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Identification of crotasin, a crotamine-related gene of *Crotalus durissus terrificus*

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Abstract

Crotamine is a cationic peptide (4.9 kDa, pI 9.5) of South American rattlesnake, *Crotalus durissus terrificus*' venom. Its presence varies according to the subspecies or the geographical locality of a given species. At the genomic level, we observed the presence of 1.8 kb gene, *Crt-p1*, in crotamine-positive specimens and its absence in crotamine-negative ones. In this work, we described a crotamine-related 2.5 kb gene, crotasin (*Cts-p2*), isolated from crotamine-negative specimens. Reverse transcription coupled to polymerase chain reaction indicates that *Cts-p2* is abundantly expressed in several snake tissues, but scarcely expressed in the venom gland. The genome of crotamine-positive specimen contains both *Crt-p1* and *Cts-p2* genes. The present data suggest that both crotamine and crotasin have evolved by duplication of a common ancestor gene, and the conservation of their three disulfide bonds indicates that they might adopt the same fold as β-defensin. The physiological function of the crotasin is not yet known.

Keywords: Animal toxin; Molecular toxinology; Molecular evolution; Toxin gene; Expression pattern; Gene duplication; Paralogous gene; Intron phase; Accelerated evolution; Protein fold

1. Introduction

Vellard (1937) reported the dependence of the physiopathological effect of *Crotalus durissus* venom on its geographical origin, reflecting the variability of the venom composition. In 1956, Gonçalves isolated the first small basic myotoxin from the venom of South American rattlesnake, *C. d. terrificus*, and described the venom polymorphism in relation to crotamine (Gonçalves, 1956).

Crotamine belongs to a closely related group of myotoxin-*a* like proteins that are widely distributed among venoms of the rattlesnake species, *Crotalus*, but its presence depends on the subspecies or the geographical locality of a given species (Schenberg, 1959; Bober et al., 1988; Ownby, 1998). Crotamine-positive subspecies of *C. d. terrificus* inhabit regions extending roughly from western part of the state of São Paulo (west of the 49th meridian) into northern and western part of the state of Paraná and 21° south (Bober et al., 1988). Dry crude venom yields approximately 10% of crotamine by weight, although intra-specific variation may occur. Crotamine shares a 92.9% peptide sequence similarity with crotamine-*a* from *Crotalus viridis viridis*, prairie rattlesnake, of Western USA (Ownby, 1998).

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Crotamine causes similar pathological effects as the most studied myotoxin-*a* does (Cameron and Tu, 1978) that is, induction of localized myonecrosis of skeletal muscle cell with a low onset of action (Fletcher et al., 1996). It has been reported that crotamine increases the voltage-activated Na^+ current causing a high depolarization of the muscle fiber membrane at the junctional region of the myocyte membrane, which is prevented by tetrodotoxin (Matavel et al., 1998). On the other hand, myotoxin-*a* that differs from crotamine by only three amino acids (92.9% amino acid sequence homology) seems to prevent the Ca^{2+} uptake by interacting to the sarcoplasmic reticulum Ca^{2+}-ATPase (Utaisincharoen et al., 1991) or by releasing calcium ions from the calsequestrin stocks (Okhura et al., 1995) or through ryanodine receptors (Yudkowisky et al., 1994). However, the basic mechanism by which these toxins, crotamine and myotoxin-*a*, act is still unresolved.

Our previous comparative studies on Northern blot analysis of poly(A^+) RNA from venom glands of a crotamine-positive specimen and total RNA and poly(A^+) RNA from a crotamine-negative rattlesnake, *C. d. terrificus*, revealed that crotamine transcripts were not expressed in the crotamine-negative specimens (Rádis-Baptista et al., 1999). In addition, Oguiura et al. (2000) devised an enzyme-linked immunosorbent assay method to detect crotamine in crude venom, and correlating the pattern of expression of crotamine in the venom of *C. d. terrificus* variants.

The crotamine gene, from *C. d. terrificus*, has 1.8 kb organized into three exons separated by a long phase-1 (900 bp) and a short phase-2 (140 bp) introns. Exon I includes the 5′-untranslated region and codes for the first 19 amino acids of the signal peptide. Exon II codifies 42 amino acids, three belonging to the signal peptide and 39 to the mature crotamine. Exon III codes for the last three amino acids of the mature toxin, including the terminal lysine, and the 3′-untranslated region. The crotamine gene, *Cts-p1*, is localized to the telomeric region of the chromosome 2 long arm of *C. d. terrificus* crotamine-positive karyotype (Rádis-Baptista et al., 2003).

Southern blotting analysis of genomic DNA performed in our laboratory indicated the presence of crotamine related sequences in crotamine-negative genome. This fact hinted the probable presence of a defective crotamine gene or a related gene in crotamine-negative specimens, and the cause of absence of crotamine expression in the venom of crotamine-negative specimen might be operating at the DNA level.

In the present work, we report the genomic sequences and analysis of crotamine gene, *Crt-p1*, present only in crotamine-positive specimens, and crotamine-related gene crotasin gene, *Cts-p2*, present in both crotamine-positive and negative specimens.

2. Materials and methods

2.1. Snake specimens

Specimens of *C. d. terrificus* crotamine-positive and negative were obtained from the Herpetology Laboratory, Instituto Butantan, São Paulo, Brazil. Crotamine-positive specimens (*Cdt* 001, *Cdt* 002, *Cdt* 9705 and *Cdt* 9706) were captured in western region of São Paulo State. Crotamine-negative ones (*Cdt* 93015, *Cdt* 9315-2, *Cdt* 00081 and *Cdt* 00083) were from eastern region of the State.

2.2. Preparation of genomic DNAs

Snakes were anesthetized with carbon dioxide and decapitated. Livers were excised and quickly frozen in liquid nitrogen. For DNA isolation, frozen livers were ground to a powder under liquid nitrogen with a mortar and pestle and purified in the presence of proteinase K and SDS, according to Ausubel et al. (1995).

2.3. Construction of the C. d. terrificus crotamine-negative genomic library

A genomic phage library from *C. d. terrificus's* liver crotamine-negative (specimen No. 93015) was constructed using the Lambda-FIXII/*Xho I* partial fill-in Kit, essentially as described by the manufacturer (Stratagene, La Jolla, CA).

2.4. Isolation sequencing and analysis of crotasin gene (Cts-p2)

Based on highly conserved crotamine gene sequence (Rádis-Baptista et al., 2003), two oligonucleotides PRO-S2 (5′-ACACTGCAAGTCCTGAGCCT-3′) and NOR-RV (5′-TAGCAGGCGGCACTCTCAGGT-3′) were synthesized, which correspond to 5′-UTR and 3′-UTR of the *Crt-p1* gene, respectively. The DNA, isolated from liver of crotamine-negative specimen *Cdt* 93015, was used in a long distance polymerase chain reaction (LD-PCR) protocol.

This crotamine-related gene, crotasin (*Cts-p2*), was amplified using 1 μg of genomic DNA or 10^7–10^8 phage particles (pfu) of amplified genomic library, 10 pmol of each primer, and 25 μl of ExLONGase enzyme mix (Invitrogen Life Technologies, San Diego), as described by the manufacturer. The LD-PCR products were purified from the gel slice and cloned into pCR2.1-TOPO (Invitrogen Life Technologies, San Diego).

The gene was sequenced with ABI Prism Big Dye Terminator (Perkin–Elmer, Foster City, CA) in an automated sequencer (ABI Prism 373 or 377, Perkin–Elmer) using synthetic oligonucleotides designed from the *Crt-p1* sequences (Rádis-Baptista et al., 2003).

The cloned crotasin gene, *Cts-p2* (accession no. *AF250212*), were compared against crotamine gene, *Crt-p1* (*AF223646* and *AF223947*), and crotamine mRNA

sequences (*AF04674* and *AF53075*) on the GenBank at NCBI (http://www.ncbi.nlm.nih.gov), using the BLAST algorithm (Altschul et al., 1997) and using the Biocomputing software Lasergene (DNAStar, Inc., Madison, WI).

2.5. RT-PCR for analysis of Cts-p2 expression

To verify the expression pattern of *Cts-p2* in crotamine-negative rattlesnake's organs, reverse transcription and PCR amplification were carried out in one tube reaction, using the Super-Script One-Step PCR Kit (Invitrogen Life Technologies, San Diego, CA). Brain, venom glands, heart, liver, kidneys and pancreas were excised from the decapitated specimen (No. 93015) and rapidly frozen in liquid nitrogen. Samples were pulverized in liquid nitrogen and total RNAs were purified using TriZol Reagent (Invitrogen Life Technologies, San Diego). One hundred nanograms of each RNA sample were transferred to PCR tubes containing the RT/*Platinum Taq* reaction mix, and 0.2 μM of *Cts-p2* gene specific primers H10 (5′-AACCAGTCTCAGCAT GAAGATC-3′) and CTS-MRV (5′-TATGCAGCAGCTCT CATACCACA-3′). After a pre-denaturation cycle of 94 °C for 3 min, the DNA amplification was achieved by 35 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 2 min, and a final extension time of 7 min at 72 °C, with a Perkin–Elmer thermal cycler (PE 2400). As control of RT-PCR, the β-actin gene with the specific primers βAC1 sense (5′-GCGGGAAATCGTGCGTGACATT-3′), and βAC2 anti-sense (5′-GATGGAGTTGAAGGTAGT TTCGTG -3′) were used (Meiri et al., 1997). The products were separated on a 2% agarose gel in TAE (Tris-acetate–EDTA buffer) and visualized with ethidium bromide, using VDS Image Master (AmershamPharmacia Biosciences, Piscataway, NJ).

2.6. AR-PCR for probing Crt-p2 in C.d. terrificus genomes

To verify the presence of *Cts-p2* gene in the genome of crotamine-positive *C. d. terrificus* specimen, an arbitrary PCR (AR-PCR) was carried out. To a total volume of 25 μl, the following reagents were added: One millimolar Tris–HCl pH 8.0, 2 mM $MgCl_2$, 1.0 U of recombinant *Taq* DNA polymerase (Invitrogen Life Technologies, San Diego), 200 ng of genomic DNA, 200 μM of each deoxynucleotide triphosphates, and 10 pmol of each, H10 forward primer and CTS-MRV reverse primer, corresponding to the leader sequence in the exon I and C-terminal of mature toxin in the exon II of *Cts-p2*, respectively. The AR-PCR was achieved by 35 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 3 min, after an initial denaturation step of 94 °C for 2 min. The AR-PCR was terminated with a final extension cycle of 72 °C for 7 min. The PCR products were resolved on 1% agarose gel in TAE, and visualized with ethidium bromide, using VDS Image Master (AmershamPharmacia Biotech, Piscataway, NJ).

3. Results and discussion

We have previously shown that crotamine gene transcripts are present only in mRNA pool isolated from venom glands of crotamine-positive specimens and not in crotamine-negative rattlesnakes, *C. d. terrificus*. Several mRNA precursors, coding for two crotamine isoforms, were also isolated (Rádis-Baptista et al., 1999). Recently, we have reported the structure and chromosomal localization of crotamine gene in *C. d. terrificus* (Rádis-Baptista et al., 2003). However, Southern blot analysis of genomic DNA, isolated from livers of crotamine-positive and negative specimens, had indicated the presence of crotamine or crotamine-related gene in both. This fact hinted the probable presence of a defective crotamine gene or a related gene in crotamine-negative specimens. To isolate the crotamine-related gene, from *C. d. terrificus* crotamine-negative, oligonucleotide primers with the sequence flanking the toxin gene *Crt-p1* were synthesized and used in a LD-PCR. This amplification process yielded a ~2.5 kb product, a gene, *Cts-p2*, longer than the ~1.8 kb from *Crt-p1* gene (Fig. 1; Table 1). A nucleotide sequence comparison of this crotamine-related gene, *Cts-p2*, designated *crotasin*, with that of *Crt-p1* gene, revealed the same overall gene organization, that is, three exons separated by two introns, one long and another short, and a high degree of divergence: exons—27.2% of bases substituted; deletions (exons—6 bases; introns—17 bases) and insertions (exons—0, introns—892 bases). Insertions, in a total of 892 bases, range from a single to hundreds of bases, notably, a repeat of the intron I segment of 760 bp. The putative TATA box, 58 bp upstream from the start codon, and splice donor and acceptor sites are intact. Fig. 2 depicts this type of gene organization and the comparison with that of a small basic toxin with unrelated physiological activity, cardiotoxin (*CTX*) gene from *Naja naja sputatrix* (Lauchumanan et al., 1998). The structural organization of crotasin and crotamine gene is very similar to that of the majority of snake toxin genes: (a) the first exon contains almost complete leader sequence, followed by a relatively long intron; (b) the second exon codes for the majority of the mature toxin, succeeded by the second intron, and (c) the third exon encompassing the sequence coding for the toxin C-terminal region and the 3′-UTR of mRNA. One peculiarity of *Crt-p1* and *Cts-p2* genes is the phase of introns which differ from other toxins: the 3′-ends of exon I, in both genes, are interrupted after the first nucleotide of the last codon (phase 1—intron) and the 3′-end of the exon II has its 3′-end codon interrupted after the second nucleotide (phase 2—intron), characterizing asymmetrical exons. In contrast, the exons of other toxins, like postsynaptic-neurotoxin and cardiotoxin genes are symmetrical, i.e. they are interrupted by introns of the same phase, in this case, phase 1. The gene organization has influence on its own evolution and on protein diversity. For instance, the phase of introns is involved in shuffling of exons and, consequently, protein domain assortment

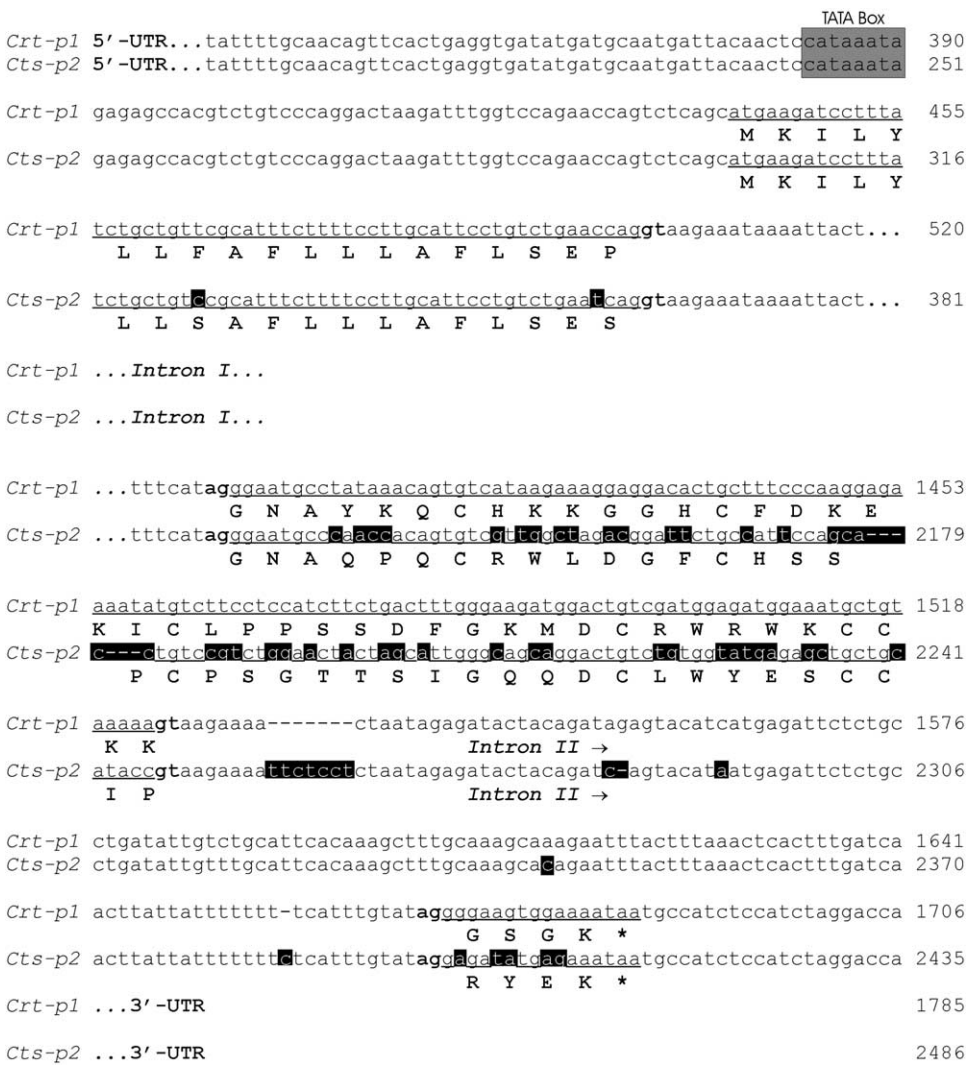

Fig. 1. Comparison of crotamine, *Crt-p1*, and crotasin, *Cts-p2*, genes. Nucleotide sequences are in lowercase. The genes are depicted without intron I for shortening. Coding sequences are underlined and amino acid sequences are in uppercase under the nucleotide sequence. The exon/intron flanking nucleotides are in bold. Nucleotides in *Cts-p2* differing from *Crt-p1* are boxed. 5′-UTR and 3′-UTR stand for 5′- and 3′-untranslated region, respectively.

(Patthy, 1996, 1999). Furthermore, it has been observed that, in *Drosophila*, the rate of recombination is associated with intron size (Carvalho and Clark, 1999). In addition, in *Conus*, signal sequence, pro-region and mature toxin region of pre−pro-peptides are also separated by relatively long introns, and such organization would allow for different rates of mutation in the three separated exons (Oliveira et al., 1999). This fact might, partially, explain why crotasin and crotamine peptides have diverged so widely. In the exon region, 27.2% of nucleotides are substituted, compared to 7.7% in the intron region, excluding insertions. In the crotamine proper region, base substitutions result in almost all non-synonymous substitutions, giving rise to crotasin peptide with only 11 conserved amino acids, including six

Table 1
Comparison between exons and introns of *Crt-p1* and *Cts-p2*

	Ctr-p1	*Cts-p2*
Gene size (bp)	1785	2486
Exon I	441 (5′-UTR) + 58 (CS)	304 (5′-UTR) + 58 (CS)
Intron I	898	1768
Exon II	124	118
Intron II	145	152
Exon III	13 (CS) + 104 (3′-UTR)	13 (CS) + 73 (3′-UTR)

CS—coding sequence; 5′-and 3′-UTR- 5′- and 3′-untranslated region.

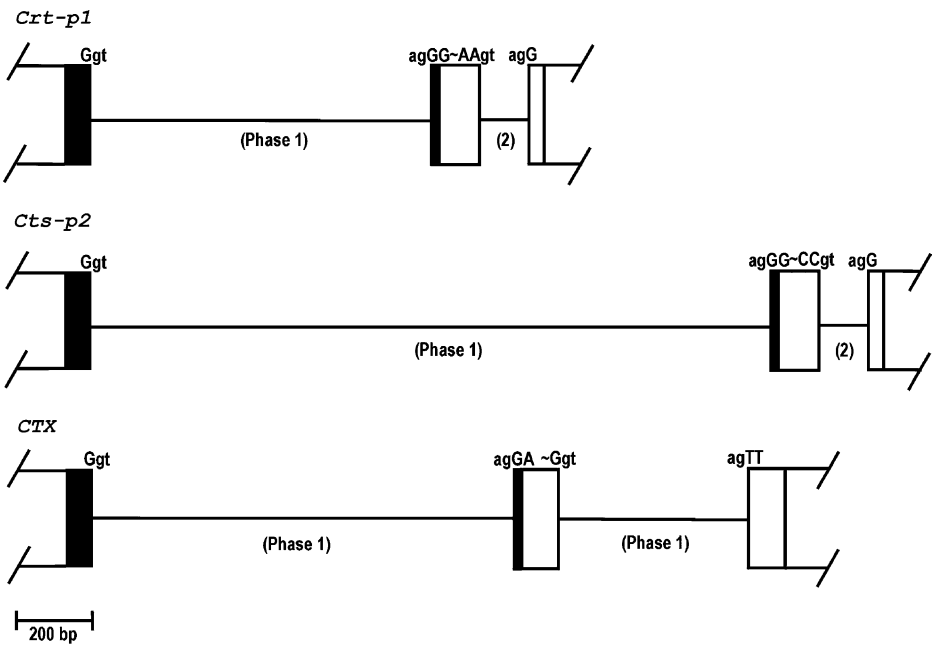

Fig. 2. Schematic structural organization of crotamine (*Crt-p1*) and crotasin (*Cts-p2*) genes from *C. d. terrificus*, and of cardiotoxin gene (*CTX*) from *N. n. sputatrix*. Boxes indicate the exons and lines, the introns. In black boxes are depicted the nucleotide sequences that correspond to the signal peptide in both genes. Open boxes indicate the exons encompassing the sequences of mature toxins. The exon/intron junctions are indicated, as well the intron phases. The bar represents 200 base pairs.

cysteine residues, out of the total of 41 in crotasin. Due to this high number of non-synonymous substitutions, 10 lysine residues present in crotamine are reduced to only C-terminal lysine in crotasin, which is, probably, eliminated by post-translational modification, as in the case of crotamine. Consequently, crotasin is not a basic peptide. Curiously enough, these non-synonymous replacements did not result in any stop codons or change in cysteines pattern. In fact, one cysteine codon was affected but the third letter had a synonymous substitutions (TGT–TGC). Although codons with observed substitutions may have changed coding amino acids, not all sites in a given observed are non-synonymous. One interesting aspect of this non-synonymous substitutions observed is the predominance of the frequency of substitutions involving two letters of codons (19 cases) compared to those involving only one base of codons (12 cases). Substitution involving all three codon nucleotides appears only once. These facts seem to imply that the divergence between crotamine and crotasin occurred rather remotely from a common ancestor.

In the case of crotamine, where different cDNA precursors presented nucleotide variations in the 5′- and 3′-untranslated regions but constancy in the coding region (Rádis-Baptista et al., 1999), the comparison of crotamine and crotasin gene nucleotide sequences shows a strikingly conserved introns (92.3%—excluding indels), signal peptide (96.9%), 5′-UTR (93.4%), 3′-UTR (100%) and highly divergent coding region (59.5%; Table 2). Various snake toxin genes have been described showing a high conservation of intron sequences, in contrast to a high number of nucleotide substitutions in the coding region, an accelerated evolution, e.g. erabutoxin C gene from the sea snake *Laticauda fasciata* (Fuse et al., 1990), PLA_2 genes from the habu snake *Trimeresurus flavoridis* (Nakashima et al., 1995), cardiotoxin and cobrotoxin genes from Taiwan cobra, *Naja naja atra* (Chang et al., 1997), cardiotoxin genes from *N. n. sputatrix* (Lauchumanan et al., 1998), α-neurotoxin genes from *N. n. sputatrix* (Afifiyan et al., 1999) and β-bungarotoxin genes from *Bungarus multicinctus* (Chang et al., 1999). A new gene with a novel function may emerge every time that nucleotide changes accumulate in coding regions. In contrast to what one observes with toxins, it is generally accepted that the evolutionary rates of introns are greater than those of the exons (Kimura, 1983). Mutations should occur at the same rate in both exons and introns, but deleterious one are more

Table 2
Similarity and divergence of *Crt-p1* and *Cts-p2*

	No. of mutated bases/ no. of total bases	Similarity (%) in relation to *Ctr-p1*
Signal peptide	2/66	96.9
Crotamine coding region	51/123	59.5
Introns	81/1043	93.4
5′-UTR	29/441	93.4
3′-UTR	0/104	100

efficiently removed from the exon but an intron is able quite freely to accumulate a variety of changes. However, in the absence of constrains imposed by physiological function performed by a protein, mutations in exons will be tolerated. This is what one observes with snake toxins. For instance, Ohno et al. (1998) investigating the evolutionary mechanism of structural conservation of a variety of toxins, indicated that the rates of mutations that occur in genes of toxins with the same fold is high, and this phenomena of accelerated evolution is largely adopted with phospholipases A2 from Viperidae. In fact, accelerated amino acid substitutions have been reported to occur also in proteins antigen recognition sites of the MHC molecules (Hughes and Nei, 1989; Hughes et al., 1990), reproductive conflict genes (Wyckoff et al., 2000), active site regions of serine protease inhibitors (Ray et al., 1994) and porcine elafin family (Tamechika et al., 1996). Interspecies comparisons in *Conus* have also revealed the preservation of the same fold by conserving cysteine residues but rather producing a diversity of toxins by varying amino acid sequences with functional diversity (Oliveira et al., 1999).

To verify whether crotasin gene is expressed in venom glands or other tissue of *C. d. terrificus*, a RT-PCR was carried out with specific primers for exon I and II. Six organs (brain, heart, kidneys, liver, pancreas, and venom glands) of crotamine-negative rattlesnake specimen were analyzed. High expression level of *Cts-p2* transcripts were detected in the pancreas, followed by heart, liver, brain and kidneys but scarcely expressed in the venom glands (Fig. 3). In opposition, crotamine transcripts were detected only in the total RNA pool from venom glands of *C. d. terrificus* crotamine-positive (data not shown). The fact of crotasin transcripts being present at high level in pancreas is noteworthy, when one considers the fact that snake venom glands are specialized salivary glands and have an evolutionary link with the pancreas. Moreover, it is an accepted notion that there is an evolutionary and functional relationship between animal toxins and non-toxic counterparts in other tissues. Many examples tend to corroborate the idea that animal toxins have evolved from endogeneous genes functioning in a normal cellular pathway: snake venom sarafatoxins and endothelins (Kochva et al., 1993), elapid snake Ly-6/neurotoxin gene family and murine gene *lynx1*, a modulator of nAChRs (Miwa et al., 1999), functional homologs to signaling molecules, like NGF from cobra venom (Inoue et al., 1991), *Bungarus* venom's acetylcholinesterase located also in the snake liver and muscle (Cousin et al., 1998), *Crotalus* phosphodiesterase finds its counterpart in cellular nucleic acid catabolism, and snake venom PLA_2 isozymes and three-fingered toxins have

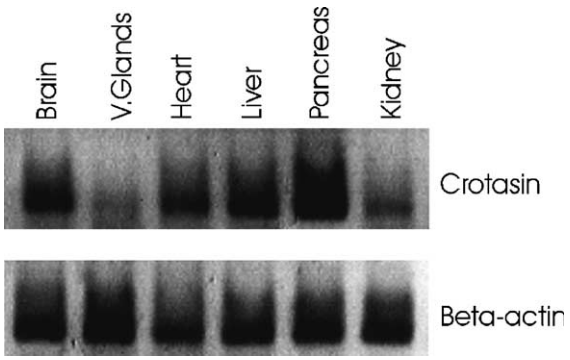

Fig. 3. Pattern of crotasin expression in different tissues of *C. d. terrificus* crotamine-negative. Total RNA from brain, venom glands, heart, liver, pancreas, and kidneys were purified as described in Section 2. Specific primers corresponding to signal peptide (exon I) and carboxi terminal of mature peptide (exon II) were used in a RT-PCR. Both reverse transcription and PCR were done sequentially in a single tube. As control, β-actin mRNA was reverse transcribed and amplified.

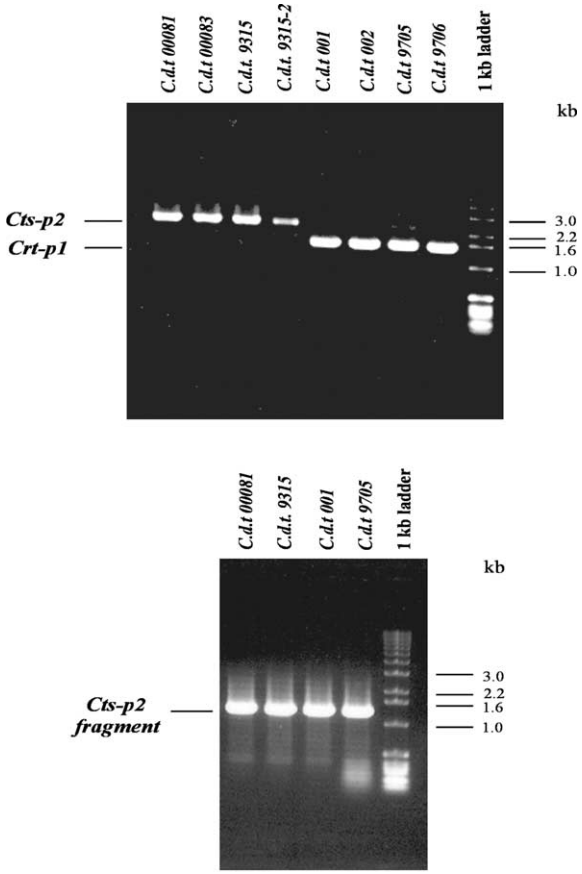

Fig. 4. Identification of crotamine (*Crt-p1*) and crotasin (*Cts-p2*) genes in *C. d. terrificus* genomes by AR-PCR. *C.d.t. 00081, 00083, 9315*, and *9315-2* (*C. d. terrificus* crotamine-negative liver genomic DNAs); *C.d.t. 001, 002, 9705*, and *9706* (*C. d. terrificus* crotamine-positive liver genomic DNAs); 1A—amplified *Crt-p1* and *Cts-p2*; 1B—amplified *Cts-p2*, in both genomes of *C. d. terrificus* variants.

Fig. 5. Common features comparison of crotamine-like cationic toxic peptides and of cationic anti-microbial peptides. Disulfide bonds are represented by pairing lines (1–5, 2–4, 3–6). Positively charged –COOH terminal amino acids are also depicted. HBD 1–3, stands for human β-defensin (GenBank accession number, *Q09753*, *0152263*, *NP 061131*); TAP, bovine tracheal anti-microbial peptides (*P25068*); LAP, bovine lingual anti-microbial peptide (*Q28880*).

various non-toxic counterparts in other tissues (John et al., 1994; Ohno et al., 1998; Fujimi et al., 2002).

The possible presence of the *Cts-p2* gene in the genome of crotamine-positive *C. d. terrificus* was also investigated. By AR-PCR, we detected its presence besides *Crt-p1*, whereas crotamine-negative specimens presented only *Cts-p2* gene. Using liver genomic DNA of several crotamine-positive and negative specimens as template, and H10 sense primer and CTS-MRV reverse primer, specific products of approximately ~1.3 kb were amplified from both genomes (Fig. 4). The product size is compatible with the actual distance between the signal peptide, in the exon I, and carboxi-terminal region of mature toxin, in the exon II, except to the duplicated segment of the intron I of *Cts-p2*. Sequencing of these ~1.3 kb major amplified product confirms the identity of *Cts-p2* homologous in both genomes. The *Cts-p2* genes isolated from both, crotamine-positive and negative specimens, presented exactly the same structure and sequence, but the absence of the duplicated intron I segment. Therefore, in *C. d. terrificus* crotamine-negative genome, at least two copies of *Cts-p2* exist.

In the present work, our data show that crotamine and crotasin share a common gene structure and conservation of the cysteine-rich consensus motif, which constitute critical determinants of the polypeptide topology. This suggests that these snake proteins might adopt a similar three-looped (three disulfide bonds) structure, a toxin-fold presented by a group of peptide antibiotics, the β-defensin family (Ganz and Lehrer, 1998; Andreu and Rivas, 1999), with which crotamine shares 36% of similarity in amino acid sequence (Fig. 5). In fact, tri-dimensional model of crotamine, based on computer calculation (Siqueira et al., 2002) and on NMR coordinates (Nicastro et al., 2003) corroborate our hypothesis of conserved fold among these peptides and β-defensin members. The common features of these peptides are not restrict to protein scaffold, the genes of β-defensin and epithelial α-defensin share the same overall organization of these toxin genes, concerning to the separation of an anionic region of signal peptide in one exon and the mature cationic peptide in other (Hancock and Diamond, 2000).

In conclusion, *Crt-p1* and *Cts-p2* are paralogous genes that evolved through accelerated evolution of exon II, and the crotamine is the most recent product of this process. The absence of crotamine toxin in the venom glands of *C. d. terrificus* crotamine-negative is due to the absence of crotamine gene itself. The basic mechanism of where and how crotamine acts is still an open question and it may indicate a diversity of its action. Obviously, it would be of a great interest to know the physiological function of crotasin peptide in snake tissues and also to search for crotasin in other *Crotalus* species.

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