Molecular cloning and sequencing of two 'short chain' and two 'long chain' K^+ channel-blocking peptides from the Chinese scorpion *Buthus martensii* Karsch

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Abstract Five full-length cDNAs encoding the precursors of two 'short chain' scorpion non-toxic peptides active on Ca^{2+}-activated K^+ channels (BmP02 and BmP03) and two novel putative long chain K^+ channel-blocking peptides (named BmTXKβ and BmTXKβ2) were first isolated from the venom gland cDNA library of the Chinese scorpion *Buthus martensii* Karsch (BmK). BmTXKβ2 showed a high similarity with AaTXKβ, while BmTXKβ was completely different in the deduced primary structure from the long chain and short chain scorpion toxins already characterized. Thus, BmTXKβ expands the scorpion long chain K^+ channel-blocking peptide family. Although little sequence similarity exists between the above two short and two long peptides, they are similar at the positions of six cysteines, suggesting that they should all share a similar scaffold composed of an α-helix and a three-stranded β-sheet.

Key words: K^+ channel-blocking peptide; Gene; *Buthus martensii* Karsch

1. Introduction

Potassium channels comprise a large family of proteins that control electrical excitability as well as the resting membrane potential in many different cell types [1]. Meanwhile, they are the putative target sites for designing therapeutic drugs [2]. To discover the function of K^+ channels in physiology and pathology, it is necessary to develop high affinity and selective probes that target specific K^+ channels. The discovery of peptidyl inhibitors in scorpions has played an instrumental role in the development of our current understanding of K^+ channels [3]. At present, the search for peptidyl inhibitors of K^+ channels has become an active area of investigation [4]. It is expected, as more novel peptides and their genes are discovered, that our understanding of the K^+ channel structure and function will be further enhanced.

Buthus martensii Karsch (BmK) is widely distributed in China. Their venom has been shown to be a rich source of various selective neurotoxins active on the Na^+ channels of mammals and/or insects [5]. Recently, Romi-Lebrun et al. isolated and characterized seven short chain K^+ channel-blocking peptides from BmK, which were named BmP01, BmP02, BmP03, BmP05, BmKTX, BmTX1 and BmTX2 [6,7]. Previous studies demonstrated that BmP01, BmP02 and BmP03 showed a weak binding to the small conductance Ca^{2+}-activated K^+ channels of rat brain and non-toxicity in mice [7]. Tong et al. newly reported that BmP02 can efficiently inhibit the transient outward K^+ current channels of rat ventricular myocytes [8]. The small non-toxic peptides, which are of close structural homology with BmP01, BmP02 and BmP03, have been isolated and characterized from the venoms of various species of scorpion venoms, e.g. peptide II Bs from *Buthus sindicus*, peptide P01 from *Androctonus mauretanicus mauretanicus*, leiuropeptide I, leiuropeptide II and leiuropeptide III from *Leiurus quinquestriatus hebraeus* [9], and all these molecules are significantly similar with sapecin B, an anti-bacterial peptide isolated from the fresh fly (data not shown) [7], which suggests that this group of conserved scorpion non-toxic peptides may be of an important biological and pharmacological function to be discovered. We have focused on studying their cDNA and gene organization and searching for new types of receptors for them [10].

In addition, long chain scorpion toxins active against K^+ channels are a group of new type toxins that contain about 60 amino acid residues with only six cysteines, which are less well been studied. Since TsTXKβ was isolated in 1994 [11] and the cDNAs encoding AaTXKβ and TsTXKβ were cloned in 1998 [12], no other new toxin and its gene have been reported.

This paper reports five full-length cDNA sequences, two of which encode the precursors of BmP02 and BmP03 and the other three encode those of two novel putative long chain K^+ channel-blocking peptides, which may be used as probes to study the function of specific K^+ channels. The probable regulation mechanism of BmTXKβ2 gene expression at the post-transcriptional level by an ATTTA motif in the 3′ untranslated region (UTR) was also discussed.

2. Materials and methods

2.1. Preparation of the total RNA and purification of mRNA from the venom glands of BmK

Scorpions of the species *B. martensii* Karsch were collected in the area of Hubei Province, China. They were killed 48 h after extraction of their venom to allow for the toxin-producing cells of the venom glands to enter the secretory phase. Total RNA was prepared from the venom gland segments (telson) using the hot phenol method [13]. Poly A^+ RNA was purified using a Poly A Tract RNA Isolation System (Promega, USA).

2.2. Construction of a BmK venom gland cDNA library

A BmK venom gland cDNA library was constructed using the Superscript Plasmid System (Gibco BRL, USA). 5 μg of mRNA was used to synthesize first strand cDNA by reverse transcription. Second strand cDNA was synthesized with *Escherichia coli* DNA polymerase and DNA ligase. After double-stranded (ds) cDNAs were filled with T4 DNA polymerase, *Sal*I adaptor with T4 DNA ligase was added and then digested with *Not*I. Excess *Sal*I adaptor was removed and small fragments were cut with a nucleon extraction

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and purification kit (Amersham, USA). We took 10 ng dscDNA to ligate with plasmid pSPORT1 and introduced the ligated cDNA into *E. coli* K12 MC1061 by electroporation.

2.3. Screening of the cDNA library with the PCR strategy

2.3.1. Design of two specific primers for PCR screening. Forward primer 1 (FP1): 5′-GTTGGTTG(TC)GA(GA)GA(GA)TG(TC)CC-3′, corresponding to the residues VGCEECP of the conserved region of BmP02 and BmP03. Forward primer 2 (FP2): 5′-TGTCACGG-NTTCAAATG(TC)AA(AG)TG(TC)-3′, corresponding to the residues CHGFKCKC of the conserved region of AaTXKβ and TsTXKβ. Reverse primer (RF): 5′-GAGCGGCCGCCCT$_{15}$-3′, the same sequence as the primer for synthesis of first strand cDNA.

2.3.2. PCR screening strategy. The transformed cells of the BmK venom gland cDNA library were plated on 10 LB/Ap$^+$ plates containing about 200 clones for each plate. 2000 Clones of 10 plates were inoculated into 2000 individual 1.5 ml Ep tubes containing 850 μl of LB/Ap$^+$ medium. After incubation, 150 μl glycerol was added and the clones were stored at −20°C for use. The PCR screening strategy consists of three steps. First, screening of 10 PCR reactions was performed by dividing the 2000 clones into 10 groups (200 clones/group). Positive group(s) were chosen from the first screening for second screening of 10 PCR reactions, in which the 200 clones were divided into 10 groups (20 clones/group). Third, screening of 20 PCR reactions was performed by dividing the 20 clones into 20 groups (one clone/group).

2.3.3. PCR reaction. The template was prepared using a boiling method [14]. The culture from single or mixed clones was centrifuged at 10 000 rpm for 30 s, the medium was decanted and an equal volume of ddH_2O added. After vortexing thoroughly, the suspension was boiled for 5 min and cooled immediately on ice, centrifuged at 15 000 rpm for 5 min and the supernatant was collected and stored at −20°C for use. PCR components included 2.5 μl 10×PCR buffer, 2 μl 2.5 mM dNTPs, 1.5 μl 2.5 mM $MgCl_2$, 1 μl 25 μM FP1 or FP2, 1 μl 12.5 μM RP, 1 μl plasmid template, 0.25 U Taq DNA polymerase (SPromega, China), ddH_2O was added to 25 μl. PCR conditions: 5 min at 95°C followed by 30–35 cycles of 95°C for 60 s, 50–55°C for 60 s, 72°C for 60 s. The samples were analyzed on 1.5% agarose gels.

2.4. DNA sequencing and computer analysis

The plasmids characterized as positive clones were sequenced using the chain termination method with an ABI PRISM DNA Sequencer. Sequence analysis was carried out with BLASTX and PC/GENE.

The cDNAs sequences of BmP02, BmP03, BmTXKβ2 (BmTXKβ2′) and BmTXKβ have been deposited in the GenBank database under accession numbers AF132975, AF156170, AF155370 (AF156173) and AF155371, respectively.

3. Results and discussion

3.1. Screening of the BmK venom gland cDNA library with a PCR strategy

From 10 ng dscDNA, 2×10^3 transformants were formed by electroporation. By three cycles of PCR screening, two and four positive clones from primers FP1/RP and FP2/RP were

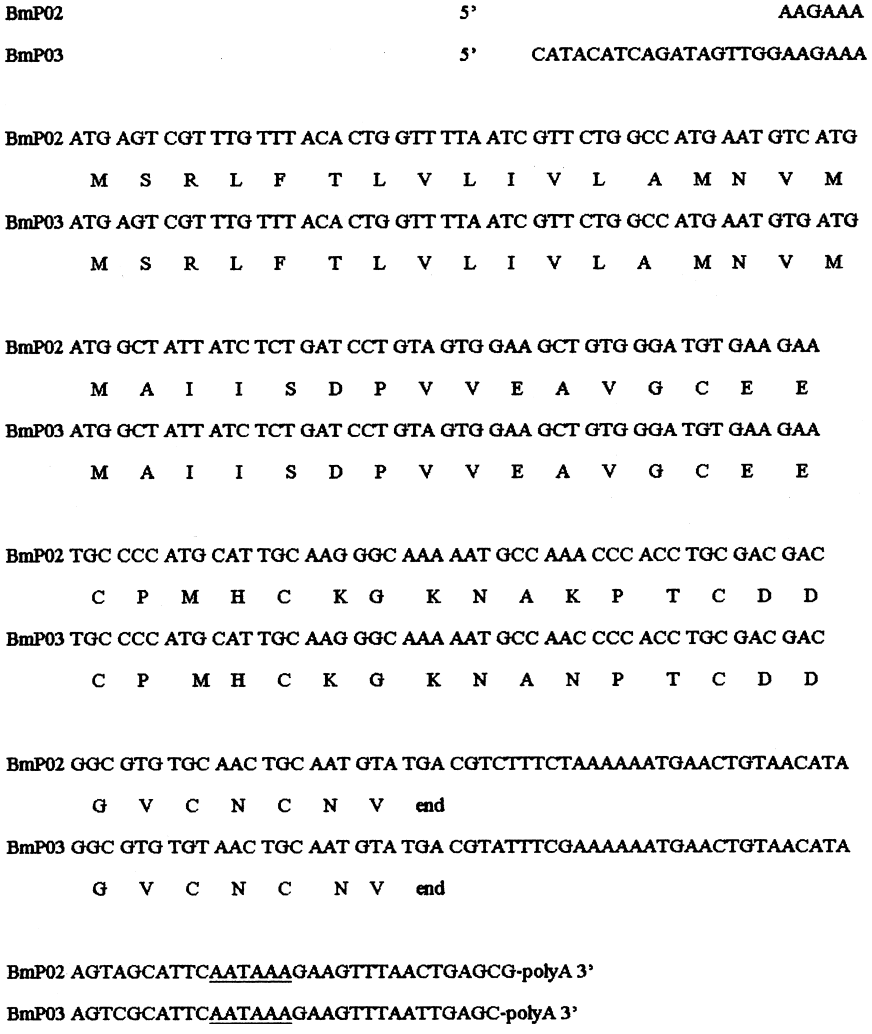

Fig. 1. Nt sequences of cDNAs and predicted amino acid sequences of BmP02 and BmP03. The putative poly(A) signals are underlined.

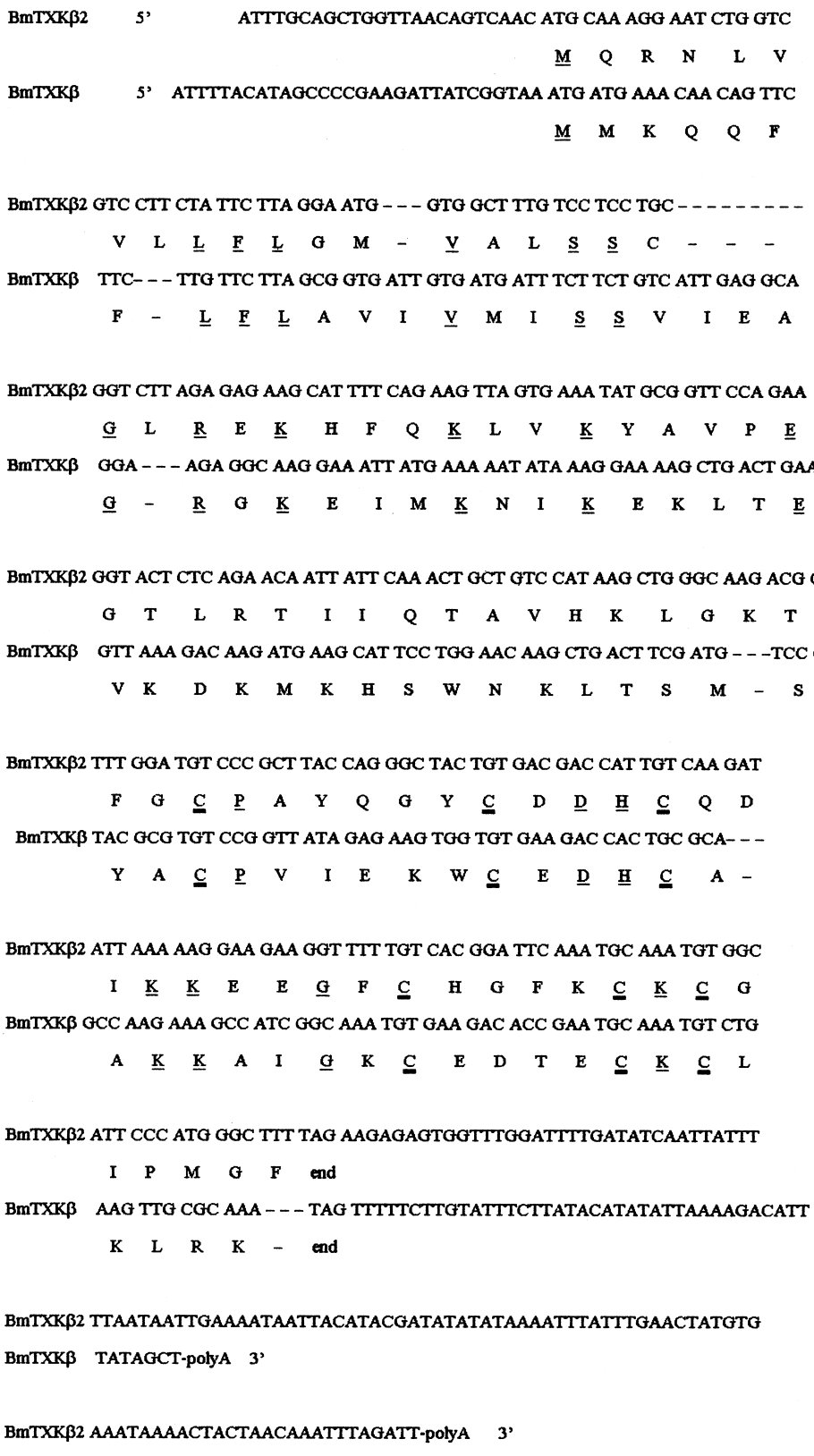

Fig. 2. Nt sequences of cDNAs and the predicted amino acid sequences of BmTXKβ2 and BmTXKβ. Alignment of amino acid sequences with cysteine residues.

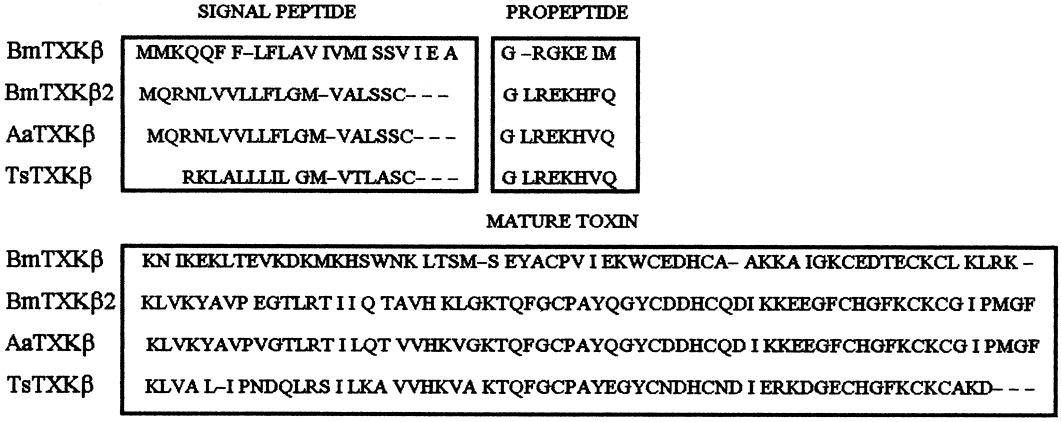

Fig. 3. Comparison of the amino acid sequences of four long chain K^+ channel blocker precursors. Alignment of amino acid sequences with cysteine residues.

obtained, respectively. Sequence analysis results showed that two positive clones screened from primers FP1/RP consisted of one copy of BmP02 and one copy of BmP03, four positive clones screened from primers FP2/RP consisted of two copies of BmTXKβ and two copies of BmTXKβ2.

Each class of scorpion toxin is a group proteins highly conserved in primary structure either in or among the species, thus, their conserved amino acid sequences help to design degenerate primers for PCR screening to obtain positive clones. Compared with traditional cloning methods [15] (e.g. screening the cDNA library using oligonucleotide probe), the PCR strategy includes all the characteristics of PCR, that is, sensitivity, specificity, rapidity, convenience and it is free from radioisotopes [16]. This strategy allows us to isolate a desired scorpion toxin cDNA clone(s) within 3 days or so and the prepared templates may be stored at −20°C and used repeatedly to isolate different toxin cDNAs.

3.2. cDNA sequence of BmP02 and BmP03

No cDNA sequences of short non-toxic peptides acting on K^+ channels were previously reported from scorpions. The BmP02 and BmP03 cDNAs were obtained by PCR screening of the cDNA library of BmK with a specific degenerate primer corresponding to their conserved amino acid region and a universal primer. The full-length cDNAs of BmP02 and BmP03 were composed of three parts, including 5′ UTR, open reading frame (ORF) and 3′ UTR (Fig. 1). Their precursors consisted of 56 amino acid residues, with a putative signal peptide of 28 residues and a mature toxin of 28 residues, and the deduced amino acid sequences of mature BmP02 and BmP03 were completely consistent with that previously determined by primary structure analysis. It has been proved that all scorpion toxin precursors contain a signal sequence with a very hydrophobic core. We found that the signal peptide of 'short chain' toxin was longer than that of 'long chain' toxins [10,17]. Whether the length of the signal peptide has relevance to the secretion level of toxin has been unknown. The signal peptide of BmP02 and BmP03 showed a low degree of similarity with that of other characterized toxins, but the residues at position −1 (Ala) and −3 (Val) are similar with the majority of reported scorpion 'long chain' toxins acting on Na^+ channels and the residues at position −2 (Glu) and −1 (Ala) are the same as the 'short chain' toxins acting on K^+ and Cl^- channels [17].

The nucleotide (nt) sequences differed between the precursors of BmP02 and BmP03 by three substitutions, one of which at position 132 A↔C led to mutation of Lys↔Asn in the amino acid sequence. In addition, substitution also existed in their 3′ UTR. The 3′ UTR of BmP02 and BmP03 contained a poly(A) signal (AATAAA) at 15–16 bp upstream from the poly(A) tail. The bases AAA at positions −1–−3 upstream from the translation initiation site were highly conserved in some scorpion toxins acting on Na^+, K^+ and Cl^- channels (Zhu, personal communication).

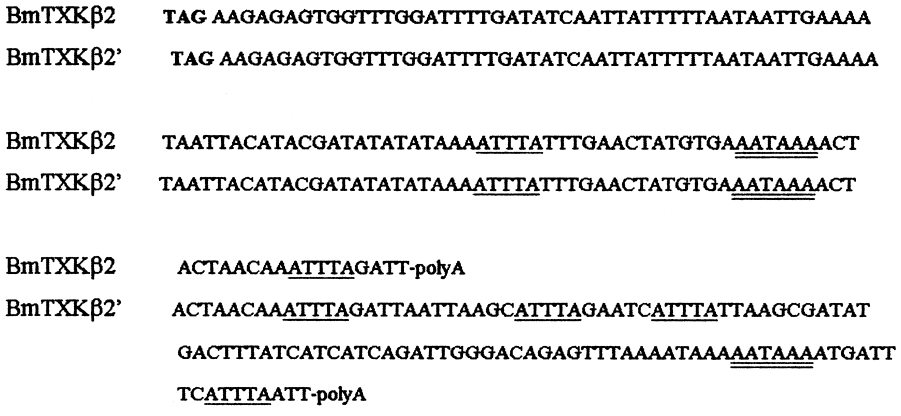

Fig. 4. Comparison of 3′ UTR nt sequences of BmTXKβ2 and BmTXKβ2′. The poly(A) signals (AATAAA) are underlined twice. The ATTTA motifs are underlined once. Note: BmTXKβ2′ is different from BmTXKβ2 only in their 3′ UTRs.

3.3. cDNA sequence of BmTXKβ and BmTXKβ2

To date, only two scorpion 'long chain' toxins active on K^+ channels and their cDNA sequences were reported. By designing a specific degenerate primer corresponding to the conserved region, which contains three cysteines, of AaTXKβ and TsTXKβ for PCR screening the BmK venom gland cDNA library to obtain new members of 'long chain' K^+ channel blockers, we obtained two novel cDNA sequences encoding putative 'long chain' K^+ channel-blocking peptides, named BmTXKβ and BmTXKβ2 (Fig. 2). Sequencing of four positive clones from the primers FP2/RP showed that templates prepared from CZ68(s) and CZ56(s) containing the insert of BmTXKβ2′ and BmTXKβ2 can perfectly match FP2, while that from BmK11(s) and C68 containing the insert of BmTXKβ cannot. But we found that FP2 can completely match its template at its 3′-end of 11 continuous bases and partly at its 5′-end, which led to a PCR product generated from the clones BmK11(s) and C68.

Sequence analysis showed that the precursors of BmTXKβ and BmTXKβ2 consisted of 90 and 91 amino acid residues with a putative signal peptide of 22 and 19 residues, a propeptide of seven and eight residues and a mature toxin of 61 and 64 residues, respectively. BmTXKβ2 shared a high similarity with AaTXKβ. Of 19 base substitutions in their ORFs, five substitutions at positions 76, 107, 127, 137 and 148 led to mutation of V↔F, V↔E, L↔I, V↔A and V↔L in the amino acid sequence. We also found that mutation at the 3′ UTR was higher than that at the 5′ UTR. At the 5′UTR, only one base substitution occurred, while at the 3′UTR, the differences existed not only in base composition but also in length and the positions of the poly(A) signal (data not shown). BmTXKβ2 and BmTXKβ2′ displayed a difference only in their 3′ UTR length (Fig. 4). In addition, by comparing with AaTXKβ and TsTXKβ, we supposed that BmTXKβ and BmTXKβ2 should also be intron-less [12].

BmTXKβ shared a low similarity at the nt and amino acid level with AaTXKβ, TsTXKβ and BmTXKβ2, but the organization of cDNA and the spacing pattern of six cysteines (Cys...CysXXXCys...Cys...CysXCys, X: variable amino acid residue) were consistent with them (Fig. 3), suggesting it should share a similar scaffold containing a cysteine-stabilized α-helical motif, which involves a CysXXXCys stretch of the α-helix bonded through two disulfide bridges with a CysXCys triplet of β-strand [18,19]. A high degree of similarity existed between BmTXKβ and the other three toxins at the positions CysXXXCys (Cys-Asp-Asp-His-Cys in AaTXKβ and BmTXKβ2, Cys-Asn-Asp-His-Cys in TsTXKβ, Cys-Glu-Asp-His-Cys in BmTXKβ) and CysXCys (Cys-Lys-Cys in all the above four toxins), but no similarity in the N-terminal segment, which might be involved in K^+ channel-blocking [20,21]. All these findings showed that BmTXKβ should represent a group of new long chain scorpion toxins acting on specific K^+ channels and should be highly valuable in searching for the functions of specific K^+ channels.

Interestingly, the positively charged residue N-terminal to the fourth cysteine (Lys), which is thought to interact directly with K^+ in the ion conduction pore [19], was preserved in the majority of scorpion K^+ channel-blocking peptides [12], but displayed differences among BmTXKβ (Lys), BmTXKβ2 (Phe), AaTXKβ (Phe), TsTXKβ (Glu), BmP02 (Thr) and BmP03 (Thr). It has been proved that BmP01, BmP02 and BmP03 are weak ligands with Ca^{2+}-activated K^+ channels and TsTXKβ is active against the voltage-sensitive 'delayed rectifier' potassium channel. Compared with TsTXKβ, the Lys-substituted Glu at that position may alter the K^+ channel-blocking specificity of BmTXKβ.

3.4. Does the 3′ UTR of BmTXKβ2 regulate gene expression itself?

Sequence analysis showed that two cDNAs encoding BmTXKβ2 and BmTXKβ2′ had the same 5′ UTR and ORF, but displayed a difference in 3′ UTR (Fig. 4). Of these two 3′ UTRs, one, which contains five copies of the ATTTA motif, was 96 nt longer than the other, only containing two copies of the ATTTA motif. It has been proved preliminarily that ATTTA motifs in the 3′ UTR of mRNA play a critical role in the regulation of gene expression by promoting mRNA rapid deadenylation and a rapid decay in eukaryotes [22,23]. Based on these two cDNAs differing only in their 3′ UTR nt length and the longer one existing two putative poly(A) signals (AATAAA), we reasonably believe that these two mRNA molecules may be generated from a common primary transcript by selecting different tailing sites (Fig. 5). This strategy was also found in other eukaryotic genes, but due to one of the poly(A) signals locating in the ORF, which leads to generation of two different products. The mechanism by which two mature mRNAs different in 3′ UTR length form from a common pre-mRNA by selecting different tailing sites, probably to regulate gene expression, has not been reported in the species scorpion.

Whether this selective tailing mechanism in the 3′ UTR regulates the scorpion toxin gene expression level, which further adapts scorpions themselves to the environment, has been unclear. Further studies to these genes may help to clarify the

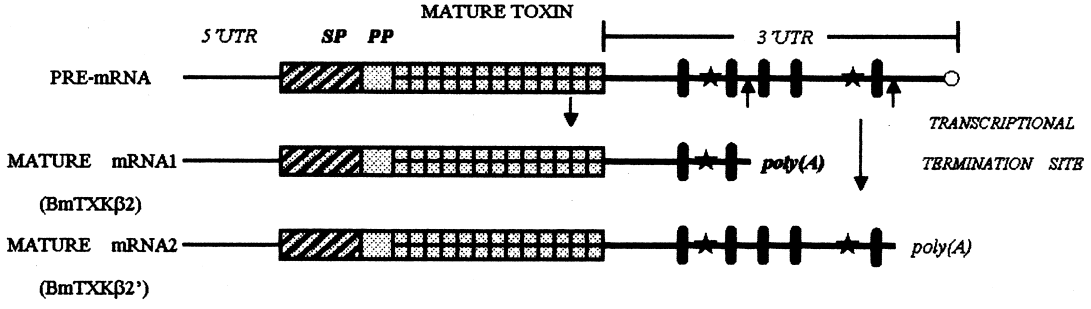

Fig. 5. The presumed post-transcriptional processing mechanism for BmTXKβ2. SP, signal peptide; PP, pro-peptide; vertical filled bar, ATTTA motif; *, poly(A) signal; ↑, cleavage and polyadenylation site.

relationship between scorpion toxin gene expression and the 3′ UTR.

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