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Genetic organization of A chain and B chain of β-bungarotoxin from Taiwan banded krait (*Bungarus multicinctus*)

A chain genes and B chain genes do not share a common origin

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β-Bungarotoxin, the main presynaptic neurotoxin purified from the venom of *Bungarus multicinctus*, consists of two dissimilar polypeptide chains, the A chain and the B chain, cross-linked by an interchain disulfide bond. In this study, A and B chain genes isolated from the liver of *B. multicinctus* encoded the A and B chain precursors, respectively. Analyses of the coding regions of the A and B chain genes revealed that both consist of three exons and two introns. The sequences of all exon/intron junctions agree with the GT/AG rule. However, sequence alignment and phylogenetic analysis did not support that the evolution of A and B chain genes are closely related. Comparative analysis of A chain genes with Viperinae and Crotalinae phospholipase A_2 genes indicated that genetic divergence of the A chain and phospholipase A_2s was in accordance with their family. Moreover, evolutionary divergence of the intron and exon regions of the A chain, as observed for phospholipase A_2 genes, was not consistent. Noticeably, the transcription of A and B chain genes may be regulated under different transcription factors as revealed by analyses of their promoter sequences. In terms of the finding that A and B chains are encoded separately by different genes, this strongly supports the view that the intact β-bungarotoxin molecules should be derived from the pairing of A and B chains after their mRNAs are translated.

Keywords: A chain; B chain; β-bungarotoxin; evolutionary divergence; genetic organization.

β-Bungarotoxin (β-Bgt), the main presynaptic neurotoxin purified from the venom of *Bungarus multicinctus* (Taiwan banded krait) [1–3], is one of the most investigated presynaptic neurotoxins from snake venoms. The toxin consists of two dissimilar polypeptide chains, the A chain with 120 amino-acid residues and the B chain with 61 residues, cross-linked by an interchain disulfide bond, and possesses a weak phospholipase A_2 (PLA_2) activity [4,5]. The amino-acid sequences of A chains are structurally homologous with those of PLA_2s from snake venoms and mammalian pancreas [2,3]. B chains share sequence similarity with the trypsin inhibitor, toxin I, toxin K and dendrotoxin [6–8]. Kondo *et al*. [3] and Kini & Iwanaga [9] suggested that the A chain is an active subunit that is responsible for the PLA_2 enzymatic activity and neurotoxic effect of β-Bgt, while the B chain may serve as a recognition subunit of the toxin to a specific target cell membrane. Nevertheless, Benishin [10] suggested that the B chain might be responsible for the blockage of certain voltage-gated potassium channels in a manner that did not involve the A chain. Recent studies on the recombinant B chain supported this suggestion [11].

The molecular structures of PLA_2s with presynaptic neurotoxicity are quite varied. Some of these structures are single polypeptide chains, such as notexin and ammodytoxin, whereas others are binary (β-Bgt, crotoxin), ternary (taipoxin) or pentanary (textilotoxin) complexes [12]. Besides β-Bgt and the two textilotoxin D subunits, the complex toxins did not possess an intermolecular disulfide bridge to link their subunits. This seems to reflect that the interchain disulfide bond between the A and B chains of β-Bgt might play a role in the biological activities of β-Bgt. The finding that the recombinant A chain exhibited lower activity than β-Bgt supported this proposition [13,14].

Crotalinae and Viperinae single polypeptide PLA_2 cDNAs and genomic DNAs have been reported previously [15–22]. The genomic structures of Crotalinae PLA_2s contained four exons and three introns [15–19,22], whereas those of Viperinae PLA_2s comprised five exons and four introns [20,21]. Phylogenetic analyses reveal that they might share a common evolutionary origin [18,22]. However, the presynaptic PLA_2 neurotoxin genes composed of multiple chains remain to be determined.

Although seven β-Bgt isotoxins have been identified previously [2,3,23,24], Chu *et al*. [25] observed that > 16 β-Bgt isotoxins could be isolated from *Bungarus multicinctus* venom. To date, four A chain cDNAs and three B chain cDNAs have been determined [11,13,14,26,27]. These observations reflect the multiplicity of β-Bgt isotoxins. In order to elucidate the evolutionary diversification of A and B chain genes and the possible pairing mechanism of A and B chains, determination of the genetic structures of the A and B chains was carried out in this study.

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Abbreviations: β-Bgt, β-bungarotoxin; PLA_2, phospholipase A_2.

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MATERIALS AND METHODS

Preparation of genomic DNA from *B. multicinctus* liver

B. multicinctus livers were ground to a fine powder in liquid nitrogen. The genomic DNA was extracted from the powder in the presence of SDS and protease K [28].

PCR amplification of A and B chain genes

The sense and antisense primers for the A and B chains were designed from the sequences on the signal peptide region, 3′-UTR and protein-coding region of A and B chain cDNAs [11,26,27]. The forward sequence 5′-GCAGTTTGTG-TCTCCCTCTTAGGA-3′ and reverse sequence 5′-TAAGTGC-CACAGTCCTTGCGCTGAAG-3′ were designed for PCR amplification of the A chain gene. The forward sequence 5′-ATGTCTTCTCGAGGTCTTCTTCTCC-3′ and reverse sequence 5′-TTAGGGATACACAAGACACTCGCTGCG-3′ for the B chain gene were synthesized. PCR amplification of A and B chain genes was performed in 50 μL with 0.5 μg genomic DNA, each dNTP at 10 mM, 50 pmol of each primer, 2.5 units of *Taq* DNA polymerase and buffer provided by the enzyme supplier (Promega). Optimization of the PCR led to the following step: initial denaturation 1 min at 94 °C, 30 cycles of 1 min at 94 °C denaturation, 1 min at 60 °C annealing and 1 min at 72 °C extension, and a final extension at 72 °C for 40 min. The resulting PCR products were ligated directly into a pCRII vector using a TA cloning kit (Invitrogen) for sequencing. The PCR-amplified A and B chain genes were employed as probes for *B. multicinctus* genomic library screening and Southern blotting analysis.

Screening of the *B. multicinctus* genomic library

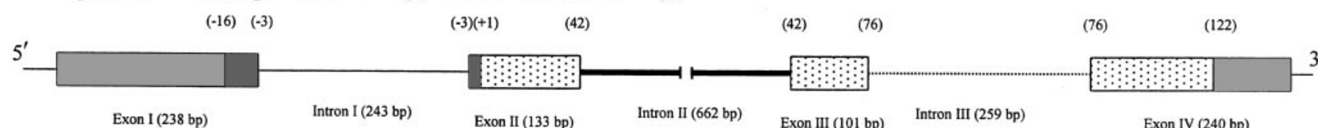
Genomic DNAs were partially digested with *Sau*3AI and filled with dATP and dGTP. The fraction composed of 15–20-kb fragments was ligated with λGEM-12 arms (Promega), and the ligated DNAs were packaged using packaging extracts (Promega) according to the manufacturer's procedures. The genomic library was amplified once in *Escherichia coli* strain KW251 and the titers were up to 10^9 plaque forming units (p.f.u.).

Probes were prepared by ^{32}P-labeling the gene encoding A1 chain precursor or B1 chain precursor using a random-priming kit (Promega). Hybridization was carried out at 42 °C for 16–18 h in hybridization buffer mixtures (CLONTECH). The Hybond-N membranes (Amersham Pharmacia Biotech) were washed successively with 2 × NaCl/Cit, 1 × NaCl/Cit and 0.5 × NaCl/Cit at room temperature for 20 min and 65 °C for 20 min. Positive clones were rescreened following the same procedure described above.

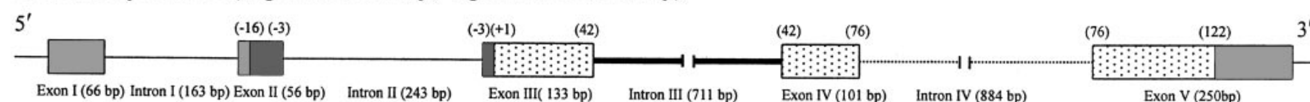
Southern blotting analysis

Phage DNA was prepared from bacterial lysates and digested with *Bam*HI, *Xba*I and *Nde*I, respectively. The resulting

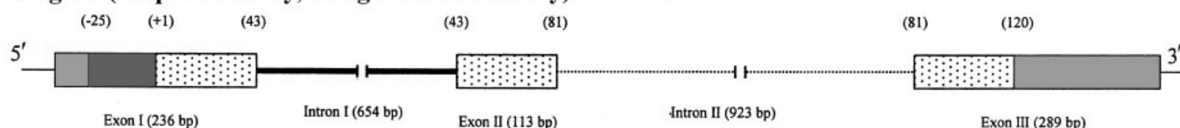
A Mojave toxin b (Viperidae family, Crotalinae subfamily)



B Ammodytoxin C (Viperidae family, Viperinae subfamily)



C A chain-like gene (Elapidae family, Bungarinae subfamily)



D

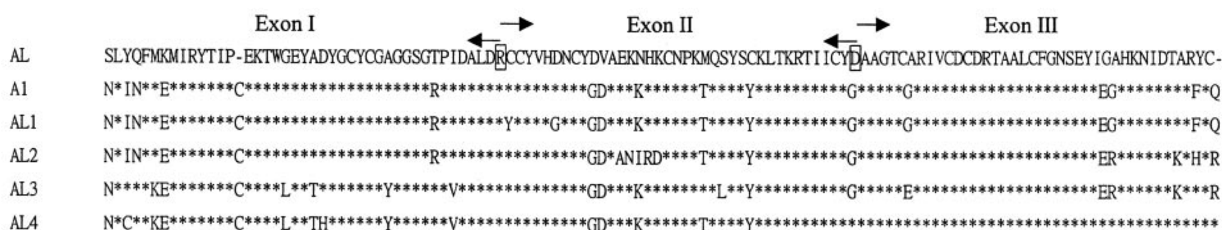


Fig. 1. Schematic representation of the structures of PLA_2 genes and the A chain-like gene. (A) Mojave toxin b gene (U01027), (B) ammodytoxin C gene (X76731), (C) A chain-like gene (AJ251226). Boxes represent exons and lines represent introns. Intron I of the A chain gene corresponds to intron III of ammodytoxin C and intron II of mojave toxin b. Intron II of the A chain gene corresponds to intron IV of ammodytoxin C and intron III of mojave toxin b. (D) Alignment of protein sequences of A chains. The conserved amino-acid residues are indicated by asterisks. AL, AJ251226; A1, AJ251360; AL1, AJ251227; AL2, AJ251220; AL3, AJ251221; AL4,AJ251222. It is worth noting that the proteins encoded by AL1, AL2, AL3 and AL4 genes have a conserved Cys15, which forms a disulfide linkage with Cys55 of the B chain. However, one more Cys residue appearing at position 3 of the AL4 protein may change the pairings of disulfide linkages, and consequently the resulting products.

fragments were separated by gel electrophoresis on a 0.8% agarose gel, then transferred to nitrocellulose membranes and hybridized with ^{35}S-labeled A1 chain gene or B1 chain gene at 42 °C. Positive genomic fragments were subcloned into pUC18 and their nucleotide sequences determined.

DNA sequencing analysis

Sequencing analysis was performed using the dideoxy method with a sequencing kit (Sequenase sequencing system, Amersham Pharmacia Biotech) labeling with [^{35}S]dATP (Amersham Pharmacia Biotech; 1000 Ci·mmol^{-1}). The reaction products were sequenced in a 6% (w/v) polyacrylamide gel, which was dried and exposed on Kodak film for 16–18 h at room temperature.

Comparison of nucleotide sequence and homology search

In the comparison and analysis of the determined nucleotide sequences, a software package (PC GENE program, Stratagene Ltd) was used for sequence alignment based on percent sequence identity. The phylogenetic tree was constructed using a SEQWEB software package. BLAST searches against nonredundant databases were carried out via the Internet using software on the website (http://www.ncbi.nlm.nih.gov/).

RESULTS AND DISCUSSION

PCR amplification of the genes encoding A and B chain precursors

PCR products of ≈ 2 kb were amplified using primers designed from A chain cDNAs (data not shown). Fragments were subcloned into a pCRII vector for DNA sequencing, and five genes encoding A chain precursors (accession nos. AJ251227, AL1; AJ251220, AL2; AJ251221, AL3; AJ251222, AL4; AJ251360, A1) were determined. Alternatively, the gene encoding B chain precursor could not be amplified by the primers designed from signal peptide and 3′-UTR of B chain cDNAs. However, a fragment estimated to be 0.8 kb was amplified successfully using a pair of primers designed from the signal peptide and the protein-coding region of the B1chain cDNA. DNA sequencing analysis showed that this fragment represented part of the B1chain precursor.

Screening of A and B chain genes from *B. multicinctus* genomic library

Because the complete A and B chain genetic structures could not be constructed by PCR amplification, screening the *B. multicinctus* genomic library with ^{32}P-labeled A1 or

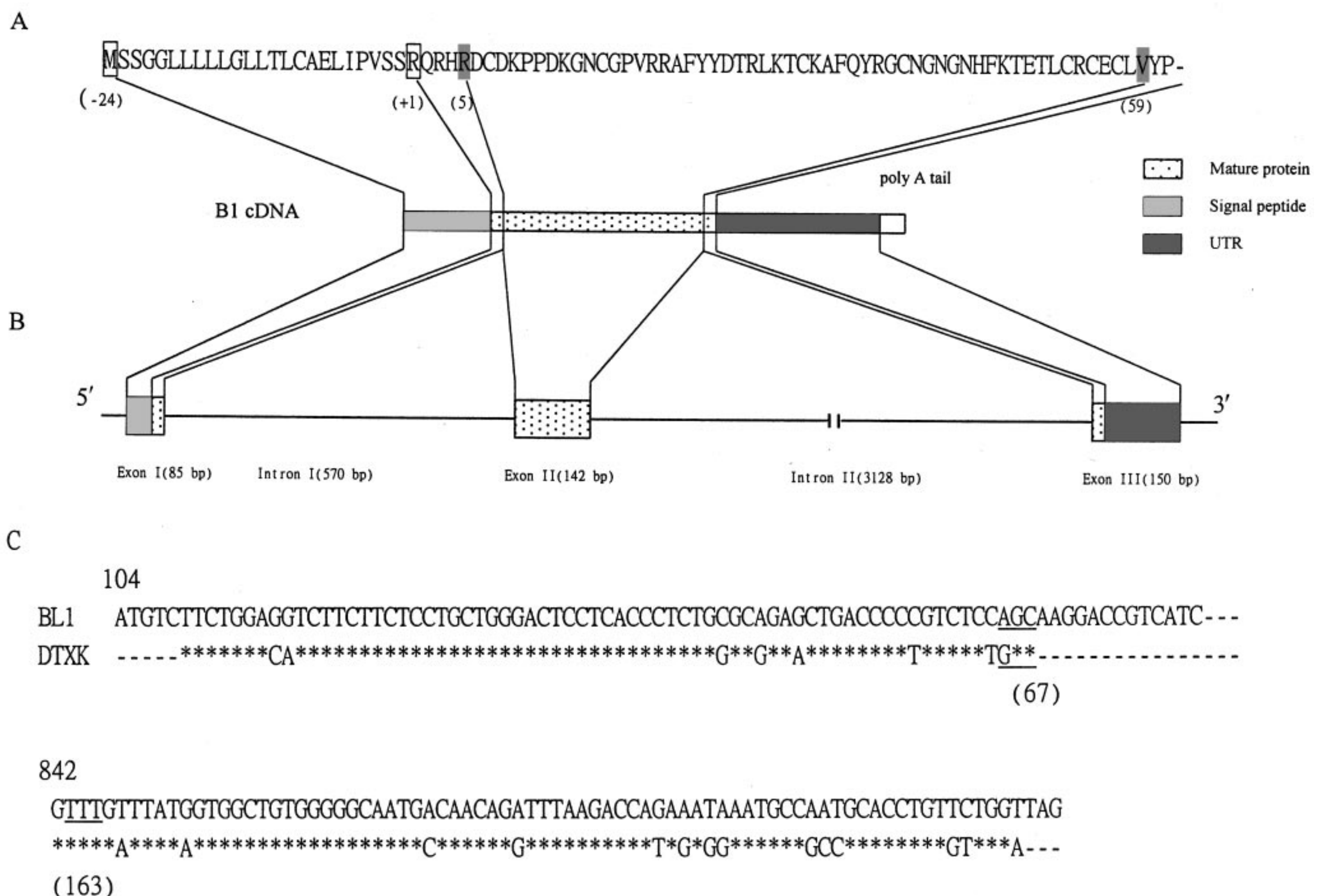


Fig. 2. Gene organization of the B1 chain. (A) The amino-acid sequence of the B1 chain. The cDNA sequence of the B1 chain was deposited in the DDBJ/EMBL/GenBank database under accession no. Y121000. The first amino acid in the signal peptide (−24) and mature B1chain (+1) is boxed. The B1chain gene interrupted by introns are at positions 5 and 59 in the mature protein. (B) Genetic structure of B1chain (AJ251223). Boxes and lines represent exons and introns, respectively. (C) Sequence alignment of the BL1gene (AJ251225) with dendrotoxin k (DTXK) cDNA (S61886). Numbers indicate the positions at the nucleotide sequence of the BL1gene and dendrotoxin k cDNA.

Table 1. Organization of A chain and PLA_2 genes.

Species and gene	Exon number	Exon length (bp)	5′ Splice donor sequence	Intron number	Intron length (bp)	3′ Splice acceptor sequence	Intron phase	Amino acid interrupted
Bungarus multicinctus	I	236	GATAGgtaag	I	654	gccagGTGCT	II	Arg43
A chain-like gene[a]	II	113	CTATGgtaaa	II	923	tccagATGCC	I	Asp81
	III	289						
Vipera ammodytes	I	66	CAGCTgtaag	I	163	tccagGTCTG		
ammodytoxin C gene	II	56	AGGCGgtgag	II	243	tgcagTCGAG	I	Val(−3)
	III	133	GACCGgtaag	III	711	tccagCTGCT	II	Arg42
	IV	101	CTGTGgtgag	IV	884	tgcagGAAAA	I	Gly76
	V	250						
Crotalus scutulatus	I	238	GGGCGgtgag	I	243	tccagTCGAG	I	Val(−3)
mojave toxin b gene	II	133	GACCGgtaag	II	662	tccagCTGCT	II	Ser42
	III	101	CTGCGgtgag	III	259	tgcagGAAAG	I	Gly76
	IV	240						
Trimeresurus gramineus	I	188	GGGCGgtaag	I	243	tccagTTGAG	I	Val(−3)
$gTgPLA_2$-V gene	II	133	GACCGgtaag	II	679	tgcagCTGCT	II	Arg42
	III	101	CTGCGgtgag	III	259	tgcagGAGAG	I	Gly76
	IV	249						
Trimeresurus okinavensis	I	242	GGGCGgtgag	I	243	tccagTTGAG	I	Val(−3)
$gPLA_2$-01 gene	II	124	GACCGgtaag	II	674	tccagCTGCT	II	Arg39
	III	101	CTGCGgtgag	III	259	tgcagGAGGG	I	Gly73
	IV	146						

[a] The accession no. is AJ251226. The first exon is assigned according to the A2chain cDNA (accession no. X53407).

B1 chain genes (the A1 and B1 chain genes were amplified by PCR reaction) was conducted. More than 20 positive phage DNAs were subjected to digestion with *Bam*HI, *Xba*I and *Nde*I, respectively. Agarose gel electrophoresis results indicated that the enzyme-digested positive phage DNAs supposedly containing A chain genes exhibited the same restriction pattern (data not shown). A positive DNA fragment ≈ 4 kb long was identified by Southern blotting. This DNA fragment was subcloned into pUC18 for sequencing. It was found that the 4 kb fragment contained an A chain-like gene encoding an

A

Sn STE12

-692 TTATAAGCTT GAGGATCCCC AGTTGTACAA ACTCAACCTG TTTCATAGCA ACACAGAACA

G6-factor

-632 ACAGAGTTGG AAGGGGCCTT GGAGGTCATC CAATCCAACC CCCCCATTTC ATCCATTCCC

-572 AGTCCACTGT AATGATAGTC CTCGACTTAC AATGACCATC AGGGACCATA ATTTCCATCA

-512 CTAAGCAAAG CGAATATGCG AAATGACTGG TGTCCAATTT TACAAGCTTG GTTTTGGGTT

-452 CCTTAGCGCA GTTCTGAAAA AGGAACTACA AAGTCCTTCC GTGAACCCAA CTTCTCCTGT

-392 TGACTTTGCT GGTCGGAAGC CGGCTGGGAA GGGGGCAAAC AGCGAGAAGT GGGCCGGTGC

-332 AAATGCATCA CCCTAGGAGA CAGATGATCT TCCAAAAAGA GGGAAGGTTC ACCCAGAGCA

-272 ATGCGGTAGC TGAGAGATAG AGCTCTCGCC TCACAATCAG GTGGCTGAGA GTTCAATCCT

GAGA-factor Sox-5

-212 AGGTAGAGGC AGATATTTCT CTCCCTCTCG GCACAAGGAC AATATATCTG CTGAACAATA

-152 CTCCGCGCTA GTGACAGGAA TGGCATCCCG CCAGTGAACA TTCTGCTAGC TCCATTCAGT

Bcd

-92 TGCCCAGACT CCACGCCGCA AGGGATTACG GGGTCTCTAA CCGCCACTTA GCAGGGAACT

LyF-1

-32 GGAATCGGCT GATGGATATT TGGGAAACAC TA

Fig. 3. Analyses of the putative regulatory elements appeared in the promoter regions of the A chain and B chain genes. (A) Promoter sequence of the A chain-like gene (B) Promoter sequence of the B1 chain gene. Putative regulatory sites and their corresponding elements are indicated by boxes, gray boxes, italic and underlining. The program used to search the putative regulatory elements was BCM gene feature searches, which is freely available at the website http://dot.imgen.bcm.tmc.edu:9331/seq-search/gene-search.html.

B

-934 GGATCCAGTA GAAGTTCAAC AGAAGTTCAA ATTTGGTGGG CTCTTGGGGA ATAACGCCAA
-874 GAGGTCAGAG TGTTACACTG AAGTCCATAA GCCCTAATAA TGGAAAGGAG AGGAGATGCT

GATA-1 myc-CF1
-814 GTTGAAGAAT GGATTTTAAA ATGGATTGAA TTGGCAGAGA TGGCAAAATG ATTAGAGATA

SRF Elk-1 TFIID CD28RC/NF-kappaB
-754 ATGGACTAGA CGGCTTTATA AGAGACTGGA AACCTTTTAT GGACTTTCTA CTTGTCAACC
-694 CCAATAGCAG ACAGTGAACA GGGGGATTTG AAGTCTAGCG TAATATTTGG TTTAAGTTGC

TBP NF-IL-2A NFAT-1/Pu_box_binding_factor CF2-II
-634 TGTCTGTTTT ATATATTTTT CCTTTTCTCT TTTGTATATA TATTAAATGC TGAGTTGATT
NFH3-1

Sox-5 HFH-1/HFH-2
-574 CGACTATTAG ATAGGGACCT CCTCATCTGA AATATTTGTT GATATAATAT ATTGTTTGTG

Elf-1 BR-C Z4
-514 TTAAAGTTTG TAACTGTTTT GTGAGAAAGT TTATAACTAT TGACAGATCC AGATAATAAA

Pit-1a HFH-2 HNF-3B Croc NF-1
-454 GAGAATTGAT CAACTGTTTA TTTTAAAGGT TTACTTTTCA GCACCCAGCC AGGGTGGGTG
MEF-2 TFIID

NF-IL-2A BR-C_Z4 NFAT-1
-394 TTTTATATGT TTAATGTTGG ATATTTCTCT TTTTCTTTTC TTCTTTTTGG TACACATTTT

BR-C_Z2 BR-C_Z4
-334 GTCATTGTTC CTACTACTGG ATCTTTTTCG TTTTGCTTTT TCTATTTTAT TTCCATTGAT

GC1/Pit-1a
-274 AAATGTAGAG ATGAAAAAAT AAAGTCCTTA AACTGCTAAT ATGTGTGCGA GTGGGACTGG
-214 AATAGGTTAA CAGGCTAACT TCCCTGCCTA ACTTCCCCCA AACTGGCCAG CCAATCCAAC
-154 TCTCAGATTC TCCAAACTTG TTCGACCACC TTCTCAAAGA GGAAGGAATT CGGATCCTCA
-94 CTGCATTATG TCACCCTGCA TCCGGGATCT GGAGGTGGGG AATGGGGAGA ATAAATAGAG
-34 CCAGCAGCCC GAGGGTGGTG TAGAGAGCTT CATC

Fig. 3. continued.

immature A chain (accession no., AJ251226; Fig. 1). The remaining positive clones also contained the same A chain-like gene. In contrast, two positive DNA fragments of ≈ 6.2 kb and 1.3 kb, respectively, were identified using Southern blotting with ^{35}S-labeled B1chain gene. DNA sequencing analysis showed that the 6.2-kb DNA fragment encoded the complete B1chain precursor (accession no., AJ251223; Fig. 2). Alternatively, the 1.3-kb DNA fragment (designated BL1gene, accession no., AJ251225) had a high level of sequence homology with B1chain genes and contained segments corresponding to exon I, intron I and exon II of the B1chain gene. The BL1gene signal peptide shares 97.2, 97.2 and 88.1% sequence homology with the B1chain cDNA, B2chain cDNA and dendrotoxin k cDNA, respectively. Moreover, the homology of intron I of the B1chain gene and BL1gene is up to 92.4% identified. Notably, the BL1gene region corresponding to the second exon of the B1chain gene contains a segment that shares 79.7% sequence homology with the protein-coding region of dendrotoxin k cDNA [29]. This was higher than that (60% homology) observed between B1(B2) cDNA and dendrotoxin k cDNA (Fig. 2).

Organization of A chain genes

Alignment of the A chain genes amplified by PCR reaction with A chain cDNA sequences revealed that the A chain genes may contain three exons and two introns (Fig. 1). This suggestion was supported by the finding that the determined A chain-like gene (AJ251226), at 3022 bp, contained three exons interrupted by two introns. Moreover, the nucleotide

sequences of the A chain genes and A chain-like gene show up to 92% similarity. Accordingly, exon I of the A chain precursors encodes the 5′-UTR, signal peptide and amino-acid residues 1–43, exon II encodes protein residues 43–81 and exon III encodes protein residues 81–120 and 3′-UTR (Fig. 1). It was found that the signal peptide of the A chain-like gene is composed of 25 amino-acid residues. This is the same as that observed with the A2 cDNA sequence (25 amino-acid residues), but different to that of the A4 cDNA sequence (27 amino-acid residues) [26,27]. However, the high level of sequence identity in the exons and introns indicates that A chain genes and the A chain-like gene arose by duplication and divergence from a common ancestral gene.

Analysis of the protein-coding region of the A chain-like gene (AJ251226) revealed that it encoded an immature A chain protein. There are base substitutions which occurred at positions 15 and 120 of the mature A chains (Fig. 1). The substitutions led to a stop codon (TGA) and were expected to produce a mRNA which would not be able to translate into a mature protein. Likewise, Nobuhisa *et al*. [19] observed that the base substitution or insertion which occurred within *Trimeresurus okinavensis* PLA_2 genes, $gPLA_2$-02 and $gPLA_2$-03, inactivated the genes. Previous studies have shown that the production of a multiplicity of PLA_2s proceeded via accelerated evolution of exon regions [16–19,22,30]. These base substitutions probably represented one of the steps taken during the evolution of A chain genes and PLA_2 genes.

The genetic organization of Viperinae and Crotalinae PLA_2s had been reported previously [15–22]. It has been shown that Viperinae PLA_2s, such as *Vipera ammodytes* ammodytoxin C, consist of five exons and four introns, while Crotalinae PLA_2s, such as *Crotalus scutulatus* mojave toxins, *T. gramineus* $gTgPLA_2$s and *T. okinavensis* $gPLA_2$s, consist of four exons and three introns. However, the A chain genes determined in this study comprised three exons and two introns. Both Viperinae and Crotalinae are subfamilies of the Viperidae family. *B. multicinctus* is a member of the Bungarinae subfamily of the Elapidae family. In terms of these observations, it is reflected that the genetic structures of PLA_2s from different families (subfamily) have a different organization (Fig. 1 and Table 1). According to the splicing sites of protein-coding regions, the first intron of A chain genes corresponds to the third intron of Viperinae PLA_2 genes and the second intron of Crotalinae PLA_2 genes (Fig. 1). A length of 654, 711 and 662 bp was observed for intron I of the A chain, intron III of ammodytoxin C and intron II of mojave toxin b, respectively. The sequence homology between intron I of the A chain and intron III of ammodytoxin C, between intron I of the A chain and intron II of mojave toxin b, and between intron II of mojave toxin b and intron III of ammodytoxin C is 50.9, 45 and 88.2%, respectively. Alternatively, the second intron of the A chain gene is located in a position homologous to that occupied by the fourth intron of Viperinae PLA_2 genes and the third intron of Crotalinae PLA_2 genes. However, a notable discrepancy in length is noted. The sizes of intron II in the A chain, intron IV of Viperinae PLA_2 and intron III of Crotalinae PLA_2 are 923, 884 and 259 bp, respectively. The sequence homology of intron II in the A chain with intron IV of ammodytoxin C, intron II of the A chain with intron III of mojave toxin b and intron III of mojave toxin b with intron IV of ammodytoxin C is 49.9, 85.3 and 89.6%, respectively. Sequence alignment of intron IV of ammodytoxin C and intron III of mojave toxin b revealed that a 600-bp fragment containing an ART2-retroposon element was inserted within intron IV of the ammodytoxin C gene [20,21]. However, BLAST searches on the sequence similarity did not show a significant element appearing in intron II of the A chain genes.

Organization of B chain genes

The B1 chain gene determined in this study was 5062 bp. It consisted of a 5′-flanking region followed by three exons and two introns and a 3′-flanking region (Fig. 2). Exon I encodes the 5′-UTR, signal peptide and amino-acid residues 1–5, exon 2 encodes amino-acid residues 5–59 and exon III encodes protein residues 59–61 and the 3′-UTR. Within the protein-coding region of A chain genes, introns I and II interrupted the reading frame in phase II and phase I (Table 1), but the interrupted introns in the B1 chain gene were phase I and phase II. The 5′-donor and 3′-acceptor splice sites in each of the introns conforms to the GT/AG rule. However, attempts at PCR amplification of B2 chain gene using primers designed from the promoter region and 3′-UTR of the B1 chain gene were not successful. Nevertheless, the gene with a size of 827 bp encoding part of the B2 chain precursor (AJ251224) was amplified using the primers designed from a signal peptide region and protein-coding region (located within the region corresponding to exon II of the B1 chain gene) of the B2 chain cDNA. The overall sequence homology between the B1 chain gene and PCR-amplified B2 chain gene was up to 90.7%. The length of intron I of the B1 chain gene and B2 chain gene was 570 and 580 bp, respectively. Based on these observations, it is inferred that the B1 chain precursor and B2 chain precursor should share the same organization.

Analysis of the promoter sequence of the A and B chain genes

Because the A chain-like gene (AJ251226) has base substitutions which result in an inactivated gene, one may inquire whether the promoter sequence of the A chain-like gene is notably different from that of A chain genes. Although *T. okinavensis* PLA_2 genes, $gPLA_2$-02 and $gPLA_2$-03, had the event of base substitutions as observed for an A chain-like gene, the promoter regions of *T. okinavensus* PLA_2 genes ($gPLA_2$-01, $gPLA_2$-02 and $gPLA_2$-03) shared a sequence homology up to 90% [19]. This observation allows us to deduce that the promoter regions of the A chain-like gene and A chain genes should be similar. Efforts were also made to amplify the complete A chain gene using PCR reaction. Unfortunately, only the A chain-like gene (AJ251226) was identified from PCR products.

Analyses of the promoter sequences of the A chain and B chain genes show that their promoter regions are susceptible to binding with different transcription factors (Fig. 3). This shows that transcription of A chain and B chain genes may be regulated by different transcription factors. Moreover, the putative regulation sites of A chain genes differed notably from those of Viperidae (Viperinae and Crotalinae) PLA_2 genes [31]. This implies that genetic regulation for the transcription of A chain genes and Viperidae PLA_2 genes is different.

Evolutionary diversity of the A and B chains of β-Bgt

Although the A and B chain genes share the same organization, sequence alignment and phylogenetic analysis do not support that the evolution of the A and B chain genes is closely related. In terms of the finding that the ‘putative’ second exon of the BL1 gene shared a high degree of sequence homology with the dendrotoxin k cDNA sequence (Fig. 2), it emphasizes the idea that the B chain and dendrotoxin may share a common

origin as proposed by Dufton *et al*. [32]. Moreover, it increases the possibility that the B chain gene may originate from sexual hybridization of banded krait and mamba. A nondivergence theory of evolution proposed by Tamiya & Yagi [33] partly supported this proposition. In view of the observation that A and B chains are encoded separately by different genes, this clearly indicates that the intact β-Bgt molecule should be derived from pairings of A and B chains after their mRNAs are translated.

A chain genes are composed of three exons and two introns, which are different from those noted with Viperinae and Crotalinae PLA_2s genes. In contrast to what is observed for A chain genes, the signal peptide region of Viperinae and Crotalinae PLA_2 precursors is interrupted by one intron (Fig. 1). Noticeably, the signal peptide of Elapidae PLA_2s and the A chain of β-Bgt consists of 25–27 amino-acid residues, while that of Viperidae (Viperinae and Crotalinae) PLA_2s consists of 16 amino-acid residues [34]. These findings reflect that the genetic divergence of snake PLA_2s evolves according to their respective families. Previous studies have suggested that snake PLA_2 genes have evolved via accelerated and regional evolution to gain diverse physiological activities [16–19,22,30]. Phylogenetic analyses of the exon and intron regions of A chain genes reveal that the evolutionary distances of the exon regions are not in consensus with those of the intron regions (data not shown). In view of the fact that PLA_2 and β-Bgt had been isolated simultaneously from *B. multicinctus* venom [35], this implies that accelerated evolution may occur during the evolution of Elapidae PLA_2 genes and A chain genes.

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