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Genetic organization of α-bungarotoxins from *Bungarus multicinctus* (Taiwan banded krait): evidence showing that the production of α-bungarotoxin isotoxins is not derived from edited mRNAs

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ABSTRACT

Two genomic DNAs with a size of ~2.8 kb, isolated from the liver of *Bungarus multicinctus* (Taiwan banded krait), encode the precursors of the long neurotoxins, α-Bgt(A31) and α-Bgt(V31), respectively. Both genes share virtually identical overall organization with three exons separated by two introns, which were inserted in the same positions in the coding regions of the genes. Moreover, their nucleotide sequences share ~98% identity. This result indicates that the two genes co-exist in the genome of *B.multicinctus*, and probably arose from gene duplication. The exon/intron structures of the α-Bgt genes were essentially the same as those reported for the short neurotoxins. This reflects that the long and short neurotoxins should share a common evolutionary origin. Comparative analyses on long neurotoxin and short neurotoxin genes showed that the protein coding regions of the exons were more diverse than the introns except for the signal peptide domain. This implies that the protein coding regions of the neurotoxins may have evolved via accelerated evolution. PCR amplification of venom gland cDNA mixtures revealed that only two amino acid sequences corresponding to α-Bgt(A31) and α-Bgt(V31) could be deduced from the cDNAs. The results of chromatographic analyses and protein sequencing again emphasized the view that, with the exception of α-Bgt(A31) and α-Bgt(V31), no other α-Bgt isotoxins with amino acid substitutions were present in *B.multicinctus* venom. In contrast to the proposition of Liu *et al.* (*Nucleic Acids Res.*, 1998, 26, 5624–5629), our findings strongly suggest that each α-Bgt isotoxin is derived from the respective gene, and that α-Bgt RNA polymorphism does not originate from one single, intronless gene by the mechanism of RNA editing.

INTRODUCTION

Venoms from the *Elapidae* and *Hydrophida* snakes contain high amounts of α-neurotoxins. The α-neurotoxins, like curare, bind specifically to the nicotinic acetylcholine receptor (nAchR) on post-synaptic membranes of skeletal muscles to block neuromuscular transmission. Approximately 100 α-neurotoxins from elapid and hydrophid venoms have been fully sequenced to date, and they can be structurally classified into two major groups, short neurotoxins (60–62 residues and four disulfide bonds) and long neurotoxins (66–74 residues and five disulfide bonds) (1). Both classes of neurotoxins share a high sequence similarity. Moreover, X-ray crystallographic and NMR analyses show that all α-neurotoxins adopt an all β-sheet three-finger loop structure, but differ in the extent of secondary structure and positioning of invariant side chains (2–6). Chemical modification and mutagenesis studies on the α-neurotoxins have provided clues to their structure–function relationships (1,7,8). These indicate that several common and invariant residues of α-neurotoxins are involved in the manifestation of their neurotoxicity. A class of toxic proteins, cardiotoxins (60–62 amino acid residues, held together by four disulfide linkages), have also been isolated from snake venoms (9). Alignments of amino acid sequences of cardiotoxins and neurotoxins reveal that their cysteine residues are located at consensus positions. Moreover, cardiotoxins have a three-loop structure as revealed by X-ray crystallographic and NMR studies (10–12). However, cardiotoxins and α-neurotoxins exhibit different pharmacological activities (9). Unlike the well-known neurotoxicity of α-neurotoxins by binding with nAchR, cardiotoxins show no defined cellular targets and pharmacological functions. cDNA sequence analyses showed that the 5′- and 3′-untranslated regions and the signal peptide region of neurotoxin and cardiotoxin cDNAs were highly conserved (13–16), suggesting that neurotoxins and cardiotoxins evolved from a common evolutionary

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+Y17693, Y17694, Y17057, Y17058

origin. Phylogenetic analyses on the amino acid sequences of neurotoxins and cardiotoxins revealed that short neurotoxins resemble cardiotoxins more closely than do long neurotoxins (17). Recent studies showed that the genes of cardiotoxins and short neurotoxins from *Naja naja atra* and *Naja naja sputatrix* shared a virtually identical overall organization with three exons separated by two introns, which were inserted in similar positions in the coding regions of the genes (18–21). Moreover, their nucleotide sequences shared >80% identity. These results strongly support the idea of an evolutionary relationship between cardiotoxins and short neurotoxins. Surprisingly, genetic characterization of the gene encoding α-bungarotoxin (α-Bgt, a long neurotoxin from *Bungarus multicinctus* venom) suggested that the gene was intronless, and that mRNA polymorphism of α-Bgt was derived from a single gene by the mechanism of RNA editing, which resulted in the production of 16 mRNAs encoding 10 α-Bgt isotoxins (22). This seems to reflect that long neurotoxins may have evolved through a different process from those observed for cardiotoxins and short neurotoxins. In order to address this problem, extensive analyses of the genomic structures, cDNAs and isotoxins of α-Bgt have been carried out in the present study. Our results are indicative of the presence of only two α-Bgt isotoxins, α-Bgt(A-31) and α-Bgt(V-31), in *B.multicinctus* venom. Moreover, the two isotoxins are derived from different genes which share virtually identical gene organizations as those reported for short neurotoxins and cardiotoxins.

MATERIALS AND METHODS

PCR amplification and cloning of genomic DNAs encoding α-Bgt precursors

Bungarus multicinctus liver was ground to a fine powder in liquid nitrogen. The genomic DNA was extracted from the powder in the presence of SDS and proteinase K (23).

Two oligonucleotide primers, P1 and P2, based on the promoter region and 3′-non-coding region of the cobrotoxin and cardiotoxin genes (18,19) with the sequences 5′-GTCCAGGTGCCCAGGTTTTGTATG-3′ and 5′-GGATGGTCCTTGATGGATGAGAGC-3′ were synthesized, respectively. In order to specifically amplify the α-Bgt genomic DNAs, gene-specific primers P3 and P4 designed from α-Bgt(A31) cDNA (22) were synthesized with the sequences 5′-AGCGCTGTGACTTGTCCACCTGG-3′ and 5′-ACTTGTCTGTTGAGCAACAGGTAAC-3′, respectively.

The PCR reaction was carried out with 100 µl reaction buffer containing 1 µg genomic DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM dNTP, 50 pmol primers and 5 U *Taq* polymerase. A thermal cycler was used for 30 cycles of reaction under conditions of denaturing at 94°C for 1 min, annealing at 61°C for 1 min and extension at 72°C for 1 min. Two PCR products were amplified by primer combinations P1 and P4 and P2 and P3, respectively.

The PCR products were cloned into vector pCRII according to the TA cloning procedures (Invitrogen, San Diego, CA).

DNA sequencing

Exonuclease III-generated deletion subclones of the genomic DNA containing α-Bgt precursor were prepared using an Erase-a-Base kit (Promega) following the manufacturer's instructions. Sequence analysis was carried out according to the dideoxy method with a sequencing kit (Sequenase Sequencing System; US Biochemical), labeling with [^{35}S]dATP (>1000 Ci/mmol; Amersham). The reaction products were sequenced in a 6% polyacrylamide gel which was dried and exposed on a Kodak film for 1 day at room temperature.

Reverse transcriptase–PCR (RT–PCR) amplification of α-Bgt cDNAs

Cellular RNA was isolated from snake (*B.multicinctus*) venom glands which were stored in liquid nitrogen immediately after killing. Two deep frozen glands from one snake were homogenized to extract RNA with a guanidinium isothiocyanate/phenol/chloroform isolation kit (Stratagene, La Jolla, CA).

Two oligonucleotide primers of sense and antisense orientation based on the signal peptide and 3′-non-coding region of α-Bgt genes with the forward sequence 5′-ATGAAAACTCTGCTGCTGACCTTGGTG-3′ and the reverse sequence 5′-GGATGGTCCTTGATGGATGAGAGC-3′ were synthesized.

RT–PCR was carried out with 100 µl reaction buffer containing 100 mM Tris–HCl (pH 8.3), 1 mM dNTP, 1 µM antisense primer and 200 ng RNA template. In the reverse transcription, the reaction was started with r*Tth* reverse transcriptase (5 U) and 2 µl of 10 mM $MnCl_2$ at 70°C for 15 min, and stopped by placing the tube on ice until needed. An 8 µl chelating buffer containing 50% glycerol (v/v), 100 mM Tris–HCl (pH 8.3), 1 M KCl and 7.5 mM EGTA, 0.5% Tween 20 was added to the reaction. After addition of 8 µl of 25 mM $MgCl_2$ and 1 µM sense primer, the amplification proceeded on a thermocycler at 94, 42 and 72°C for 1 min each, for a total 30 cycles.

The PCR products were cloned into vector pCRII according to the TA cloning procedures (Invitrogen, San Diego, CA).

Primer extension analysis

The synthetic oligonucleotide (5′-CTAAGTCCAGGCACACGATTGTCACC-3′) corresponding to the antisense sequence in exon 1 encoding the signal peptide of α-Bgt was used in the primer extension analysis. Total RNA from *B.multicinctus* venom glands was isolated with a guanidinium isothiocyanate/phenol/chloroform isolation kit. The primer (50 pmol) was hybridized to 6 ng of total RNA in reverse transcription buffer (50 mM NaCl, 34 mM Tris–HCl and 5 mM DTT, pH 8.3) at 85°C for 10 min, followed by 45°C for 12 h. dATP, dGTP and dTTP, each at a final concentration of 0.25 mM, and 8 µCi of [α-^{32}P]dCTP were added to the annealing mixture and incubated with 3 U of MMLV reverse transcriptase in a final volume of 10 µl at 42°C for 15 min. An aliquot of 1 µl of dNTP mixture (2 mM each) was then added to the reaction mixture and chased for another 15 min at 42°C. The reaction was stopped by adding 5 µl of 98% formamide containing 10 mM EDTA, 0.3% xylene cyanol and 0.3% bromophenol blue. The α-Bgt gene served as template for dideoxynucleotide sequencing using the same oligonucleotide primer. The sequencing reaction products were run in parallel with the primer-extended fragments on a 6% polyacrylamide–urea sequencing gel.

Comparison of the nucleotide sequences and homology searches

In the comparison and analysis of the determined nucleotide sequences, a software package (PC/GENE; Stratagene) was

used for sequence alignment based on percent sequence identity. BLAST searches (24) against non-redundant databases were carried out via the Internet using the GCG software package.

Characterization of α-Bgt isotoxins from *B.multicinctus* venom

α-Bgt from *B.multicinctus* venom was isolated and purified as previously described (25). The homogeneity of α-Bgt was verified by SDS–PAGE and native gel electrophoresis. Amino acid sequencing was carried out on an Applied Biosystem 477A protein sequencer. Reduction and *S*-carboxymethylation of α-Bgt was performed essentially according to the method described (19). The reduced and *S*-carboxymethylated (RCM) proteins were then subjected to automated Edman degradation to obtain the partial N-terminal sequence of α-Bgt up to 40 residues. Moreover, RCM-α-Bgt was digested with *Staphylococcus aureus* V8 protease (protein:enzyme, 30:1 w/w) in 0.2 M ammonium bicarbonate (pH 7.8) at 37°C for 3 h. The hydrolysates were separated by HPLC on a SynChropak RP-P column (4.6 × 250 mm) equilibrated with 0.1% trifluoroacetic acid (TFA) and eluted with a linear gradient of 2.5–35% acetonitrile for 70 min. Each peptide fraction derived from the hydrolysates of RCM-α-Bgt was subjected to amino acid composition and sequence determination.

Southern hybridization of chromosomal DNA

After overnight digestion of chromosomal DNA (20 μg) from *B.multicinctus* liver with the restriction enzyme *Pst*I or *Bam*HI, the DNA fragments were separated by electrophoresis in 0.8% agarose gels and transferred onto nylon membranes. Hybridization was carried out according to the method of Sambrook *et al.* (23) with ^{32}P-labeled α-Bgt cDNA.

RESULTS AND DISCUSSION

cDNA sequence analyses on short neurotoxins, long neurotoxins and cardiotoxins suggest that they are evolutionarily related (13–16,22). Meanwhile, comparative analyses on the genetic structures of short neurotoxins and cardiotoxins from *N.n.atra* and *N.n.sputatrix* show that the nucleotide sequences of the promoter and 3′-non-coding regions are highly conserved. Thus, two primers, P1 and P2, were designed from the conserved regions for amplification of genomic DNA encoding α-Bgt precursor. Moreover, in order to specifically amplify α-Bgt genomic DNA, gene-specific primers, P3 and P4, designed from α-Bgt(A31) cDNA (22) were synthesized. PCR amplification of *B.multicinctus* genomic DNA with primer combinations P1 and P4 and P2 and P3 achieved the isolation of two PCR fragments estimated to be ~2.7 and 0.8 kb, respectively (data not shown). The DNA fragments were then subcloned with a TA cloning kit (Invitrogen), and more than 40 clones were selected for nucleotide sequencing. Two genomic DNAs composed of the promoter regions and the exon–intron organizations of the α-Bgt(A31) and α-Bgt(V31) genes were unequivocally constructed from the nucleotide sequences of the PCR products (Fig. 1). Comparing the cDNA sequence of α-Bgt(A31) with the genomic structures allowed us to assign intron/exon boundaries. Alignment of the deduced cDNA sequences of α-Bgt(A31) and α-Bgt(V31) revealed that the one amino acid substitution (Ala→Val) results from one base substitution (GCA→GTA). The genomic DNAs of α-Bgt(A31) and α-Bgt(V31) are 2924 and 2925 bp long, respectively. The transcription initiation sites of the α-Bgt genes were further determined by primer extension analysis. It was found that the transcription initiation site of both genes should be 29 bp upstream from the ATG start codon (data not shown). The nucleotide sequence TATAAATAA, corresponding to the consensus sequence of the TATA box, is located 25–33 bp upstream from the transcription initiation site. These are exactly the same as those reported for the *N.n.atra* cobrotoxin gene (a short neurotoxin genomic DNA from a land snake) and the *Laticauda semifasciata* erabutoxin c gene (a short neurotoxin genomic DNA from a sea snake) (18,19,26).

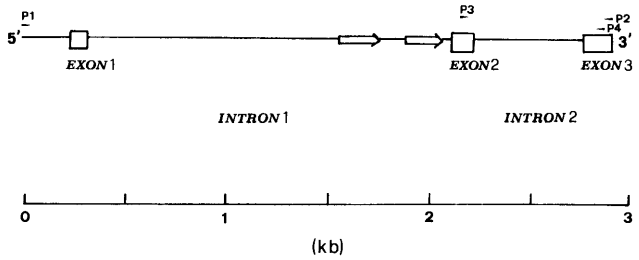

Figure 1. Schematic drawing showing the genetic organization of α-Bgt. The sequence data of genomic structures of α-Bgt(A31) precursor and α-Bgt(V31) precursor have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession nos Y17694 and Y17693, respectively. The tandem repeats are indicated by arrows. P1, P2, P3 and P4 represent the primers used for PCR amplification of genomic DNAs.

The genes encoding the α-Bgt precursors have three exons, and the three exons are interrupted by two introns. The sequences of all the exon/intron junctions agree with the GT/AG rule (27,28). Exon 1 of the α-Bgt genes encodes a large part of the signal peptide. Exon 2 of the α-Bgt genes encodes the latter part of the sequence of the signal peptide (three amino acids) and the N-terminal half of mature α-Bgt (1–32 out of 74 amino acids). Exon 3 encodes the C-terminal half of the toxin and the following 3′-non-coding region. Structurally, the genomic DNAs of the short neurotoxins from *N.n.atra*, *N.n.sputatrix* and *L.semifasciata* are also comprised of three exons and two introns (18,19,21,26). Moreover, the genes of the short neurotoxins were similar to the α-Bgt genes in that the introns interrupted the coding regions in the same positions (according to their amino acid sequences). However, the number of amino acid residues encoded by the exon 2 region of the α-Bgt genes was less than those of the short neurotoxins by one residue (Table 1). The long C-terminal tail observed with long neurotoxins appeared in exon 3. Although the 5′-splice donor was highly conserved (Table 1), a slight difference in the 3′-splice acceptor sequence was observed between the short neurotoxin genes and the α-Bgt genes. Similar sizes for intron 2 were noted [537, 533, 551, 553 and 538 bp for the α-Bgt(A31), α-Bgt(V31), cobrotoxin, ntx-1 and erabutoxin c genes, respectively], but the size of intron 1 of the α-Bgt genes was notably longer than that observed for the short neurotoxin genes (Table 1). Intron 1 of the α-Bgt(A31), α-Bgt(V31), cobrotoxin, ntx-1 and erabutoxin c genes was 1794, 1800, 1269, 1203 and 197 bp, respectively. Obviously, during the evolution of snake neurotoxin genes

Table 1. Comparison of structural organization of short neurotoxin and long neurotoxin genes

	Exon no.	Exon length[a]	5′-splice donor sequence	Intron no.	Intron length	3′-splice acceptor sequence
Bungarus multicinctus α-Bgt(A31)	1	84 (55)	CTTAGgtaag	1	1793	tccagGATAT
	2	105 (105)	ATTCTgtaag	2	537	tgtagGTTCC
	3	157 (125)				
Bungarus multicinctus α-Bgt(V31)	1	84 (55)	CTTAGgtaag	1	1800	tccagGATAT
	2	105 (105)	ATTCTgtaag	2	533	tgtagGTTCC
	3	157 (125)				
Naja naja atra cobrotoxin	1	84 (55)	CTTAGgtaag	1	1269	tccagGATAC
	2	108 (108)	CCGTGgtaag	2	551	cacagGATAT
	3	143 (86)				
Naja naja sputatrix ntx-1	1	84 (55)	CTTAGgtaag	1	1203	tccagGATAC
	2	108 (108)	CCGTGgtaag	2	553	cacagGATAT
	3	301 (86)				
Laticauda semifasciata erabutoxin c	1	84 (55)	CTTAGgtaag	1	197	tccagGATAC
	2	108 (108)	CCGTGgtaag	2	538	cacagGATAT
	3	301 (86)				

[a]The numbers in parentheses indicate the amino acid residues encoded by the exons. The shorter size of the exon 3 regions of α-Bgt and cobrotoxin precursors is due to only a part of the 3′-untranslated region having been determined in the present study.

intron 2 was more conserved than intron 1. This seems to imply that intron 2 may play a more critical role in pre-mRNA stability and/or processing. Although a notable variation in the size of intron 1 was observed for these neurotoxin precursors, it was found that the nucleotide sequences neighboring the splice sites were highly conserved. As listed in Table 2, the sequence identity of intron 1 for the long neurotoxin and short neurotoxin genes was up to 83%. Previous studies revealed that the difference in size of intron 1 of cobrotoxin and erabutoxin c was due to deletion of a part of the intron 1 sequence from the presumed erabutoxin c gene (or addition of these sequences to the cobrotoxin gene) occurring after the evolutionary divergence of the land and sea snakes (18). Moreover, it was found that the inserted segment within the intron 1 sequence of land snake neurotoxins exhibited the structural hallmarks of small nucleolar RNAs (snoRNAs) (18,29,30), which form long sequences (12 and 15 nt) complementary to invariant sequences in eukaryotic 5S rRNA (31–34). Likewise, intron 1 of the α-Bgt(A31) and α-Bgt(V31) genes also retained the structural characteristics of snoRNA genes [at positions 750–854 and 752–856 for the α-Bgt(A31) and α-Bgt(V31) genes, respectively]. Apparently, intron insertions and/or deletions of snoRNA gene sequences occurred in the evolution of land and sea snake venom neurotoxins, but the snoRNA gene sequences were not deleted in the evolution of land snake neurotoxins. The finding of the presence of a snoRNA gene in *N.n.sputatrix* neurotoxin genes (21) again supports this suggestion. Alignment of intron 1 of the cobrotoxin and α-Bgt genes showed that an inserted segment of ~530 bp appeared exclusively in close proximity to the 3′-splice acceptor site of the α-Bgt genes. The inserted segment is composed of two tandem repeats with a length of ~200 bp [Fig. 1, positions 1565–1764 and 1886–2089 of the α-Bgt(A31) gene, positions 1566–1766 and 1893–2096 of the α-Bgt(V31) gene] and sequence identity up to 98%. However, BLAST sequence similarity searches against the GenBank database showed that the sequence of tandem repeats did not have any significant similarity to known genes.

Table 2. The similarity level in α-Bgt(A31), cobrotoxin and erabutoxin c genes

Gene segment[a]	Similarity (%)		
	α-Bgt/CBTX[b]	CBTX/ETXC[b]	α-Bgt/ETXC[b]
5′-Flanking region	90.3	89.3	91.7
Exon 1	98.8	97.6	98.8
Intron 1	83.5	91.4	96.4
Exon 2	65.7	74.1	58.1
Intron 2	88.5	87.5	85.1
Exon 3	66.4	79.0	63.6

[a]Comparison of the related parts of *B.multicinctus* α-Bgt(A31) gene sequences with gene sequences from *N.n.atra* cobrotoxin and *L.semifasciata* erabutoxin c. The intron and exon designations correspond to the α-Bgt(A31) gene.
[b]CBTX and ETXC represent cobrotoxin and erabutoxin c, respectively.

In order to further characterize the presence of isoformic cDNA sequences of α-Bgt, two primers designed from the signal peptide and 3′-untranslated regions of the α-Bgt genes (Fig. 1) were used to amplify the α-Bgt cDNAs. PCR amplification of venom gland cDNA mixtures with the designed primers achieved the isolation of a PCR fragment estimated to be ~300 bp. The DNA fragments were then subcloned with a TA cloning kit, and more than 100 positive clones were

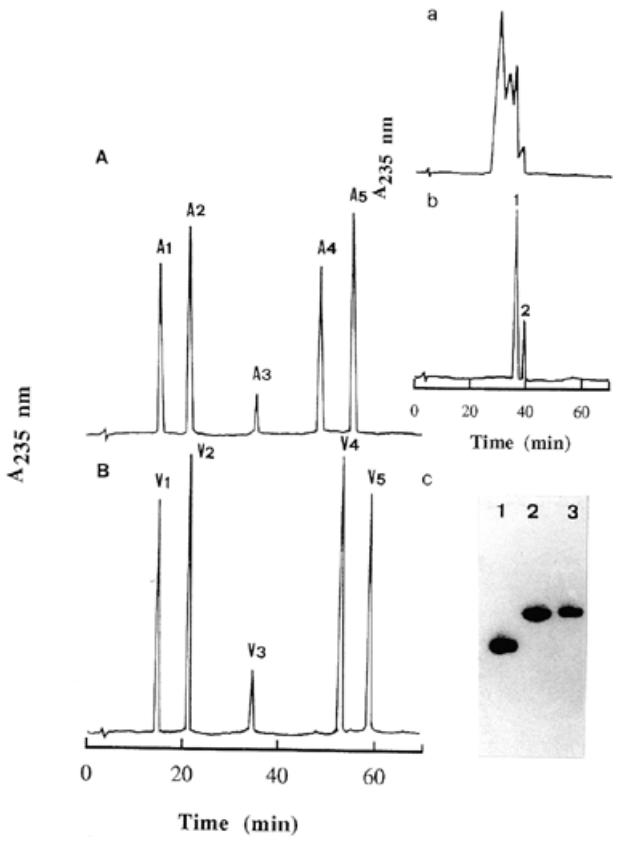

Figure 2. Separation of the peptide fragments of RCM-α-Bgt(A31) and RCM-α-Bgt(V31) after hydrolysis with *S.aureus* V_8 protease. The proteolytic hydrolysates of (**A**) RCM-α-Bgt(A31) and (**B**) RCM-α-Bgt(V31) were applied to a SynChropak RP-P column (4.6 × 250 mm) equilibrated with 0.1% TFA and eluted with a linear gradient of 2.5–35% acetonitrile for 70 min. The flow rate was 0.8 ml/min and the effluent was monitored at 235 nm. (Inset a) HPLC analysis on the purified α-Bgt from a SP-Sephadex C-25 column (25). (Inset b) Separation of RCM-α-Bgt. For both insets (a) and (b) the samples were applied to a SynChropak RP-P column equilibrated with 0.1% TFA and eluted with a linear gradient of 5–40% acetonitrile for 70 min. The flow rate was 0.8 ml/min and the effluent was monitored at 235 nm. (Inset c) Native gel analyses of native α-Bgt (lane 1), RCM-α-Bgt(A31) (lane 2) and RCM-α-Bgt(V31) (lane 3).

selected for nucleotide sequencing. Only two amino acid sequences corresponding to α-Bgt(A31) and α-Bgt(V31) could be deduced from the cDNAs. The cDNA sequences (DDBJ/EMBL/GenBank accession nos Y17057 and Y17058) are essentially the same as those deduced from the α-Bgt genes.

Purification of α-Bgt from *B.multicinctus* venom was carried out essentially according to the procedure described previously (25). Although the homogeneity of purified α-Bgt was verified by SDS–PAGE and native gel electrophoresis analysis, α-Bgt did not elute from a reverse phase column as a single peak (Fig. 2, inset a). This probably arose from a fast exchange conformational process of α-Bgt in the reverse phase HPLC column (35). After α-Bgt was subjected to reduction and *S*-carboxymethylation, two RCM proteins were separated by HPLC on a SynChropak RP-P column (Fig. 2, inset b). Each fraction was subjected to amino acid sequencing to obtain their partial N-terminal sequences. The determined N-terminal sequences up to 40 residues revealed that peaks 1 and 2 were α-Bgt(A31) and α-Bgt(V31), respectively (Fig. 2, inset b). Native gel analyses showed that no significant differences in electrophoretic mobility of RCM-α-Bgt(A31) and RCM-α-Bgt (V31) were observed (Fig. 2, inset c). The RCM proteins were digested with *S.aureus* V8 protease, then the hydrolysates were separated into five peptide fractions (Fig. 2). The results of amino acid composition and sequence determinations revealed that A1, A2, A3, A4 and A5 were the amino acid residues at positions 57–74, 42–56, 1–20, 21–41 and 1–41 of α-Bgt(A31), respectively. Likewise, V1, V2, V3, V4 and V5 were the segments at positions 57–74, 42–56, 1–20, 21–41 and 1–41 of α-Bgt(V31), respectively.

In the present study, two genomic DNAs were identified encoding the α-Bgt(A31) precursor and the α-Bgt (V31) precursor, respectively. Since the genes for the α-Bgt isotoxins were present independently in the *B.multicinctus* genome, the genetic structures reported here clearly exclude the possibility that the α-Bgt mRNAs are produced by alternative splicing. The yield of α-Bgt(V31) was ~30% of that of α-Bgt(A31), as revealed by HPLC analysis on the preparation of RCM-α-Bgt (Fig. 2, inset b). This suggests that the production of α-Bgt(A31) and α-Bgt(V31) is differently regulated at the transcriptional or/and translational level. The findings that the α-Bgt(A31) and α-Bgt(V31) genes share the same exon–intron organization and a high degree of nucleotide sequence identity (98%) suggests that the two genes arose from a common ancestor by gene duplication and probably co-exist in the snake genome. Analyses of cDNAs revealed the presence of two mRNA species encoding α-Bgt(A31) and α-Bgt(V31), respectively. The results of protein sequencing further supported this finding. These clearly indicate that each α-Bgt isotoxin should be derived from the respective gene. Apparently, our findings are not in agreement with the results of Liu *et al.* (22) showing that the production of 10 α-Bgt isotoxins and 16 polymorphic mRNAs arose from one single and intronless gene by the mechanism of RNA editing. Notably, the 10 α-Bgt isotoxins deduced from cDNA sequences were not verified by protein sequencing in their study (22). Only two α-Bgt isoforms, α-Bgt (A31) and α-Bgt(V31), had been isolated from *B.multicinctus* venom previously (5). Although a 'high fidelity' *Pfu* DNA polymerase was employed for PCR amplification in the studies of Liu *et al.* (22), it is worth noting that *Pfu* enzyme has only a 12-fold higher fidelity of DNA synthesis compared to *Taq* polymerase (36). Thus, the finding of 16 polymorphic cDNAs in the study of Liu *et al.* (22) may be attributed to PCR cloning artifacts or DNA sequencing artifacts. Analyses of the restriction maps of the α-Bgt(A31) and α-Bgt(V31) genes show that they share a common one. Southern blot analysis of *Bam*HI-digested chromosomes revealed the appearance of a single band with a size of ~4 kb (Fig. 3). Alternatively, two bands with sizes of ~2 and 7 kb were visualized on Southern blot analysis of *Pst*I-digested chromosomes. This is in agreement with the restriction maps of the α-Bgt genes, which have *Pst*I sites in the first intron [at positions 973 and 984 of the α-Bgt(A31) gene and at positions 975 and 986 of the α-Bgt(V31) gene], but do not contain a *Bam*HI site within the region encoding the α-Bgt precursors. In view of the fact that the α-Bgt cDNAs and 16 polymorphic cDNAs that Liu *et al.* (22) characterized do not contain *Pst*I sites, the results of Southern blotting again emphasize that the α-Bgt genes should have an exon/intron

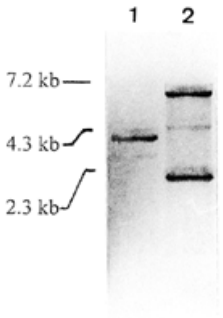

Figure 3. Southern blot analysis of *Bam*HI or *Pst*I digests of *B.multicinctus* genomic DNA with α-Bgt cDNA as probe. Lane 1, *Bam*HI digests of genomic DNA; lane 2, *Pst*I digests of genomic DNA.

organization. The genetic organization of α-Bgt determined in the present study is essentially the same as those reported for short neurotoxins and cardiotoxins, which were organized as three exons and two introns. Obviously, the proposal of Liu *et al*. (22) that the protein coding region of α-Bgt was not interrupted by introns is incorrect. In terms of these observations, it is evident that the production of α-Bgt(A31) and α-Bgt(V31) in *B.multicinctus* venom glands is not from edited mRNAs.

The common genetic organization of long neurotoxins and short neurotoxins supports the view that they share a common evolutionary origin. As listed in Table 2, the exon regions of the short neurotoxin and long neurotoxin genes were more diverse than the intron regions. This reflects the fact that the exon and intron regions evolved via different processes. Previous studies on the genes encoding snake venom phospholipase A2 from different species revealed that the protein coding regions of phospholipase A2 isozyme genes were unusually variable, and that the introns of venom gland phospholipase A2 isozyme genes had evolved at a similar rate and were highly conserved (37–42). Our data indicate that the genes encoding neurotoxin precursors are in good agreement with this observation. These results probably suggest that intron regions are superior to exon regions for assessing the evolutionary relationships of homologous proteins.

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