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Alternative splicing contributes to K^+ channel diversity in the mammalian central nervous system

(genetic diversification/tissue-specific gene expression/developmental gene expression)

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ABSTRACT **In an attempt to define the molecular basis of the functional diversity of K^+ channels, we have isolated overlapping rat brain cDNAs that encode a neuronal delayed rectifier K^+ channel, K_v4, that is structurally related to the *Drosophila* Shaw protein. Unlike previously characterized mammalian K^+ channel genes, which each contain a single protein-coding exon, K_v4 arises from alternative exon usage at a locus that also encodes another mammalian Shaw homolog, NGK2. Thus, the enormous diversity of K^+ channels in mammals can be generated not just through gene duplication and divergence but also through alternative splicing of RNA.**

The electrical excitability of cells of the nervous system is regulated, in part, by voltage-sensitive K^+ channels (1). The structure of neuronal K^+ channels that have been cloned has been conserved from *Drosophila* to humans and consists of proteins containing six hydrophobic, putative transmembrane domains, one of which, S4, has been implicated in sensing changes in the membrane potential (2–4). Although they are structurally similar, these K^+ channels can be divided into four distinct classes by virtue of their sequence homology to channels encoded at the *Drosophila* Shaker, Shab, Shal, and Shaw loci (5–11). In *Drosophila*, diversity of the K^+ channels within a given class arises, in part, from differential splicing of RNA encoding the channels (5–11). In contrast, mammalian K^+ channel genes that have been described to date have no introns in the coding region (12–14) and the diversity of these channels appears to have resulted from gene duplication and divergence. We describe here, however, the cloning and expression of a cDNA encoding a mammalian Shaw-type K^+ channel, K_v4, which arises from alternative splicing of a transcript from a gene that also encodes NGK2 (15), another mammalian channel related to Shaw.‖

MATERIALS AND METHODS

K_v4 cDNA Clones. An oligo(dT)-primed cDNA library, constructed from mRNA isolated from the brains of 2-week-old rats, was screened using a probe prepared from clone K41 (12), encoding part of a Shaker-type channel, labeled to a specific activity of $>1 \times 10^9$ by random hexamer-primed labeling (17). The filters were hybridized at 60°C in 5× SSPE (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA), 5× Denhardt's solution, 0.5% SDS, and 1% glycerol with 200 μg of salmon sperm DNA per ml and probe at 1×10^6 cpm/ml and were washed to a final stringency of 0.2× SSPE/0.1% SDS at 22°C. A cDNA (5A) containing a 2.6-kilobase (kb) insert was isolated and subcloned into pBS+ for sequencing. To obtain the 3′ end of the coding region, another oligo(dT)-primed cDNA library, constructed from mRNA isolated from the brains of 14-day-old rats, was screened using an oligonucleotide probe (O123) that included bases 1260–1359 from 5A (Fig. 1). The probe was labeled to a specific activity of $>1 \times 10^8$ cpm/pmol by filling in two overlapping oligonucleotides with the Klenow fragment of DNA polymerase I and all four [α-^{32}P]dNTPs. Filters were hybridized at 42°C in 5× SSPE, 50% formamide, 5× Denhardt's solution, and 0.5% SDS containing 100 μg of salmon sperm DNA and probe at 3×10^6 cpm/ml and were washed to a final stringency of 0.1× SSC/0.1% SDS (1× SSC = 0.15 M NaCl/15 mM sodium citrate) at 65°C. The clone (273-3) containing the largest insert (1.6 kb) was subcloned into the *Eco*RI site of pKSII+.

Construction of a cDNA Containing the Full-Length K_v4 Open Reading Frame. Clone 5A in pBS+ was digested with *Hin*dIII followed by partial digestion with *Nco* I, and the 1.7-kb fragment corresponding to bases −431 to 1337 was isolated. The fragment was ligated to *Hin*dIII/*Nco* I-digested 273-3 to generate a clone containing the full-length open reading frame and 0.4 kb and 1.1 kb of 5′ and 3′ untranslated sequence, respectively.

Following base 570 (Fig. 1, first open arrow), the 5A cDNA contained a 72-base-pair (bp) insertion with termination codons in all three reading frames. The insertion was not present in the bulk of K_v4 mRNA as determined by Northern blot analysis and polymerase chain reaction (PCR) amplification (data not shown). To determine the correct sequence in this region, oligo(dT)-primed cDNA was amplified by PCR using primers upstream (position −50 to −32) and downstream (position 1748–1767) from the insertion, and the amplified products were cloned into pKSII+. The products of three independent amplification reactions were sequenced and none contained the insertion but, instead, had the sequence shown in Fig. 1. Since the sequence at the 5′ end of the insertion (GTAAG) exactly matches the consensus sequence for a splice donor (21), it is likely that the insertion is the product of an incomplete splicing event. To correct the sequence of clone 5A, the insertion was excised from the cDNA by digestion with *Nar* I and *Sfi* I and the region was replaced with the corresponding fragment from PCR products that did not contain the insertion.

The extensive 5′ and 3′ untranslated regions were truncated by the PCR to remove sequences that might interfere with expression in the oocyte. The 5′ PCR primer contained an *Eco*RI site and seven bases of the translational initiation

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. M37845).

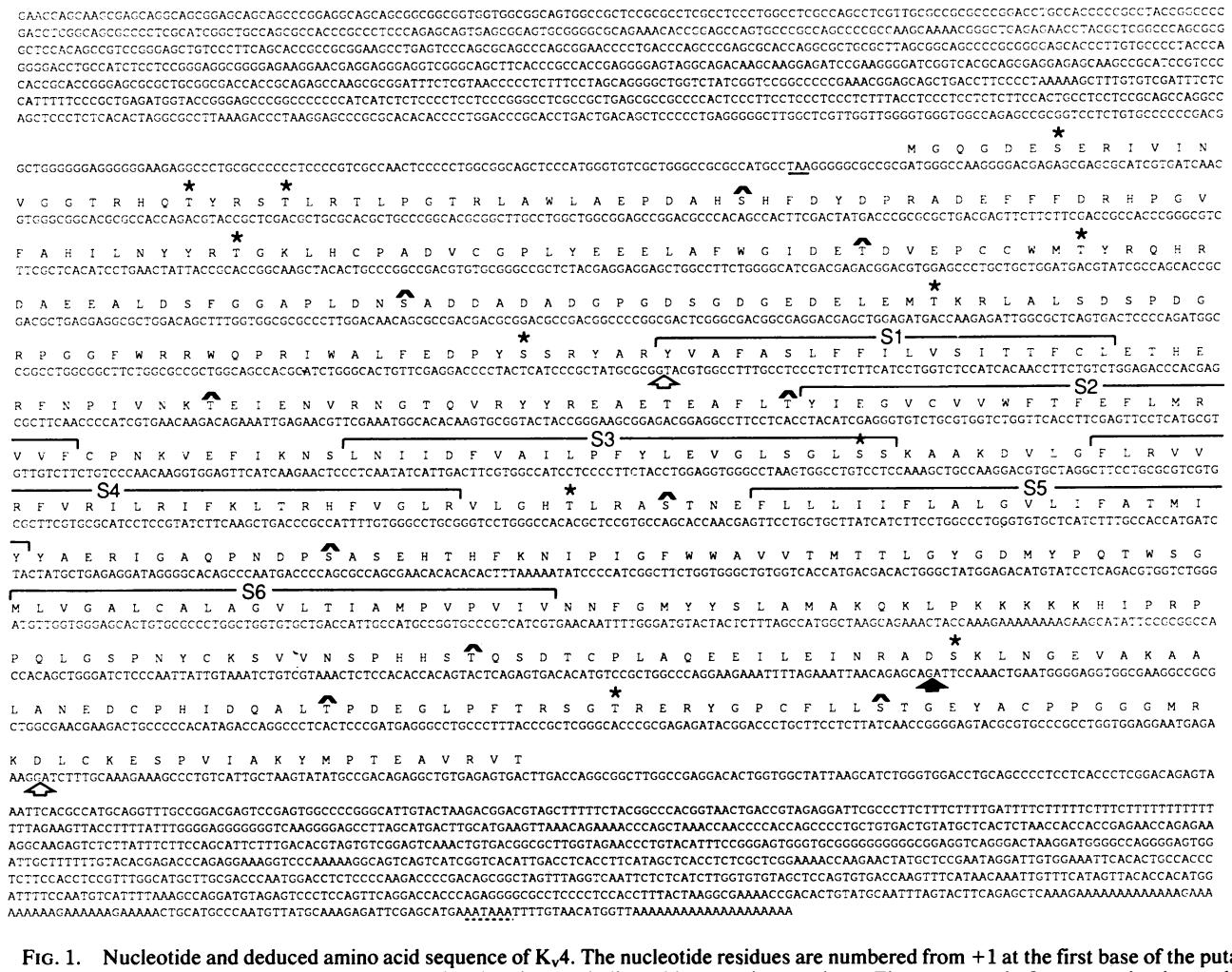

FIG. 1. Nucleotide and deduced amino acid sequence of K_v4. The nucleotide residues are numbered from +1 at the first base of the putative initiation codon and nucleotides in the 5′ untranslated region are indicated by negative numbers. The upstream in-frame termination codon is underlined. The hydrophobic, putative transmembrane, regions are overlined and consensus sites for phosphorylation by casein kinase II (^) (18) and protein kinase C (*) (19, 20) are indicated. Splice junctions that have been identified by comparison with the sequence of the genomic DNA clones are indicated by arrows; the splice junction that is the point of divergence of NGK2 and K_v4 is marked by a solid arrow. A consensus signal for polyadenylylation (16) is underlined with a dotted line.

consensus sequence (24) upstream from Met-1. The 3′ primer was located just downstream from the termination codon and contained a *Hin*dIII site. The PCR products were digested with *Hin*dIII and *Eco*RI and the insert was ligated into *Eco*RI/*Hin*dIII-digested pGEM-A (12). The construct was sequenced in its entirety on both strands and found to contain no errors that affected the amino acid sequence of the protein.

Northern Blot Analysis of RNA. Poly(A)$^+$ RNA (6 μg), purified from rat tissues by standard methods (22), was analyzed by Northern blotting as described (12). Probes O384 (Fig. 1, bases 1828–1903), O123 (see above), or O790 (ref. 15; bases 1523–1567) were labeled to a specific activity of $>1 \times 10^8$ cpm/pmol as described above. An oligonucleotide probe for actin (ref. 23; bases 259–287) was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Blots of mRNA from different tissues or from different ages were hybridized at 42°C in 50% formamide, 5× SSPE, 5× Denhardt's solution, and 0.5% SDS containing 50 μg of *Escherichia coli* tRNA per ml and were washed to a final stringency of 0.1× SSC/0.1% SDS at 50°C. Blots of rat brain mRNA probed with region-specific oligonucleotides O123, O384, or O790 were hybridized as above and were washed to a final stringency of 0.1× SSC/0.1% SDS at 50°C (O384, O790) or at 65°C (O123).

Isolation of K_v4 Genomic Clones. A size-selected rat genomic DNA library in λCharon 4A (Clontech) was screened with probes derived from the K_v4 sequence. The library was probed with a restriction fragment of clone 5A that contained the coding sequence from just upstream from S2 to just downstream of S6 (bases 681–1361). The probe was labeled by random hexamer priming (17), and the filters, which contained DNA from 5×10^5 plaques, were hybridized at 42°C in 5× SSPE, 5× Denhardt's solution, and 0.5% SDS containing 100 μg of salmon sperm DNA and probe at 1×10^6 cpm/ml. They were washed to a final stringency of 0.1× SSC/0.1% SDS at 65°C. One positively hybridizing plaque contained an insert of 15 kb, of which a 1.4-kb *Eco*RI fragment hybridized to the probe; the fragment was subcloned into pKSII+ and sequenced. A second probe, O384, was labeled as above and was used to screen 1×10^6 plaques. One of the positive plaques contained an insert of 10 kb, of which a 9-kb *Eco*RI fragment hybridized to the probe. The *Eco*RI fragment was subcloned into pKSII+ and the clone was sequenced using primers from K_v4 to determine the boundaries of the exons.

RESULTS

cDNA Isolation and Sequence Analysis. Clone K41, encoding part of a Shaker-type K^+ channel (12), was used to screen a rat brain cDNA library as described and a clone (5A) was isolated that encodes the amino terminus and hydrophobic domains of a protein with homology to the *Drosophila* Shaw K^+ channel (9–11). To obtain the carboxyl terminus of the protein, another

cDNA library was screened with an oligonucleotide probe derived from the 3′ end of 5A. A clone was isolated that overlapped 5A and extended 1.5 kb in the 3′ direction to a poly(A)$^+$ tail. The two cDNAs were digested at a restriction site in the region of overlap and ligated together to construct K_v4, encoding the entire open reading frame (Fig. 1).

We have designated Met-1 as the start of translation because it is the first methionine downstream from an in-frame termination codon, and sequence surrounding it (GGCGCCGCGATGG) agrees well with the consensus for translation initiation (GCCGCCRCCATGG; ref. 24). The open reading frame encodes a protein of 585 amino acids with a predicted size of 65,860 daltons. K_v4 has an unusually long 5′ untranslated region of 1161 bp and a 1061-bp-long 3′ untranslated region that contains a consensus poly(A) addition signal (AATAAA; ref. 16) 21 bp upstream from the poly(A) tail (150–200 adenosines in our cDNA). Based on the size of the mRNA to which the clone hybridizes (see below), K_v4 is nearly full length.

The deduced K_v4 protein sequence is 48% identical to the *Drosophila* Shaw K^+ channel (9–11) and 74% identical to RKShIIIA (25), a rat brain Shaw-like channel. The K_v4 protein is even more similar to NGK2, a Shaw-type K^+ channel cloned from NG108-15 cells (15). The K_v4 sequence differs from the published sequence of NGK2 at only 50 of the first 1504 bp of the coding sequence, and none of these nucleotide changes affects the amino acid sequence. We have cloned NGK2 from rat brain mRNA by amplification of first-strand cDNA by the PCR using a NGK2-specific 3′ primer (ref. 15; bases 1569–1586) and a 5′ primer from the region common to NGK2 and K_v4 (Fig. 1, bases 1109–1126). The sequences of rat NGK2 and K_v4 are identical up to base 1504 (amino acid 501) but thereafter, they diverge completely. After the point of divergence, K_v4 contains an additional 84 amino acids, whereas NGK2 contains only 10. Like other neuronal voltage-gated K^+ channels, K_v4 contains six hydrophobic, putative transmembrane, domains including a S4-type region that contains six positively charged residues rather than the seven found in the Shaker-type channels (2, 12–14, 26–30).

K_v4 shares with NGK2 the two consensus N-linked carbohydrate addition sites (at Asn-220 and Asn-229; ref. 31), nine potential sites for phosphorylation by protein kinase C (19, 20), and eight potential sites for phosphorylation by casein kinase II (18). In its longer carboxyl-terminal domain, however, K_v4 contains two additional consensus sites for phosphorylation by casein kinase II and two for phosphorylation by protein kinase C; the carboxyl-terminal protein kinase C sites also fit the consensus for Ca^{2+}/calmodulin-dependent protein kinase (32).

Expression of K_v4 in *Xenopus* Oocytes. K_v4 encodes a functional channel when expressed in *Xenopus* oocytes. One day after injection of the cRNA, large noninactivating outward currents could be measured when the oocyte membrane was depolarized from −80 mV to potentials of greater than −20 mV (Fig. 2 *Inset*). The K_v4 current was K^+ selective (data not shown) and activated at relatively depolarized potentials. The conductance–voltage relationship for the currents was well fit by a Boltzmann isotherm with a midpoint of activation at 15 mV and a slope factor of 10 (Fig. 2). Charybdotoxin, noxiustoxin, and mast cell degranulating peptide had no effect on K_v4 currents at concentrations as high as 1 μM. Tetraethylammonium ions and 4-aminopyridine were effective blockers with IC_{50} values of 150 μM and <300 μM, respectively.

Tissue Distribution and Developmental Regulation of K_v4 mRNA Expression. The pattern of expression of K_v4 RNA was examined by Northern blot analysis as described. Blots of poly(A)$^+$ RNA isolated from neonatal, 2-week-old, or adult rat brain were hybridized to a K_v4-specific oligonucleotide probe, O384, which is derived from the 3′ untranslated region of K_v4 (see Fig. 5 for the position of the probe). The major species detected was 4.5 kb; another less abundant doublet of 10–11 kb was seen in some RNA preparations (Fig. 3*A*). The abundance of the major species increased developmentally, in a pattern of expression similar to that found previously for K_v1 and K_v2 mRNAs in brain (12). Unlike K_v1, however, K_v4 was not found in the adult rat in any tissue other than the brain (Fig. 3*B*).

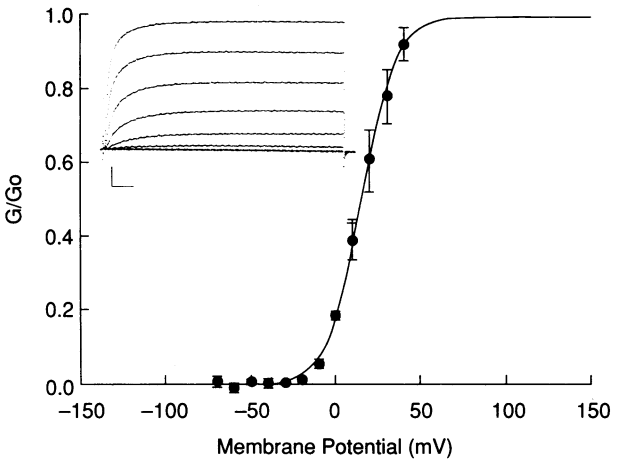

FIG. 2. Normalized conductance–voltage relationship for K_v4 currents. Conductance values were obtained as described (12). The curve represents the nonlinear least squares fit of the Boltzmann isotherm: $G = G_{max}/[1 + \exp(V - V_{1/2}/a)]$. (*Inset*) Voltage clamp currents obtained by depolarizing the membrane for 200 msec from a holding potential of −80 mV to +40 mV in 10-mV steps. (Scale bars = 4 μA and 20 msec.)

Expression of Multiple, Related Transcripts in the Brain. As discussed above, NGK2 and K_v4 are closely related in sequence. To determine whether there are additional related species in rat brain mRNA, an oligonucleotide probe (O123) containing sequences derived from a region common to

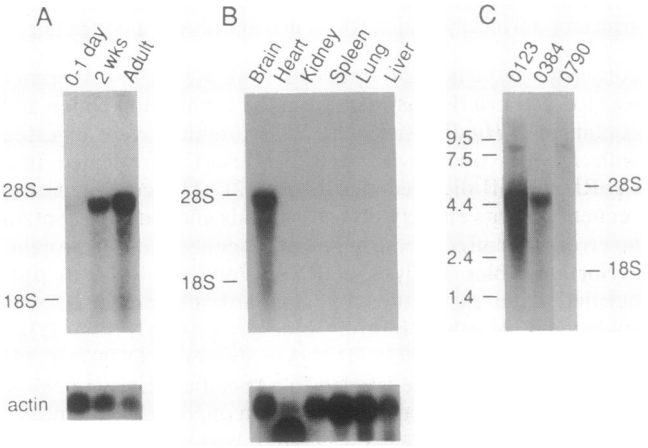

FIG. 3. Northern blot analysis of K_v4 transcripts. (*A*) Developmental expression of K_v4. Northern blots of poly(A)$^+$ RNA from the brains of rats of the indicated ages were hybridized to the K_v4-specific probe O384 (bases 1828–1903) and washed as described in the text. The blot was reprobed for actin mRNA (bottom) as a control for the amount of RNA loaded on the gel. (*B*) Tissue distribution of K_v4 mRNA. Northern blots of poly(A)$^+$ RNA from the indicated tissues were hybridized to O384 as above. The actin controls are shown below. (*C*) Detection of multiple transcripts related to K_v4. Northern blots of poly(A)$^+$ RNA from adult rat brain were hybridized with O123 (a probe containing sequence from the region common to NGK2 and K_v4), O384 (a K_v4-specific probe), or O790 (a NGK2-specific probe). Sizes are indicated in kb.

NGK2 and K_v4 was used as a probe on Northern blots of adult rat brain mRNA. The probe hybridized at high stringency not only to the 4.5-kb and 10- to 11-kb species but also to transcripts of 6 kb and 8 kb, indicating the presence of a family of related mRNAs (Fig. 3*C*). The 4.5-kb K_v4 transcript was at least 10-fold more abundant than the 8-kb and the 10- to 11-kb transcripts. A probe unique to the 3′ untranslated region of NGK2 (O790) hybridized to the transcript of 8 kb, as reported for RNA from NG108-15 cells (15), and to the 10- to 11-kb transcripts. The presence of K_v4 and NGK2 mRNAs was also confirmed by amplification of each from brain cDNA using a 3′ primer specific for either NGK2 (ref. 15; bases 1569–1586) or K_v4 (Fig. 1, bases 1748–1767) and a 5′ primer in the region of common sequence (bases 1109–1126) in the PCR. Both expected products were amplified in this manner (data not shown). Probes specific for each mRNA do not cross-hybridize with the other transcript, indicating that the 8-kb (NGK2) and 4.5-kb (K_v4) RNAs do not have a precursor–product relationship.

Characterization of the K_v4 Gene. The multiple mRNA species detected with the probe from the region common to NGK2 and K_v4 could arise either by alternative splicing of a primary transcript or by transcription from separate but homologous loci. To differentiate between these two possibilities, the structure of the genes encoding the channels was analyzed. A Southern blot of rat genomic DNA digested with several enzymes was probed with O123, the probe in the region common to NGK2 and K_v4, and was washed at high stringency. Only a single restriction fragment hybridized to the probe in digests with four different enzymes (Fig. 4), indicating that the region encoding the common sequence is represented only once in the rat genome.

To further investigate the structure of the gene encoding the K_v4-related mRNAs, a rat genomic DNA library was screened with a probe that encompassed the hydrophobic domains S2–S6 of K_v4. The probe hybridized to a genomic clone (λG1) that is identical in sequence to K_v4 from the beginning of S1 to the 3′ end of the clone just downstream from S6 (see Fig. 5). The genomic and cDNA sequences diverge upstream from base 571 in the cDNA. Based on Southern blot analysis, the additional 13.5 kb of DNA in λG1 contained no other sequence found in the cDNA. At the break of colinearity, the genomic DNA sequence (CCCA-CAG) agrees well with the consensus sequence for a splice acceptor site (YYYNCAG; ref. 21). Taken together, the data indicate the presence of a large intron upstream from the splice site at base 571.

The structure of the gene downstream from S6 was determined in two ways: by amplification of that region from genomic DNA using the PCR and by isolation of an additional genomic DNA clone containing K_v4 sequences. A 5′ primer in the region shared by K_v4 and NGK2 (Fig. 1, bases 1109–1126) and a 3′ primer in the region unique to either NGK2 (ref. 15; bases 1569–1586) or K_v4 (bases 1563–1580) were used to amplify genomic DNA. Sequencing of the PCR product obtained using the NGK2-specific 3′ primer (clone PCR-G3) revealed that the exon encoding the 3′ end of NGK2 is contiguous with the exon encoding the core S1–S6 regions (Fig. 5). The product does not represent amplification of trace amounts of contaminating NGK2 cDNA since PCR amplification from genomic DNA using a 5′ primer in the intron preceding S1 and the NGK2-specific 3′ primer gave a product that spans the core (S1–S6) exon (clone PCR-G4, Fig. 5). In addition, the sequence of NGK2 at the point of divergence (AG GTAGG) agrees well with the splice donor consensus sequence (AG GTAAG; ref. 21), suggesting that it could function as a legitimate splice site. No product was obtained with the K_v4-specific 3′ primer, suggesting that the priming sites are too far separated in genomic DNA to allow amplification of the intervening region. A genomic clone (λG2, Fig. 5) containing two additional exons encoding the unique 3′ end of K_v4 was, however, isolated from a λ library using a K_v4-specific probe, O384 (see Fig. 5). One exon has its 5′ end at the point of divergence of K_v4 and NGK2 at position 1505 and extends 3′ only 187 bp. The second exon extends from base 1693 of the cDNA 3′ to the poly(A) site at base 2817 (see Fig. 1). The sequence of the intron separating these two exons has not been determined.

DISCUSSION

The data presented here demonstrate that a mammalian Shaw-type K^+ channel, K_v4, arises by alternative splicing of a transcript from the same gene that encodes NGK2. mRNAs encoding both splicing variants are expressed in rat brain and can be detected by Northern blot analysis (Fig. 3) and PCR amplification. Both mRNAs encode functional channels, as demonstrated by their expression in *Xenopus* oocytes (Fig. 2 and ref. 15). The amino acid and nucleotide sequences of both

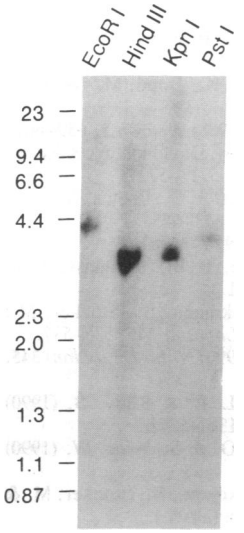

FIG. 4. Southern blot analysis of rat genomic DNA. Southern blots of rat genomic DNA digested with the indicated enzymes were hybridized to probe O123 (which contains sequence common to K_v4 and NGK2) and washed to a final stringency of 0.1× SSC/0.1% SDS at 65°C. Only a single restriction fragment hybridizes to the probe in each digest, indicating that the region of DNA common to NGK2 and K_v4 is represented only once in the genome. Sizes are indicated in kb.

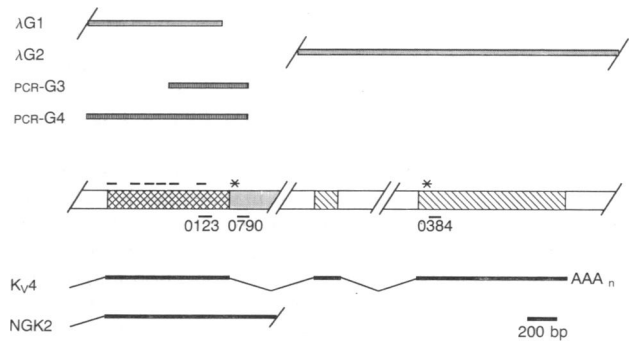

FIG. 5. Partial structure of the K_v4/NGK2 gene. Two λCharon 4A genomic clones encoding part of K_v4 (λG1 and λG2) are shown at the top, above their corresponding sequences. PCR-G3 and PCR-G4 are clones generated from genomic DNA by PCR. They span the K_v4/NGK2 alternatively used splice site. The composite map of the K_v4/NGK2 gene is shown schematically. Exons are shaded according to their usage: the exon that is common to NGK2 and K_v4 is crosshatched (▨), the NGK2-specific exon is stippled (▩), and the K_v4-specific exons are shaded diagonally (▧); introns are unshaded. The termination codons at the end of the NGK2 and K_v4 open reading frames are indicated by asterisks above the map. The positions of the hydrophobic domains S1–S6 are indicated by the black bars above the map. The positions of oligonucleotide probes used in Northern and Southern blots are shown below the map. The splicing pattern of the transcripts for NGK2 and K_v4 are shown at the bottom. Heavy bars indicate sequences found in the cDNA clones. The length of the 3′ untranslated region of NGK2 has not yet been determined.

channels are identical in the amino-terminal and putative transmembrane domains of the proteins, after which they diverge completely. The exon encoding the common core of each of the variants is represented only once in the rat genome (Fig. 4), and the point of divergence of the K_v4 and NGK2 sequences coincides with a splice junction at the 3′ end of this exon. In rat genomic DNA, the nucleotides encoding the unique carboxyl terminus of NGK2 are contiguous with the exon encoding the hydrophobic domains S1–S6 of NGK2 and K_v4. Two exons encoding the unique carboxyl-terminal domain of K_v4 are located farther downstream in the genomic DNA. From these data, we conclude that NGK2 and K_v4 are the products of alternative splicing of RNA transcribed from a single gene.

The alternative use of splice junctions has been demonstrated in several genes that use either a read-through of a splice junction or alternate 3′ exons to encode multiple mRNA species (33–35). We have demonstrated a similar splicing pattern in the K_v4/NGK2 gene. Unlike the Shaker locus, where different splice variants encode channels with different functional properties (36, 37), the functional consequences of the splicing of the K_v4/NGK2 gene are not yet known. It is interesting to note, however, that the longer variant of the channel (K_v4) has a carboxyl-terminal domain with additional potential sites for phosphorylation by protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase, and casein kinase II. The alternative splicing may, therefore, generate two forms of the channel with different susceptibilities to regulation by cellular kinases.

Several mechanisms have been demonstrated to contribute to the generation of diversity in mammalian K^+ channels. (*i*) Gene duplication and nucleotide sequence divergence have generated distinct structural classes of K^+ channels and more subtle variants within each class (2, 9–15, 25–30, 38). The different channels vary in their cellular distribution *in vivo* (39) and in their activation and inactivation kinetics, voltage dependencies, and pharmacological sensitivities when expressed *in vitro* (2, 11, 12, 14, 15, 25–30, 38). (*ii*) Although each distinct protein is capable of forming functional K^+ channels when expressed by itself, it has been demonstrated that, within a class, different channel proteins can assemble into heteromultimeric species with properties that are intermediate to those of each of the individual components (40–43). (*iii*) Posttranslational modifications of the assembled channel proteins may alter their functional properties (44, 45). Our findings demonstrate that another mechanism, that of alternative splicing of transcripts from a single gene, can also add to the diversification within at least one class of mammalian K^+ channels. These mechanisms, in concert, could provide pathways for the synthesis and modulation of a large number of functionally distinct K^+ channels. The selective expression and modulation of different populations of these channels in different cell types would enable precise control over each cell's electrical excitability and response to stimuli.

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