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Genomic organization of three neurotoxins active on small conductance Ca^{2+}-activated potassium channels from the scorpion *Buthus martensi* Karsch[1]

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Abstract According to the known primary sequences of three neurotoxins active on small conductance Ca^{2+}-activated potassium channels from the scorpion *Buthus martensi* Karsch, their corresponding cDNAs were cloned and sequenced using 3′- and 5′-RACE. All of them encoded a signal peptide composed of 28 residues and a mature toxin of 29, 28 and 33 residues, respectively. Their cDNA deduced sequences were totally consistent with those determined, and the C-terminal amidation of one neurotoxin was confirmed. The genomic DNAs of these three toxins were also amplified by PCR, cloned and sequenced. They all consisted of two exons disrupted by a small single intron. All of these introns were inserted within the signal peptide at the same −10 position upstream from the mature toxin, consisting of 94, 78 and 87 bp, respectively.

Key words: Scorpion neurotoxin; Potassium channel; RACE; Gene

1. Introduction

Scorpion venoms are a library of potassium channel toxins. Since the first K^+ channel toxin NTX was purified from *Centruroides noxius* in 1982 [1], a variety of other K^+ channel toxins have been characterized from different scorpions. These toxins are composed of 28–40 amino acids in length and share some conserved regions. According to the primary sequence and the specificity toward each corresponding subtype of K^+ channels, they could be classified into at least five groups [2–4] as follows: (1) Toxins active on high conductance Ca^{2+}-activated K^+ channels, including ChTX, Lq2 from *Leiurus quinquestriatus hebraeus* [5,6], IbTX from *Buthus tamulus* [7], and BmTX from *Buthus martensi* Karch [3], their N-terminal groups are characterized with a pyroglutamic acid. (2) Toxins active on both intermediate conductance Ca^{2+}-activated K^+ channels and voltage-dependent K^+ channels, including KTX from *Androctomus* scorpions [8,9], AgTX from *L. quinquestriatus hebraeus* and BmKTX from *B. martensi* Karch [3], they share 80–90% sequence similarity, and ended with a C-terminal residue Lys. (3) Toxins active on small conductance Ca^{2+}-activated K^+ channels, and able to compete with apamin for binding to the same receptor, including LeTX from *L. quinquestriatus hebraeus* [10], P05 from *Androctomus mauretanicus mauretanicus* [11] and BmP01, BmP02, BmP03 and BmP05 from *B. martensi* Karch [12], they have smaller size than others and their amino acid sequences also share less homology with those of the other group toxins. (4) Toxins active on voltage-dependent K^+ channels, including NTX from *C. noxius* [1,13], MgTX from *Centruroides margaritatus* [14], CllTX1 from *Centruroides limpidus limpidus* Karsch [15] and Ts Kα from *Tityus serrulatus* [16], they share 60–80% sequence identity. (5) Toxins active on voltage-dependent K^+ channels, including HsTX1 from *Heterometrus spinnifer* [17], maurotoxin (MTX) from *Scorpio maurus* [18] and Pi1, Pi2, Pi3 from *Pandinus imperator* [19,20], their primary structures are unique in having four pairs of disulfide bonds instead of three pairs found in other group toxins, and their C-terminal residues are all amidated, they display 50–70% sequence identity with each other.

Recently, two other new toxins CoTX1 and CoTX2 from *C. noxius* Hoffman active on voltage-dependent K^+ channels were reported [21]. According to their amino acid sequences, they can hardly fall into any group mentioned above. Therefore they are proposed to belong to a new subfamily.

Up to now around 40 different short chain toxins [22] against various subtypes of K^+ channels were reported on their amino acid sequences, structure conformations, chemical synthesis, physiological or pharmacological functions and their possible binding sites at their corresponding receptors. However, there have been scant data about their gene structures, only of one toxin, namely KTX_2 from *Androctonus australis*, both its cDNA and genomic DNA sequences have been elucidated [9,23]. Besides, the cDNA sequences of Ts κ [24] and the new toxin CoTX1 [21] were also reported. Therefore, it is worth studying and comparing these toxins at gene level, which can not only further verify their determined primary structures, but also provide a definite evidence for whether the C-terminal residue of a toxin is amidated or not. Furthermore the structure-function relationship of these toxins could be better understood by using mutagenesis to obtain different mutants for the study of the interaction of these toxins with their target receptors.

In the present paper, we describe the gene cloning and sequencing of the cDNAs of BmP01, BmP03 and BmP05. Their deduced amino acid sequences are consistent with those determined. The amidation of the C-terminal residue His of BmP05 is also verified as the Gly residue followed by a basic residue Lys that was removed during post-translation process-

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ing. As well known, the residue Gly is inevitably required for C-terminal amidation of any protein by peptidylglycine α-amidating monooxygenase. Based on the elucidated cDNA sequences of these toxins, their corresponding genomic DNAs were also cloned and sequenced. There was a 94, 78 and 87 bp intron inserted in the signal peptide of BmP01, BmP03, and BmP05, respectively.

2. Materials and methods

2.1. Materials

Scorpions *B. martensi* Karsch were collected in Henan Province, China. Their tails were cut off and immediately frozen in liquid nitrogen. TRIzol reagent and the 3′- and 5′-RACE kits were purchased from Life Technology Co., Nick Translation kit from Boehringer, Taq DNA polymerase, T4 DNA ligase and restriction enzymes from Promega, the DNA sequencing kit, (α-^{32}P)dATP and Hybond N$^+$ membrane from Amersham. Acrylamide, bisacrylamide, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), IPTG (isopropylthio-β-D-galactoside), and other chemical reagents were of analytical reagent grade.

2.2. Preparation of total RNA

About 100 mg of tail glands was ground into fine powder in liquid nitrogen. The total RNA was then extracted using TRIzol reagent according to the manual.

2.3. 3′-RACE

About 5 μg total RNA were taken to convert mRNAs into cDNAs using Superscript II reverse transcriptase and a universal oligo(dT)-containing adapter primer with a *Sal*I restriction site (5′-GGCCACGCGTCGACTAGTAC(dT)$_{17}$-3′). The synthesized cDNAs were then used as a template for PCR amplification. A gene specific primer corresponding to the known amino acid sequence was paired with an abridged universal amplification primer containing an additional *Eco*RI restriction site (5′-CGGAATTCGGCCACGCGTCGACTAGTAC-3′) which is similar to the adapter primer but devoid of the poly dT tail. The amplified products were then precipitated, digested with corresponding restriction enzymes and cloned into the M13 vector for sequencing.

Based on the preferential codon usage in other BmK neurotoxins [25–27], the gene specific primer 1 for BmP01 was designed and synthesized with a *Hin*dIII restriction site (5′-CGAAGCTTGCTACATGCGAAGA(T/C)TG-3′), corresponding to its known amino acid sequence right from the first N-terminal residue Ala-Thr-Cys-Glu-Asp-Cys, the codon for the residue Asp was degenerated. The gene specific primer 2 for BmP03 with a *Bam*HI restriction site (5′-CGGGATCCGTAGGATGCGAAGA(A/G)TG-3′) corresponded to the known amino acid sequence Val-Gly-Cys-Glu-Glu-Cys also starting from the N-terminal residue, the codon for the residue Glu was degenerated. The gene specific primer 3 for BmP05 with a *Hin*dIII restriction site (5′-CGAAGCTTGGTAAATGCATAGG(A/T/C)GA-3′) corresponded to the known amino acid sequence Gly-Lys-Cys-Ile-Gly-Asp at the 19–24 position, the codon for the residue Gly was degenerated.

2.4. 5′-RACE

Based on the partial cDNA sequences determined by 3′-RACE, the anti-sense primers were designed and synthesized. Gene specific primer 4 of BmP01 with a *Bam*HI restriction site (5′-CGGGATCCTCATTTCGGTTCACATA-3′) corresponded to the 29–25 residues Lys-Pro-Glu-Cys-Val and a stop codon of TGA, and its nested primer 5 with a *Hin*dIII restriction site (5′-CGAAGCTTCGCATTTGTCATTATC-3′) corresponded to the 25–20 residues Val-Cys-Lys-Asp-Asn-Asp. Gene specific primer 6 of BmP03 with a *Bam*HI restriction site (5′-CGGGATCCTCATACATTGCAGTTAC-3′) corresponded to the 28–24 residues Val-Asn-Cys-Asn-Cys and a stop codon of TGA, and its nested primer 7 with a *Hin*dIII restriction site (5′-CGAAGCTTCACGCCGTCGTCGCAGGT-3′) corresponded to the 23–18 residues Val-Gly-Asp-Asp-Cys-Thr. Gene specific primer 8 of BmP05 with a *Hin*dIII restriction site (5′-CGAAGCTTGTTATTTGCCGTGTTT -3′) corresponded to the 33–30 residues Lys-Gly-His-Lys and a stop codon of TAA, and its nested primer 9 with a *Hin*dIII restriction site (5′-GCAAGCTTAACACATTCGCATTTATC-3′) correspond to the 29–24 residues Val-Cys-Glu-Cys-Lys-Asp.

Using 1 μg total RNAs as a template, the first strand cDNAs of BmP01 were synthesized with its gene specific primers 4. After the cDNAs were purified on a Glassmax column, homopolymeric dC tails were then added to their 3′-ends by terminal deoxynucleotidyl transferase. The dC tailed cDNAs were first amplified with nested primer 5 and an abridged anchor primer (5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIG-3′) complementary to the dC tails. In order to obtain a higher yield of the specific cDNAs, the first PCR product was diluted and used as a template for the second PCR amplification with its nested primer 5 and another anchor primer devoid of dG and containing an *Eco*RI restriction site (5′-CGGAATTCACGCGTCGACTAGTAC-3′). Thus the final PCR product of BmP01 was purified, digested with *Eco*RI and *Hin*dIII, and cloned into M13 vector for sequencing.

With the same strategy described above, the 5′-end cDNAs of BmP03 and BmP05 were also obtained using their own gene specific primers and nested primers, respectively, and digested with the corresponding restriction enzymes. Then they were cloned into M13 vector for sequencing.

2.5. Amplification of genomic DNA

The total genomic DNAs were isolated from scorpion tail glands using a $NaClO_4$ extraction procedure, followed by RNase treatment. Using 5 μg total genomic DNAs as a template, the genomic DNA of BmP01 was amplified with two primers. Gene specific primer 10 with an *Eco*RI restriction site (5′-CGGAATTCGGAAAATATACAAAAATG-3′) corresponding to the determined sequence of 5′-untranslated region upstream right from the initiation codon ATG was paired with gene specific primer 4 used for 5′-RACE of BmP01.

For amplification of the genomic DNA of BmP03, gene specific primer 11 with an *Eco*RI restriction site (5′-CGGAATTCGATAGTTGGAAGAAAATG-3′) corresponding to the determined sequence of 5′-untranslated region at position 4 bases upstream from the initiation codon ATG was paired with gene specific primer 6 used for 5′-RACE of BmP03.

For amplification of the genomic DNA of BmP05, gene specific primer 12 with an *Eco*RI restriction site (5′-CGGAATTCAATAATTATTACCTAGC-3′) corresponding to the determined sequence of 5′-untranslated region upstream right from the initiation codon ATG was paired with gene specific primer 7 used for 5′-RACE of BmP05. The above PCR amplified products were then purified, digested with corresponding restriction enzymes, and cloned into the M13 vector for sequencing.

2.6. Preparation of a specific probe

In order to verify that the amplified products of 5′-RACE and genomic DNAs were really the gene of interest and to simplify screening of a positive clone, the amplified products or the uncertain clones were subjected to Southern blot with their specific probes, respectively. The specific probe of BmP01 was prepared as follows: using a BmP01 clone obtained by 3′-RACE as a template, which had been proved correct by sequencing, gene specific primer 1 of BmP01 was paired with its 5′-RACE nested primer to amplify this specific probe. The amplified product of PCR was then purified and labeled with (α-^{32}P)dATP using Nick Translation kit. With the same approach, the specific probes of BmP03 and BmP05 were also prepared using their own 3′-RACE clones, 3′-RACE gene specific primers and 5′-RACE nested primers, respectively.

3. Results

3.1. cDNA and genomic DNA of BmP01

Using 3′- and 5′-RACE, the cDNA of BmP01 was cloned, encoding a signal peptide of 28 amino acids and a mature peptide of 29 amino acids. The polyadenylation signal aataaa was found in the 3′-untranslated region, located at 39 bp downstream from the stop codon. The genomic DNA of BmP01 was then amplified from the BmK scorpion genomic DNA. The gene consisted of two exons disrupted by a 94 bp

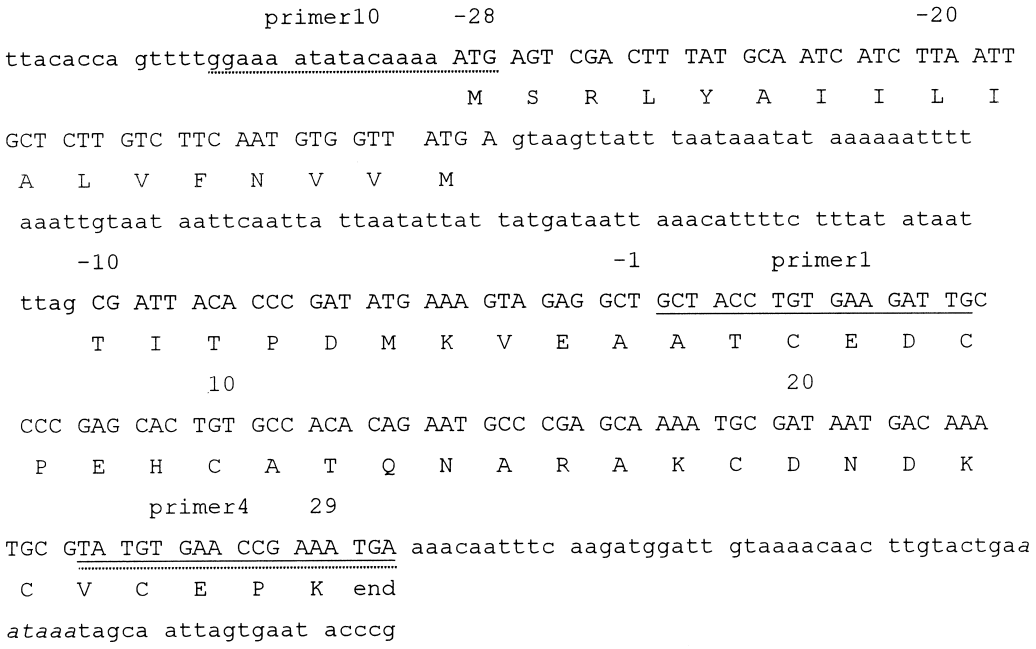

Fig. 1. Nucleotide sequences of the cDNA and genomic DNA of BmP01. Two exons are written in capital letters, while the untranslated region and the intron are written in small letters. The polyadenylation signal is written in italics. The deduced amino acid sequence is given below the nucleotide sequence and numbered. Primer 1 and 4 for 3′-RACE and 5′-RACE are underlined, and primer 4 and 10 for the genomic DNA amplification are indicated by dotted line.

intron. The intron was within the signal peptide, inserted in the codon of Thr at the −10 position upstream of the mature toxin. The intron was flanked by a gt/at donor-acceptor pair, in accordance with the features of introns in eukaryotic genes. The AT content was up to 89%.

Comparing the determined gene sequence of the residues 1–6 (GCT ACC TGT GAA GAT TG) with that of designed primer 1 (GCT ACA TGC GAA GAT TG), there were two bases mismatched as underlined (see Fig. 1).

3.2. cDNA and genomic gene of BmP03

The cDNA of BmP03 encoded a signal peptide of 28 amino acids and a mature peptide of 28 amino acids. The location of the polyadenylation signal was the same as in BmP01 at 39 bp downstream from the stop codon. The genomic DNA of BmP03 was amplified from the BmK scorpion genomic DNA. The gene consisted of two exons disrupted by a 78 bp intron. The intron flanked by a gt/at donor-acceptor pair was also within the signal peptide, inserted in the codon of Ala at

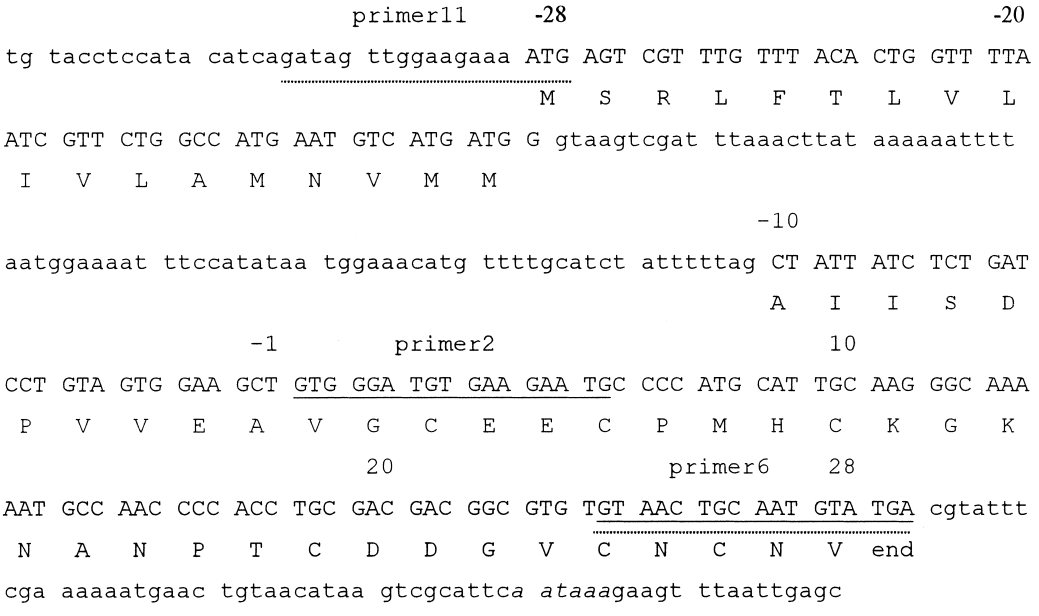

Fig. 2. Nucleotide sequences of the cDNA and genomic DNA of BmP03. Two exons are written in capital letters, while the untranslated region and the intron are written in small letters. The polyadenylation signal is written in italics. The deduced amino acid sequence is given below the nucleotide sequence and numbered. Primer 2 and 6 for 3′-RACE and 5′-RACE are underlined, and primer 6 and 11 for the genomic DNA amplification are indicated by dotted line.

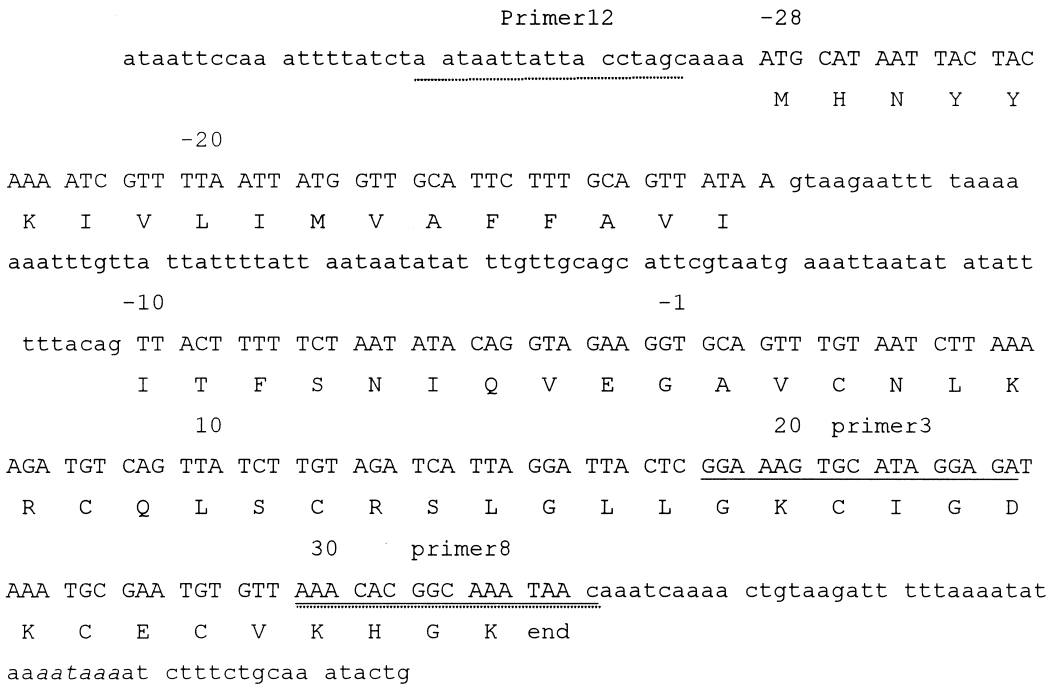

Fig. 3. Nucleotide sequences of the cDNA and genomic DNA of BmP05. Two exons are written in capital letters, while the untranslated region and the intron are written in small letters. The polyadenylation signal is written in italics. The deduced amino acid sequence is given below the nucleotide sequence and numbered. Primer 3 and 8 for 3′-RACE and 5′-RACE are underlined, and primer 8 and 12 for the genomic DNA amplification are indicated by dotted line.

the −10 position upstream from the mature toxin. The AT content was up to 78%.

Compared with the determined gene (GTG GGA TGT GAA GAA TG) two mismatched bases were found in the designed primer 2 (GTA GGA TGC GAA GAA TG) as underlined (see Fig. 2).

3.3. cDNA and genomic gene of BmP05

The cDNA of BmP05 encoded a signal peptide of 28 amino acids and a mature peptide of 31 amino acids. The two additional residues Gly-Lys were inevitable required for the C-terminal amidation of the mature toxin. The polyadenylation signal was located at 32 bp downstream from the stop codon. The genomic DNA of BmP05 was also composed of two exons disrupted by a 87 bp intron. The intron inserted in the codon of Ile was the same as in BmP01 and P03 located at −10 position upstream from the mature toxin. The AT content was up to 83%.

Compared with the determined gene (GGA AAG TGC ATA GGA GA), two mismatched bases were found in the designed primer 3 (GGT AAA TGC ATA GGA GA) as underlined (see Fig. 3).

4. Discussion

Potassium channels are present in almost every living cell and play a very important physiological role. They are involved in a number of physiological processes such as muscle contraction, secretory processes, cell proliferation and cell volume regulation [28–30]. Scorpion potassium channel toxins were successfully used as tools for K^+ channel study. They are useful probes for investigating the mechanisms of K^+ conduction, channel selectivity, the architecture of the pore region and the physiological role of individual subtype of channels [31–35]. For example, ChTX was used as a probe to elucidate the interaction between the toxin and its receptors, the high conductance Ca^{2+}-activated K^+ channel or the shaker K^+ channel, by using point mutagenesis [36–38], while AgTX2 was used to prove the structure conservation of voltage-dependent K^+ channels in prokaryotic and eukaryotic organisms [39]. Now the cDNAs of the toxins blocking small conductance, Ca^{2+}-activated K^+ channels have been cloned, which will provide an approach to study another subtype of K^+ channels.

In this paper three gene sequences of the BmK scorpion toxins were described, and it is the first report on gene structures of the low conductance, Ca^{2+}-activated K^+ channel toxins. The cDNA deduced amino acid sequences of BmP01, BmP03 and BmP05 were consistent with their determined primary sequences [12]. Two additional residues Gly-Lys were present in the deduced cDNA sequence of BmP05, and removed during post-translation, which strongly verified the amidation of the C-terminal residue His of the toxin as the residue Gly being a donor of the amide group is absolutely required for peptidylglycine α-amidating monooxygenase. The following basic residue Lys might play a role in facilitating this enzymatic amidation reaction. The C-terminal amidation was supposed to strengthen this toxin affinity for its receptor [40].

All precursors of these three toxins have a signal peptide of 28 residues, the same size of CoTX1 from *C. noxius* Hoffman [21], but longer than those of other channel toxins of scorpion, which are usually around 20 residues [23–25,41–44]. The genomic DNAs of all BmK K^+ channel toxins consisted of two exons disrupted by a small intron at the same −10 position upstream from the mature toxin. The size of intron was less than 100 bp, similar to that of another K^+ channel toxin KTX2 from *A. australis* [23], but much less than those of

other long chain Na^+ channel toxins of scorpion with over more than 400 bp [23,25,43,44]. All these introns of K^+ channel toxins shared more than 57% homology with each other. Though the BmK K^+ channel toxins are different from KTX2 in targeting their own corresponding subtype of K^+ channels, their genomic organizations were similar to each other.

Compared with the determined gene sequences, the gene specific primers 1, 2 and 3 for 3′-RACE each had two mismatched bases. Therefore they were successful for efficient and specific amplification. Besides gene specific primers 4, 6 and 8, three corresponding nested primers 5, 7 and 9 were also designed and synthesized for 5′-RACE. They were very essential for effective PCR. Because the 'primer-dimer' amplification of the residual gene specific primer can be avoided, the PCR amplification becomes more specific.

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