b (Fig. 4A). Further removal of sequence from −655 to −283 reduced reporter levels to those observed in the original (−1182 to +173) construct. Elimination of 283 bp surrounding the KCNE3b TSS resulted in basal levels of reporter activity, indicating that the core promoter activity for KCNE3b is contained within this region (Fig. 4B). Multiple conserved transcription factor sites (NFAT, AP4, ETS) were predicted upstream of the TSS, but no TATA box was present (Table 1).

KCNE4 promoter

Alignment of human and mouse genomic sequence surrounding the *KCNE4* locus revealed regions of significant conservation immediately 5′ of the untranslated exon and two conserved regions near the 3′-UTR (Fig. 5A). We tested the putative promoter region surrounding the TSS (−1249 to +56) containing highly conserved 5′ sequences for transcriptional activity in CosM6 and HL-1 cells. This putative *KCNE4* promoter region produced 40- and 27-fold increases in reporter activity in CosM6 and HL-1 cells, respectively. Stepwise removal of sequence from the 5′ end of the promoter region resulted in decreases in luciferase activity in both cell types, implicating the importance of the entire cloned region for reporter gene expression. A deletion construct with 435 nucleotides removed from the region surrounding the TSS retained a limited ability to drive expression (Fig. 5B). Consensus CAAT and TATA boxes were identified 84 and 31 bases upstream of the TSS, respectively, and two conserved MYOD sites were predicted within the promoter region (Table 1).

KCNE5 promoter

Comparison of human and mouse sequence surrounding the *KCNE5* locus revealed an extensive region of highly conserved noncoding sequence immediately upstream of the 5′-UTR (Fig. 6A). Expression of the complete putative promoter region (−1381 to +105) resulted in substantially increased reporter activity in CosM6 (60-fold) and HL-1 cells (30-fold) compared to the promoterless vector. Truncation of 494 bp of the promoter region from the 5′ end reduced activity in HL-1 cells, but not in CosM6 cells. However, removal of a 441-bp region surrounding the TSS eliminated reporter activity in both cell types, suggesting that the core promoter region is contained within this sequence (Fig. 6B). It is notable that this region corresponds well with the highly conserved noncoding region between human and mouse (Fig. 6A) and multiple conserved transcription factor binding sites were predicted in this region (Table 1).

Discussion

Potassium channels are involved in a wide range of important physiological processes including maintenance of the resting membrane potential, action potential duration,

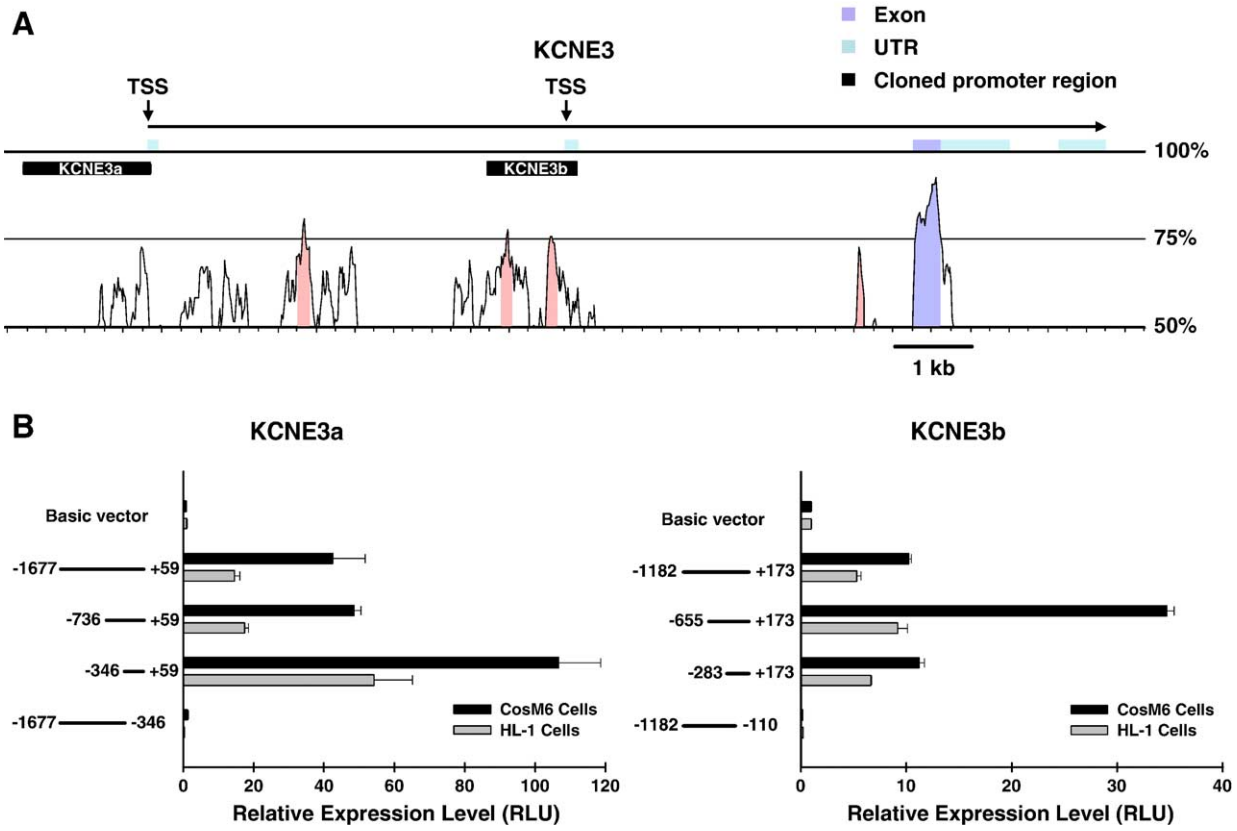

Fig. 4. KCNE3 sequence conservation and promoter analysis. Human and mouse genomic sequence surrounding the KCNE3 locus was compared using VISTA (A). Peaks represent regions of conservation greater than 50%, while regions of conservation of greater than 75% for 100+ bp are shaded orange. The locations of untranslated exons (blue) and the coding exon (purple) are depicted above the alignment. The full length cloned promoter regions for both KCNE3a and KCNE3b are represented by black boxes. Full length and truncated promoter regions (described relative to the transcription start site) for both KCNE3a and KCNE3b were subcloned into pGL3basic (Promega) and assayed for activity in CosM6 and HL-1 cells (B). Nonrecombinant vector (promoterless pGL3basic vector) expression was normalized to a value of one. Data were compiled from three independent transfections assayed in triplicate.

epithelial transport, and cellular proliferation. Heterologous expression of pore-forming α subunits often require coexpression of accessory β subunits to recapitulate biophysical properties, pharmacological responses, and normal trafficking associated with native potassium channels [2]. The KCNE genes represent one class of accessory subunits that modulate a variety of α subunits and are associated with multiple inherited human diseases.

The promiscuity of KCNE subunits in heterologous systems complicates the interpretation of their physiologic relevance. In addition to KCNQ1, studies indicate that KCNE1 may interact with HERG [22] and Kv4.3 [23]. KCNE2 has been implicated as an accessory subunit for HERG [15] channels and may modulate hyperpolarization-activated cation channel-4 [24], Kv4.2 [25], and Kv4.3 [23]. KCNE3 can also modulate HERG [8] and Kv3.4 [26]. KCNE4 has recently been shown to inhibit heterologously expressed Kv1.1 and Kv1.3 channels but to have no effects on a variety of other voltage-gated potassium channels (Kv1.2, Kv1.4, Kv1.5, and Kv4.3) [27]. To date, KCNE5 has been demonstrated only to interact biophysically with KCNQ1 [10]. The list of potassium channels that may interact with the various KCNE subunits continues to grow and fuel further speculations about their multiple physiological roles. Regulation of a single α subunit by multiple KCNE family members creates another level of channel complexity. The potential for complex modulation of potassium channels by KCNE subunits motivated our survey of relative KCNE expression across a number of human tissues and an effort to characterize the transcriptional regulation of these genes.

Expression profiling of KCNE genes

Examination of relative KCNE gene expression across human tissues revealed patterns of expression that are not evident by examination of single KCNE family members. Some tissues, such as the heart and the testes, express similar relative levels of multiple KCNEs, emphasizing the potential for convergent regulation of α subunits by multiple KCNE subunits. Other tissues such as brain, uterus, liver, and leukocytes express one predominant KCNE. Examination of these expression patterns in human tissues is informative and important as previous studies have demonstrated a species-specific pattern of expression of ion channel subunits [28].

The human KCNE1a transcript has been described [29], but to our knowledge this is the first evidence of an alternative *KCNE1* transcript expressed in human heart. KCNE1b appears to be cardiac specific, while KCNE1a was expressed at high levels in heart, kidney, testes, and uterus. Expression of KCNE1a was always accompanied by prominent expression of *KCNQ1*. In the uterus, KCNE1a and *KCNQ1* are expressed

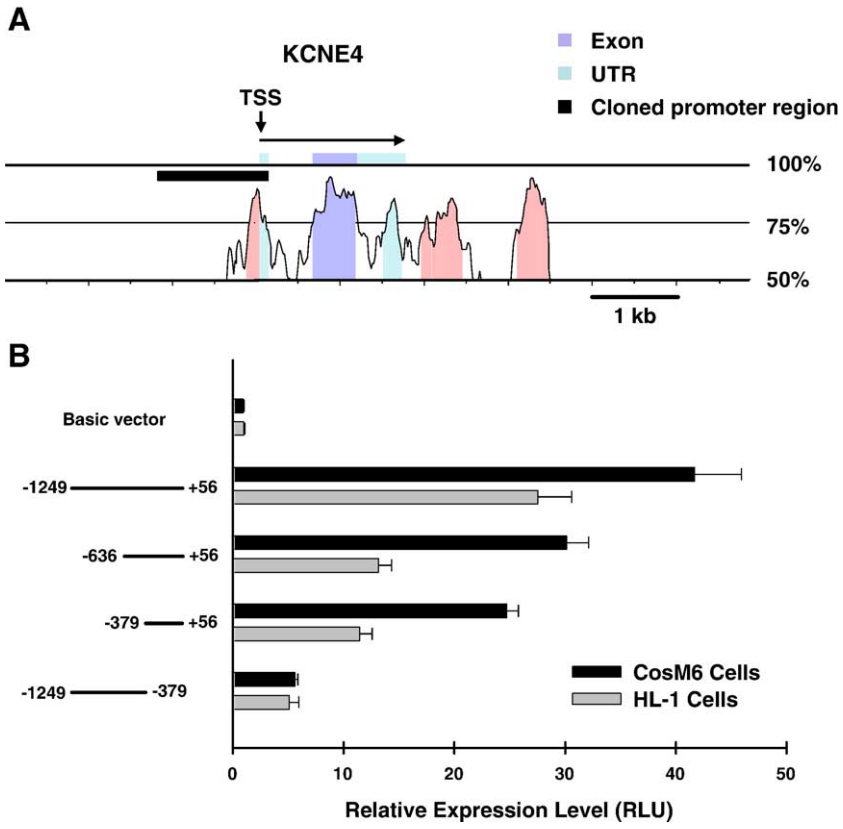

Fig. 5. KCNE4 sequence conservation and promoter analysis. Human and mouse genomic sequence surrounding the KCNE4 locus was compared using VISTA (A). Peaks represent regions of conservation greater than 50%, while regions of conservation of greater than 75% for 100+ bp are shaded orange. The locations of untranslated exons (blue) and the coding exon (purple) are depicted above the alignment. The full length cloned promoter region for KCNE4 is marked by the black box. Full length and truncated promoter regions (described relative to the transcription start site) for KCNE4 were subcloned into pGL3basic (Promega) and assayed for activity in CosM6 and HL-1 cells (B). Nonrecombinant vector (promoterless pGL3basic vector) expression was normalized to a value of one. Data were compiled from three independent transfections assayed in triplicate.

in the relative absence of other KCNE genes, suggesting that an I_{Ks} channel complex may be generated that has some role in uterine physiology.

Significant expression of *KCNE2* was limited to the colon and small intestine, while *KCNE3* was the most highly expressed family member across all tissues studied. In the kidney, colon, prostate, and leukocytes, high levels of *KCNE3* expression coincided with expression of *KCNQ1,* indicating the existence of the molecular components needed to generate a constitutively active channel as observed by coexpression of these two proteins in vitro [8]. However, in the liver and the ovaries significant expression of *KCNE3* occurred in the absence of KCNQ1, implying interaction with a different α subunit. Accordingly, in skeletal muscle KCNE3 interacts with Kv3.4, and a mutation in *KCNE3* has been implicated in familial periodic paralysis [17].

KCNE4 was expressed in a variety of human tissues. Previous studies demonstrated that KCNE4 can inhibit several potassium channel α subunits including KCNQ1, Kv1.1, and Kv1.3 [9,11,27], which are coexpressed in many of the same tissues as KCNE4. High levels of *KCNE4* expression were consistently accompanied by at least one additional member of the KCNE family, often *KCNE3*. KCNE3 and KCNE4 typically have stimulatory and inhibitory effects on α subunits, respectively, generating the potential for variable modulation of pore-forming subunits through expression of multiple accessory subunits [11].

High levels of *KCNE5* expression were observed in the brain, thymus, placenta, and ovaries, mostly in the absence of KCNQ1. These results suggest that KCNE5 interacts with another α-subunit. Notably, *KCNE5* is expressed at significantly higher levels in the brain compared to other KCNE family members. *KCNE5* is located on the X chromosome and recent evidence identified an unusually large number of genes coding for brain function on this chromosome [30].

Transcriptional control of KCNE genes

Mapping the TSS for each KCNE gene revealed that all family members other than *KCNE5* contain a noncoding exon in the 5′-UTR that is separated from the coding region by a large intron. Such noncoding exons are quite common, with a frequency of >35% in human genes [31]. The sequence surrounding each TSS closely resembled the *Inr* consensus sequence: Py-Py-A_{+1}-N-T/A-Py-Py (where A_{+1} is the transcription start site). The *Inr* element surrounds the TSS and is sufficient to direct accurate initiation in both TATA-containing and TATA-deficient promoters [32].

Alignment of human and mouse sequence in the vicinity of the coding region of each KCNE revealed segments of

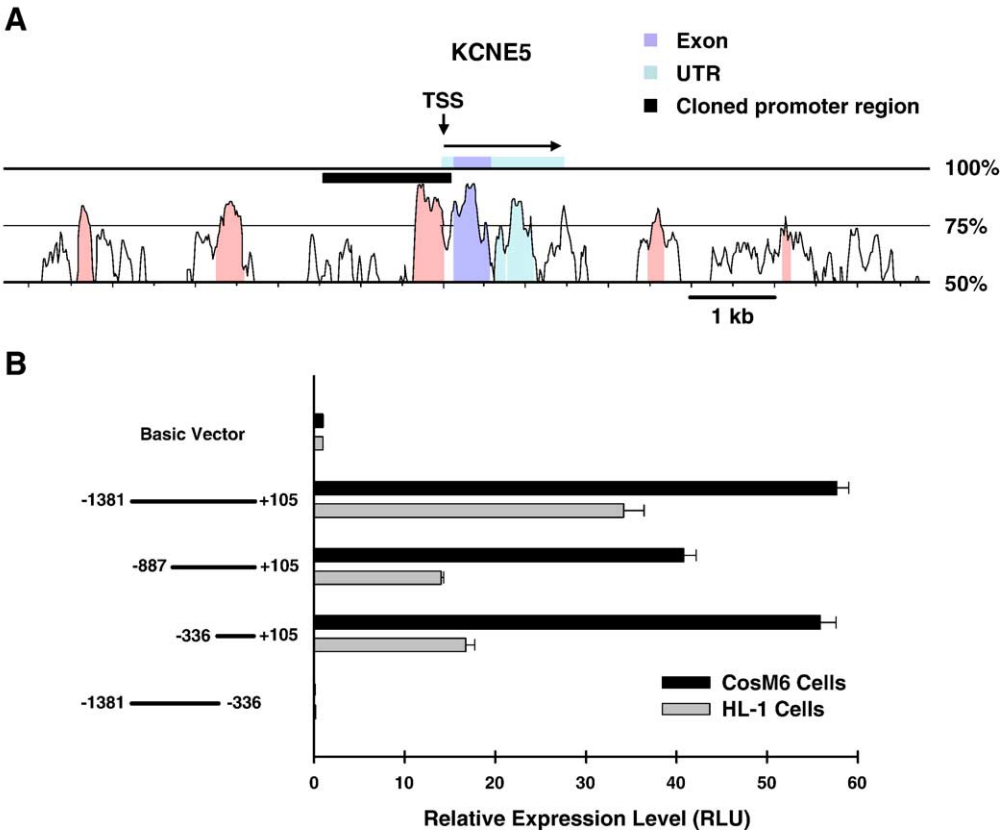

Fig. 6. KCNE5 sequence conservation and promoter analysis. Human and mouse genomic sequence surrounding the KCNE5 locus was compared using VISTA (A). Peaks represent regions of conservation greater than 50%, while regions of conservation of greater than 75% for 100+ bp are shaded orange. The locations of untranslated exons (blue) and the coding exon (purple) are depicted above the alignment. The full length cloned promoter region for KCNE5 is marked by the black box. Full length and truncated promoter regions (described relative to the transcription start site) for KCNE5 were subcloned into pGL3basic (Promega) and assayed for activity in CosM6 and HL-1 cells (B). Basic vector (promoterless pGL3basic vector) expression was normalized to a value of one. Data were compiled from three independent transfections assayed in triplicate.

increased conservation in noncoding regions that clustered around the experimentally defined transcription start sites. This conservation suggests that important regulatory elements are contained within the sequence. Prediction of regulatory motifs in silico has been successful for several genes [33–35]. Using this approach, we identified several predicted transcription factor binding sites immediately 5′ of the transcription start site for each KCNE gene (Table 1), whereas conserved binding sites were largely absent from intronic regions.

Several conserved muscle- and cardiac-specific transcription factors were identified in the promoter region of the cardiac-specific transcript KCNE1b. Serum response factor (SRF) may play a role in the commitment of cardiac progenitors and is abundant in embryonic and adult cardiac, skeletal muscle, and smooth muscle cells [36,37]. Additional evidence indicates that combinatorial interactions between SRF and GATA-6, also conserved in the KCNE1b promoter region, may facilitate expression of heart-restricted genes, implicating these factors in the cardiac-specific expression of KCNE1b [38]. The *KCNE4* promoter exhibited consensus CAAT and TATA boxes and two conserved MYOD binding sites, consistent with the high relative expression of *KCNE4* in skeletal muscle compared to the other KCNE genes. The *KCNE5* core promoter region exhibited conserved binding sites for SP1, GC, and AP2, as well as two neuronal transcription factors (OLF1 and ROAZ), which may influence the high relative expression of *KCNE5* in the brain.

Further studies will be necessary to determine the functional significance of these predicted transcription factor binding sites. In this study we took a conservative approach by annotating only those sites that were conserved between mouse and human and met a high stringency for matrix similarity. It is possible that other important binding sites exist that did not satisfy these stringent criteria.

The identification of the TSS also facilitated the testing of putative promoter regions surrounding the TSS containing conserved transcription factor binding sites for the ability to drive reporter activity. Significant luciferase activity was identified for all reporter constructs tested, except those for *KCNE2.* These results suggest that cloned genomic regions for *KCNE1* and *KCNE3–5* contain core promoter elements. It is likely that other regulatory elements such as enhancer and suppressor regions exist outside the core promoter for each of these genes. The absence of more distant regulatory regions controlling tissue-specific expression may help explain why reporter gene activity in transfected kidney-derived cells does not exactly parallel mRNA levels observed in kidney. Regardless, knowledge of the core promoter regions provides

a useful starting point for investigating tissue-specific expression and for screening KCNE genes for genetic variants that alter mRNA expression in disease susceptibility states.

In summary, we have characterized the 5′ genomic structure for each of the KCNE genes, identifying multiple human transcripts for *KCNE1* and *KCNE3*. We compared relative expression patterns of all KCNE genes across a variety of human tissues to characterize the complement of accessory subunits in each tissue. Expression results were consistent with many previous studies implicating KCNE genes in both normal physiology and disease and suggested additional human tissues in which KCNE genes may have important functions. We identified functional core promoter elements in genomic regions highly conserved between human and mouse for each of the KCNE genes, except *KCNE2*. These data provide highly valuable knowledge of core elements related to tissue-specific regulation and identify targets for studies of genetic variants in diseases associated with the KCNE genes.

Materials and methods

Transcription start site mapping

Total RNA was extracted from normal human ventricle using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA), quantified by UV spectrophotometry, and examined for integrity using denaturing agarose gel electrophoresis. Messenger RNA (poly(A)$^+$) was isolated from 10 μg of total RNA using Oligotex resin (Qiagen, Inc., Valencia, CA, USA). Ventricular poly(A)$^+$ RNA (250 ng) was processed according to the RLM-RACE protocol (Ambion, Inc., Austin, TX, USA) to create random hexamer-primed cDNA anchored to a known 5′ oligonucleotide sequence. Transcription start sites were identified by PCR amplification using a gene-specific reverse primer (located approximately 200 bp 3′ of the ATG start codon) and a forward anchor primer. Specific products were identified by Southern blot hybridization using biotinylated sense oligonucleotide probes (15 bp, beginning at the ATG start codon) and a chemiluminescence detection system (North2South; Pierce Biotechnology, Inc., Rockford, IL, USA). Southern blot positive products were subcloned into pCR2.1-TOPO (Invitrogen) and sequenced. Multiple independent clones (6–10) were sequenced for each identified transcript.

Real-time quantitative RT-PCR

Complementary DNAs synthesized using RNA isolated from normal human tissues were obtained from a commercial source (MTC Panels I and II; BD Biosciences–Clontech, Palo Alto, CA, USA). Each cDNA sample in these panels was synthesized using pooled RNA isolated from at least two normal adult Caucasians (males and females) and the concentration was standardized according to expression data of four housekeeping genes (α-tubulin, β-actin, GAPDH, phospholipase A2). Human uterine tissue was obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA, USA).

Fluorescently labeled TaqMan (Applied Biosystems, Foster City, CA, USA) probes for *KCNQ1* and *KCNE1–4* were designed to span an exon–exon boundary within the coding region and 5′-untranslated regions, respectively, while a probe for *KCNE5* targeted a continuous region of coding sequence in the single exon. We developed assays specific to KCNE1a and KCNE1b, but were unable to assay for expression of KCNE3a and KCNE3b separately because there were no distinct exon–exon boundaries. Primer sets amplifying 99- to 104-bp segments surrounding each probe were designed using PrimerExpress (Applied Biosystems). Sequences for all amplification primers and gene-specific probes were published previously [11]. No amplification was observed after 45 cycles of PCR in control reactions containing water as the template. For each tissue, results were compared to a gene-specific standard curve and normalized to expression of GAPDH, in the same samples. Template used for determining standard curves consisted of plasmid DNA containing the expected target sequence quantitated by PicoGreen fluorescence (Molecular Probes, Eugene, OR, USA).

Reporter-gene promoter assays

For each KCNE gene, a region surrounding the TSS was amplified by PCR from human genomic DNA. Putative promoter regions were subcloned into the promoterless luciferase vector, pGL3basic (Promega Corp., Madison, WI, USA), upstream of the firefly luciferase coding region. Nested truncations of each promoter construct were created by using native restriction endonuclease sites. Monkey kidney cells (CosM6) and murine cardiac myocytes (HL-1) [18] were seeded in 24-well plates and transfected with 1 μg of each promoter construct or the nonrecombinant pGL3basic vector. In the same cells, 0.1 μg of pRL-TK (Promega), containing the coding region of *Renilla* luciferase driven by the thymidine kinase promoter, was cotransfected in each well to provide an index of cell viability and transfection efficiency. Forty-eight hours after transfection, cells were washed in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and lysed. Cell lysates were assayed sequentially on a LumiCount luminometer (Perkin–Elmer, Meriden, CT, USA) for firefly luciferase activity followed by *Renilla* luciferase activity according to the Dual-Luciferase protocol (Promega). Firefly luciferase values were normalized to *Renilla* activity, and normalized values were scaled to depict fold increase in activity over the nonrecombinant pGL3b vector. Data were compiled from three independent transfections assayed in triplicate.

Annotation of transcription factor binding sites

Human and murine genomic sequences surrounding the coding region of each KCNE gene were compared using VISTA [19] to identify noncoding regions with nucleotide conservation greater than 75% over a 100-bp window. Conserved transcription factor binding sites in the vicinity of the transcription start site were predicted by rVista [20] utilizing the vertebrate TRANSFAC transcription factor library. Search parameters were optimized for identification of functional sites with a matrix similarity of 85% or greater to the consensus binding sequence of a given transcription factor.

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