Volume 301, number 2, 168–172 FEBS 10934 April 1992

I_{SK}, a slowly activating voltage-sensitive K^+ channel

Characterization of multiple cDNAs and gene organization in the mouse

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Received 3 February 1992; revised version received 3 March 1992

mI_{SK} is a protein consisting of 129 amino acids with a single putative transmembrane domain. The injection of mI_{SK} cRNA into *Xenopus* oocytes directs the expression of a voltage-gated K^+ current. A heart mRNA blot probed with mI_{SK} DNA revealed at least two transcripts. The messenger diversity of mI_{SK} was investigated by cloning and characterization of multiple cDNAs of one genomic clone, and by performing primer extension experiments. All cDNAs characterized have the same protein-coding sequence, and heterogeneity of the transcripts arises from alternative splicing, and multiple sites of transcription start and polyadenylation. I_{SK} is encoded by a single gene in the mouse genome. The gene organization reveals the existence of an exon containing the whole protein-coding sequence and of two alternative exons corresponding to the 5′ untranslated sequences. We failed to detect the presence of another exon capable of extending the protein-coding sequence. The diversity of mI_{SK} messengers is not associated with a diversity of the mI_{SK} protein.

Delayed rectifier

1. INTRODUCTION

The I_{SK} protein was originally cloned in rat kidney by functional expression [1]. This protein consists of 130 amino acid residues and has a single putative transmembrane domain. In *Xenupus* oocytes the I_{SK} protein mRNA directs the expression of a very slowly activating voltage-dependent and K^+-selective outward current. The localization of the rat I_{SK} protein in epithelial cells supports the view that this protein plays a role in the epithelial K^+ transport [2].

Expression of the I_{SK} protein is not restricted to epithelia. It is also present in myometrium [3,4], in heart [4,5] and in lymphocytes [6]. In mouse heart, the I_{SK} message is particularly abundant at the early stages of development. Northern blot analysis of neonate mouse heart mRNAs with an I_{SK} cDNA probe shows at least two hybridizing signals, indicating the presence of various related mRNAs species. It was important to know whether this mRNA diversity reflects an I_{SK} protein diversity. This paper analyzes the diversity of I_{SK} cDNAs and characterizes the gene organization. Results suggest that no more than one I_{SK} protein species is expressed in mouse tissues.

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2. MATERIALS AND METHODS

2.1. *Northern blot analysis and isolation of cDNA clones*

Northern blot analysis was carried out as described previously [5]. An oligo(dT)-primed cDNA library, derived from poly(A)$^+$ RNA isolated from newborn mouse hearts was constructed by cloning into the *Eco*RI site of phage λZAPII (Stratagene). Recombinant phages were screened by plaque hybridization with the previously characterized mouse I_{SK} cDNA clone C_{III} [5]. The probe was ^{32}P-labelled by random primer synthesis (Amersham). Hybridization-positive clones were isolated from about $3 \cdot 10^5$ clones. The λZAPII recombinant phages were converted to plasmid cDNA by rescue excision (Stratagene). The cDNA inserts were characterized by complete or partial sequencing [7] and by restriction enzyme analysis.

2.2. *Isolation of genomic clones*

The labelled cDNA insert of C_{III} was used to screen a mouse genomic DNA library constructed into λEMBL3 phage vector. After screening approximately $2 \cdot 10^5$ plaques, 22 positive clones were isolated. One clone, G_{SII}, containing a genomic insert of 16 kb and covering the mouse I_{SK} gene was characterized. The physical map of the I_{SK} gene was established by restriction mapping, Southern blot hybridization and sequencing of selected restriction fragments of G_{SII} which had been subcloned into pBluescript SK$^-$ plasmid (Stratagene).

2.3. *Genomic blot hybridization*

Mouse genomic DNA was prepared from C_2C_{12} cells [8] and digested with the restriction enzymes *Bam*HI, *Apa*I and *Eco*RI. After electrophoresis in 0.8% agarose gel and transfer to Hybond-N filters (Amersham), the blots were probed with ^{32}P-labelled insert of C_{III} and with a ^{32}P-labelled 0.95 kb *Pst*I fragment of C_{SII} (see Fig. 4) corresponding to the 3′-untranslated region of the clone.

2.4. *Primer extension analysis*

Primer extension analysis was performed using total RNA isolated from mouse neonate heart as template. Two oligonucleotides were 5′-end labelled with [γ-^{32}P]ATP using T_4 kinase and served as primers: PR1 (22-mer), 5′-GAATTGGGCAGGCTCATCCTGG-3′, comple-

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mentary to nucleotides −5/+17 from the initiation codon of I_{SK} mRNA and PR2 (29-mer), 5′-TGCCTGGTCCTGGACGGTGCAGAGG-3′, complementary to nucleotides −43/−64 (see Fig. 6). For each experiment, approximately $5 \cdot 10^5$ cpm of primer was incubated with 5 μg of RNA for 5 min at 85°C and subsequently annealed at 42°C overnight in 80% formamide, 0.4 M NaCl, 1 mM EDTA, pH 8.0 and 40 mM PIPES, pH 6.4. Reverse transcription was performed according to Sambrook et al. [8]. The final products were analyzed by electrophoresis in 7 M urea, 6% polyacrylamide gel.

3. RESULTS

3.1. *Isolation and characterization of mI_{SK} cDNAs*

Northern blot analysis of neonatal mouse heart mRNA hybridized with a probe containing the entire coding sequence of the mI_{SK} protein indicates the presence of at least two bands of size estimated to 0.9 and 3.4 kb (Fig. 1). The presence of these different transcripts in mouse heart prompted us to isolate new cDNAs corresponding to these mRNAs. A mouse neonate heart cDNA library was screened at high stringency. Approximately 50 positive clones were obtained from $6 \cdot 10^5$ plaques, and eight cDNAs were characterized. Sequences are presented in Fig. 2. It appears that the main difference between these clones consists in the length of their 3′ untranslated terminal sequence which varied from 83 (clone C111) to 1790 (clone C511) base pairs. Our analysis showed that at least 3 alternative polyadenylation sites are used to generate the different 3′ termini. When the 3′ untranslated sequence of the C511 clone was used to probe the heart mRNAs only the large-size transcripts were revealed confirming that these large mI_{SK} transcripts are generated by an elongation in their 3′ untranslated sequences. Minor differences were also found in the 5′ termini which probably

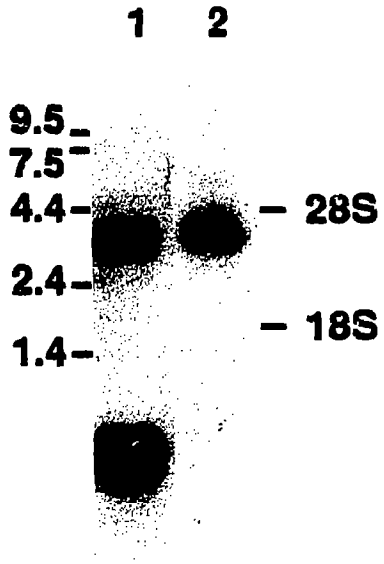

Fig. 1. Specific expression of I_{SK} in mouse heart. Poly(A)$^+$ (5 μg) from neonate mouse heart were probed with ^{32}P-labelled insert of C111 clone (lane 1) or by ^{32}P-labelled 0.95 kb *Pst*I fragment of C511 clone (lane 2).

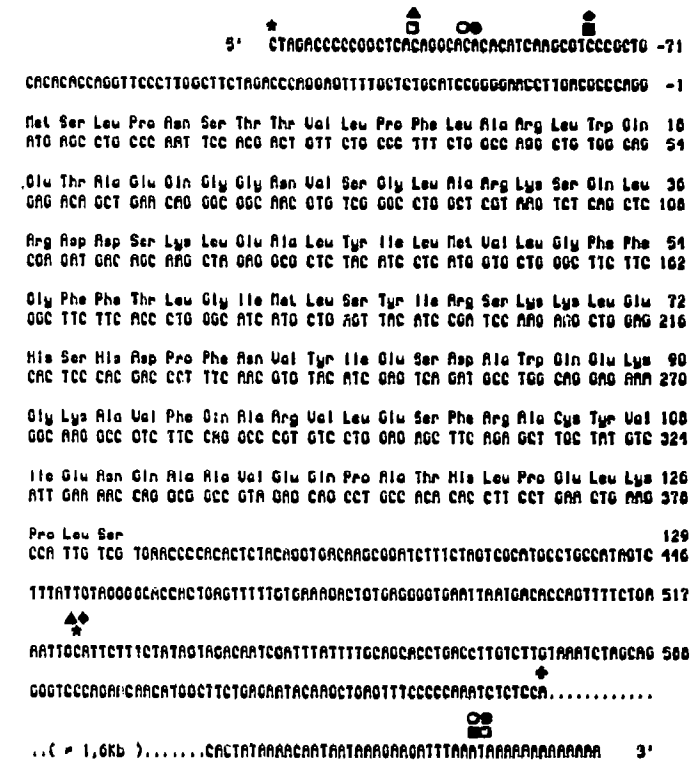

Fig. 2. Nucleotide sequence of mI_{SK} cDNAs. Nucleotides are numbered from the first residue of the translation initiation codon. The symbols indicated are as follows: ★ ▲ □ ○ ● ♦ the 5′ and 3′ termini of the cDNA insert sequences of clones C371, C381, C361, CI521, C511, C391 and C111, respectively. The symbol + corresponds to the 3′ termini of the cDNA insert of the clone C311.

reflect the use of multiple transcription start sites (see below).

Despite their differences, these 8 cDNAs encode an identical protein. Moreover, cDNA synthesized with the shorter (C111) or the longer (C511) clone as template were both capable of inducing the same K^+ channel activity when expressed in *Xenopus* oocytes (data not shown).

3.2. *Physical map of the mI_{SK} protein gene*

In order to determine the structural organization of the mouse I_{SK} gene, we isolated a genomic clone denoted G511. The 16 kb insert was digested by several restriction enzymes, and fragments hybridizing positively with the C111 insert were identified, sub-cloned and subjected to sequence determination.

It was found that the whole protein-coding sequence as well as the 3′ untranslated termini of cDNAs are encoded by a single exon (exon 2) whereas the 5′ termini upstream of the residue 39 are encoded by an other exon, exon 1, located approximately 10.3 kb upstream from exon 2. All the clones isolated in this study showed the same exon 1 with variable lengths corresponding to multiple transcription start sites (Fig. 2).

In a recent study, the rat I_{SK} protein gene organiza-

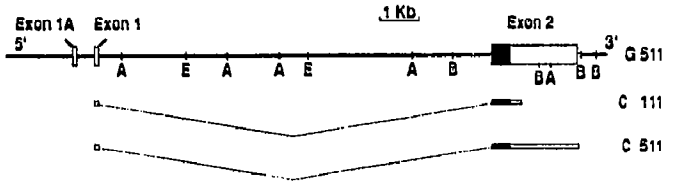

Fig. 3. Physical map of the I_{SK} genomic clone G511. The exons are boxed. Their location was assigned by comparison of the sequence determinations for the genomic insert of G511 to those of cDNA inserts of clones C111 and C511. The protein coding region is depicted by solid boxes. The restriction enzyme sites shown are A: *Apa*I, B: *Bam*HI, E: *Eco*RI.

tion has been reported [9]. It was found that the rat I_{SK} protein mRNAs were initiated from 2 alternative exons, called exon 1A and exon 1B. Our mouse genomic clone contains a nucleotide sequence that is 88% homologous with the rat exon 1A. This sequence is located approximately 400 bp upstream of exon 1. The sequences at the exon–intron boundaries (exon 1/intron/exon 2, putative exon 1A/intron/exon 2) are consistent with the consensus sequence of the splice junction [10]. The initiation of transcription from this putative exon 1A is discussed below. The physical map of the mI_{SK} protein gene and nucleotide sequences of exons and their surrounding regions are presented in Figs. 3 and 4.

3.3. *Genomic Southern analysis*

Blots of mouse genomic DNA digested with several restriction enzymes were probed with the C111 (Fig. 5) insert. The revealed bands have the sizes predicted from the partial restriction map of the G511 genomic clone. Interestingly, no other bands were observed under low-stringency conditions of hybridization. Therefore, it was concluded that there is only one copy of the I_{SK} gene in the mouse haploid genome, and that this gene does not belong to a family of related genes. This result was fully confirmed by hybridizing the blots with a probe derived from a 3′ untranslated part of the C511 clone (not shown).

3.4. *Characterization of 5′ termini of mI_{SK} mRNAs*

Primer extension analysis was performed in order to identify the 5′ termini of I_{SK} mRNAs. Two oligonucleotides were utilized for reverse transcription of mouse neonate heart total RNA. One primer was complementary to a nucleotide sequence of exon 2 (residues −5 to +17 around the AUG codon), whereas the other primer PR2 was complementary to a sequence of putative exon 1A (residues −43 to −64 upstream of the AUG codon) (Fig. 6).

The extension from PR1 gave rise to 4 major bands corresponding to sizes of 95, 110, 132 and 152 nucleotides (Fig. 6). The extended DNAs of 95, 110 and 132 nucleotides have sizes in good agreement with those predicted from the cDNA clones C111, C511 and C371, respectively. The PR1 extended DNA of 152 nucleotides

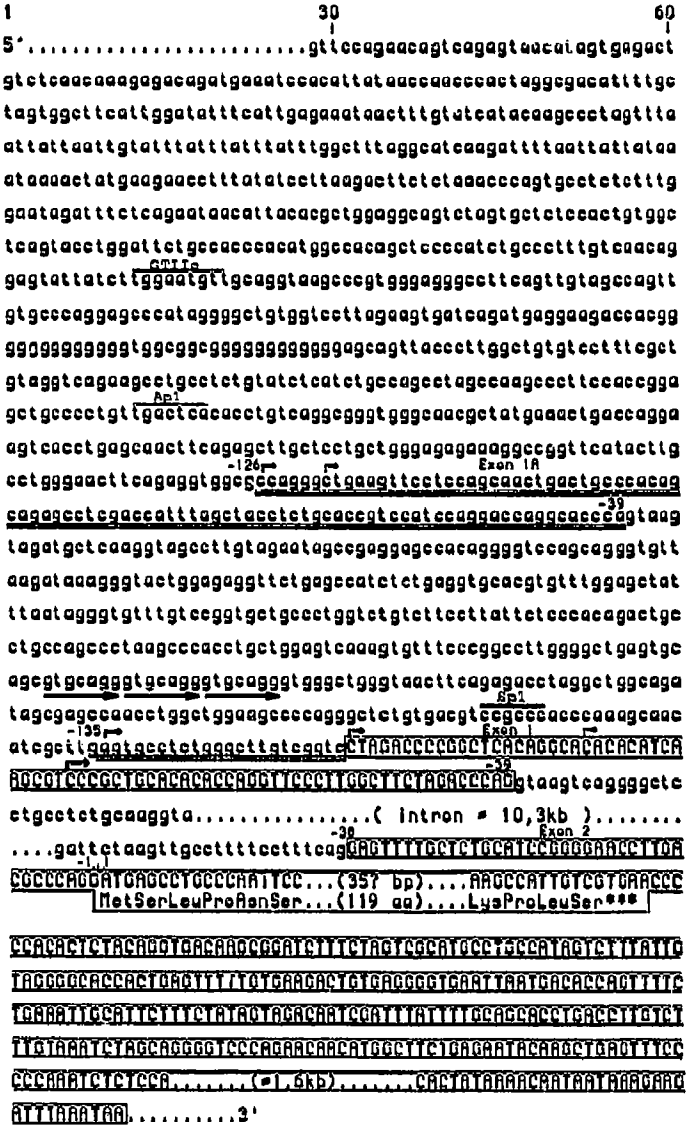

Fig. 4. Nucleotide sequence of exon 1A, exon 1 and exon 2 and of the flanking regions of these three exons. Exonic sequences deduced from cDNA sequences are boxed. The nucleotides are numbered relative to the first nucleotide of the translation initiation codon. 5′ termini sequences of exon 1A and exon 1 predicted by primer extension are indicated by lines under the sequences. The start sites of transcription revealed by analysis of the 5′ termini of I_{SK} mRNAs are indicated by arrows above exon 1A and exon 1. The location of sequences homologous to the promoter and regulating elements are shown above the nucleotide sequence. A repeated sequence is underlined by arrows.

does not correspond to any cDNA clone characterized and is probably due to another transcription start site not yet described.

The extension from PR2 gave rise to two weak bands of sizes of 83 and 76 residues. This result indicates that at least two species of mRNA are initiated from sites located in the putative exon 1A and that the largest PR2 extended DNA of 63 nucleotides probably corresponds to the 5′ end of exon 1A.

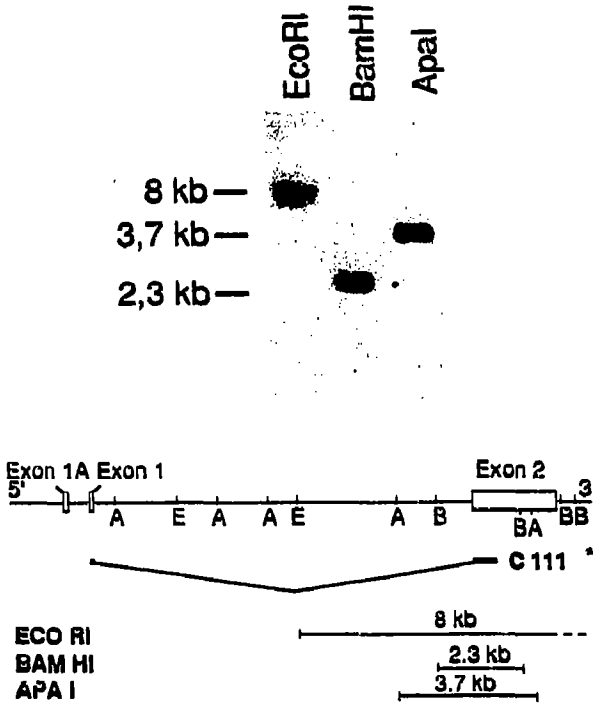

Fig. 5. Mouse genomic DNA analysis by Southern blot. Mouse cellular DNA was digested by *Eco*RI, *Bam*HI and *Apa*I and probed with the C111 insert. The sizes of hybridization-positive fragments are indicated on the left side of the autoradiograph. The restriction fragments of the I_{SK} protein gene, which were expected to hybridize with the cDNA probe, are indicated below the map of the genomic clone G511.

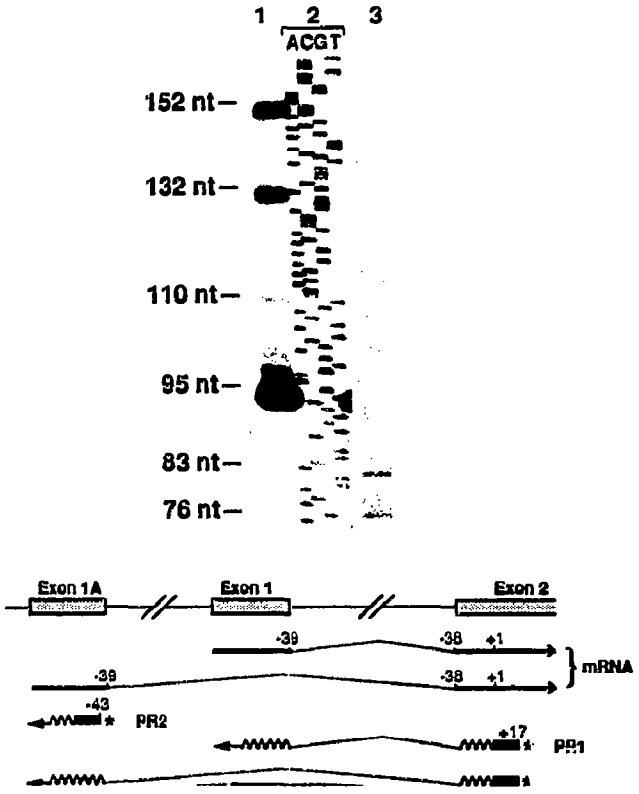

Fig. 6. Analysis of 5' termini of the I_{SK} mRNAs. The mouse neonate heart total RNA (5 μg) were annealed with two 5' end-labelled oligonucleotides (the asterisks denote the ^{32}P-labelled site PR1 (lane 1) and PR2 (lane 3)), and transcribed by reverse transcriptase. The transcription products were subjected to electrophoresis on 6% polyacrylamide urea gels. The autoradiographic exposures of lanes 1 and 2, and lane 3 were conducted for 18 and 48 h, respectively. The sizes of extended products were deduced from a plasmid sequence (lane 2).

These results were confirmed with RNAse protection experiments by using a cRNA probe consiting of exon 1 and exon 2 connected adjacently and the genomic sequence preceeding exon 1 (not shown). One protected band had a size equivalent to the PR1 extended DNA of 152 nt. This length probably corresponds to the 5' end of exon 1. Another protected band had the size expected for a fragment digested up to the diverging sequences between exon 1 and the putative exon 1A.

Hence, primer extension experiments as well as RNAse protection analysis indicate that an exon equivalent to the rat exon 1A also exists in the mouse I_{SK} protein gene. This conclusion was further supported by polymerase chain reaction experiments in which a DNA fragment containing exon 1A and exon 2 from position −48 on exon 1A to +387 in exon 2 was amplified from mouse neonate heart cDNA (not shown).

Taken together, all these results demonstrate that multiple transcription start sites are used in both exon 1 and exon 1A to generate the I_{SK} message with a marked preferential use of exon 1 in mouse.

The analysis of mRNAs was carried out with mRNAs of neonate heart since this tissue is the most abundant source of I_{SK} transcripts. Similar results were obtained with adult mouse heart and kidney mRNAs (of course, signals were much weaker in these cases). It then seems that the developmental stage of the mouse does not influence the choice of transcription start sites in both exons.

3.5. *Nucleotide sequence of the upstream region of exon 1A and exon 1*

The nucleotide sequences around exon 1A and exon 1 were determined on the basis of the assignment of the two upstream exons of the mI_{SK} gene. There are 470 bp between the 3' termini of exon 1A and exon 1. These regions have structural features typical of a housekeeping gene promoter. First, neither TATA, nor CAAT promoter elements were present in the 5' flanking region to exon 1A and exon 1 (Fig. 3). Second, the G+C content was high, it averaged for example 61 and 63% in the 250 bp immediately 5' to exon 1A and exon 1 respectively compared to about 40% for the entire mammal genome. Third, multiple transcription start sites were observed. Finally there are many potential promoter, enhancer and regulating DNA elements in these regions including GTIIc, Sp1, AP1 consensus sequences and repetitive sequences (Fig. 3).

4. DISCUSSION

The purpose of this work was to know whether there is a diversity of I_{sK} genes and of I_{sK} proteins. The analysis of 8 independent cDNA clones ranging from 0.6 to 2.3 kb revealed that the large differences in size between these clones mainly resulted from the length of their 3′ untranslated sequence due to at least three sites of polyadenylation. The 8 clones presented the same protein coding sequence. The minor differences observed in the 5′ region of the cDNA clones probably result from the use of different transcription start sites. Primer extension and RNAse protection experiments confirmed this conclusion.

Cloning of the mI_{sK} protein gene from a genomic library and analysis by genomic Southern blot revealed the existence of only one I_{sK} gene in the mouse haploid genome. The structural organization of this gene is unusual. It consists of at least two exons separated by an intron of approximately 10 kb. There are two alternatively used first exons containing only the 5′ untranslated sequence while the second exon encodes the whole protein and the 3′ untranslated sequence.

A search of a possible exonic sequence in the intron between exons 1 and 1A was carried out by probing heart and kidney mRNA blots with restriction fragments of this intron (data not shown). No signal corresponding to the possible presence of another exon capable of extending the protein coding sequence of I_{sK} was detected.

The different reasons for the diversity of I_{sK} mRNAs have been elucidated. These include alternative splicing, and multiple sites of transcription start and polyadenylation. Despite this heterogeneity of transcripts, only one I_{sK} protein species is found in the mouse. The putative physiological role of the messenger heterogeneity remains to be understood.

I_{sK} mRNA directs in the *Xenopus* oocyte the expression of a slow voltage-gated K^+ channel. Whether I_{sK} is the K^+ channel by itself, or a regulator of a K^+ channel endogenous to the oocytes and not normally active, remains a question of debate. However, recent reports using mutagenesis [11,12] seem to indicate that the I_{sK} protein is a K^+ channel. Our own results support the same conclusion [5]. If mI_{sK} is a K^+ channel, then it is interesting to note that the I_{sK} protein is a very peculiar ion channel when compared to other known structures of this kind. The structure of I_{sK} differs completely from that of the other known ion channels [13] and the I_{sK} protein is encoded by a single gene. Up to now the diversity of genes coding for a given voltage-sensitive ion channel has been the rule. There exist at least five genes encoding Na^+ channels [14,15], five genes also for Ca^+ channels [16] and probably more than ten for voltage-sensitive K^+ channels related to the *Drosophila Shaker*, *Shaw*, *Shab* and *Shal* genes [17]. The diversity observed for the *Shaker* superfamily is known to produce a very large panel of different K^+ currents with different biophysical properties, different regulations and different pharmacology that will play a crucial role in signal integration and plasticity [18,19].

The unique biophysical properties displayed by the single I_{sK} putative K^+ channel might suggest for this protein a role in a number of basic physiological processes.

Acknowledgements: We are indebted to Dr. C. Sardet (Centre de Biochimie, Nice, France) for providing the mouse genomic DNA library. The excellent technical assistance of M.-M. Larroque, C. Roulinat and F. Aguila is greatly acknowledged. This work was supported by the 'Association pour la Recherche sur le Cancer' and the 'Centre National de la Recherche Scientifique'. B. Attali is also a recipient of an ARC fellowship.

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