PERGAMON

Toxicon 37 (1999) 973–984

TOXICON

Nucleotide sequence of crotamine isoform precursors from a single South American rattlesnake (*Crotalus durissus terrificus*)

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Received 1 June 1998; accepted 15 October 1998

Abstract

A cDNA phage library was constructed from venom glands of a single adult specimen of crotamine-plus *Crotalus durissus terrificus* (South American rattlesnake) captured in a known region. Fifteen crotamine positive clones were isolated using a PCR-based screening protocol and sequenced. These complete cDNAs clones were grouped for maximal alignment into six distinct nucleotide sequences. The crotamine cDNAs, with 340–360 bases, encompass open reading frame of 198 nucleotides with 5′ and 3′ untranslated regions of variable size, signal peptide sequence, one crotamine isoform message, and putative poly(A^+) signal. Of these six different crotamine cDNA precursors, two predict the identical amino acid sequence previously described by Laure (1975), and the other four a crotamine isoform precursor where the Leucine residue at position 19 is replaced by isoleucine by a single base change. On the other hand, nucleotide variation was observed in the 5′ and 3′ untranslated regions, with one interesting variant containing an 18 base pair deletion at the 5′ untranslated region which results in the usual ATG initiator being replaced by the rarely used GUG start codon.

Comparison by Northern blot analysis of poly(A^+) RNA from venom glands of a crotamine-plus specimen to total and poly(A^+) RNA from a crotamine-minus snake

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0041-0101/99/$ - see front matter
PII: S0041-0101(98)00226-8

indicated that crotamine transcripts were not expressed in the crotamine-minus specimen.

1. Introduction

Crotamine, a myonecrotic polypeptide, is one of four main toxins in the venom of the South American rattlesnake, *Crotalus durissus terrificus*. The others are convulxin – a convulsion-inducing component (Brazil, 1972; Lee, 1972), crotoxin – a presynaptically acting neurotoxin (Slotta and Fraenkel-Conrat, 1938; Chang and Lee, 1977; Hagwood and Santana de Sá, 1979), and gyrotoxin – an enzyme with thrombin-like activity (Barrio, 1961; Alexander et al., 1988). Crotamine belongs to a group of closely related small, nonenzymatic, basic polypeptides which cause myonecrosis on snake envenomation (Bieber and Nedelkov, 1997). Crotamine shows a high degree of homology with other venom myotoxins, such as myotoxin-*a* from *C. viridis viridis* (Ownby et al., 1976; Fox et al., 1979), myotoxin I and II from *C. v. concolor* (Engle et al., 1983; Bieber et al., 1987), peptide C from *C. v. helleri* (Maeda et al., 1978), CAM-toxin from *C. adamanteus* (Mebs and Kornarlik, 1984; Samejima et al., 1987). Myotoxin isoforms have been observed in pooled venom as well as in the venom of a single adult *Crotalus* specimen (Griffin and Aird, 1990; Aird et al., 1991). Furthermore, studies have shown geographical variation, independent of morphological differences, in the composition of snake venom, with respect to crotamine (Schenberg, 1959a,b) and myotoxin-*a* (Bober et al., 1988; Straight et al., 1991). Thus, *C. durissus terrificus* may be classified as crotamine-plus or crotamine-minus, depending on the region of capture (Schenberg, 1959a,b).

The mechanism by which crotamine exerts its toxicity is similar to that of its most studied counterpart, myotoxin-*a* (Cameron and Tu, 1978; Fletcher et al., 1996). It is known to cause reduction in resting membrane potential and increase in membrane conductance by a tetrodotoxin-sensitive, i.e., Na^+-channel mediated mechanism (Hong and Chang, 1985; Brazil and Fontana, 1993). It also induces Ca^{2+} release from the heavy fraction of the sarcoplasmic reticulum, by interacting with calsequestrin (Furukawa et al., 1994; Ohkura et al., 1994, 1995), resulting in swelling of the sarcoplasmic reticulum and necrosis of skeletal muscle.

Cloning and nucleotide sequence of crotamine genes of *C. durissus terrificus* has been reported by Smith and Schmidt (1990). Their data indicated the presence of polymorphic variants of crotamine. Among four clones analyzed by them, two contained deletions in the 5′ end and leader sequence of the genes, making its mRNA's non-functional. One curious fact is that the coding regions of all four clones encoded crotamine with amino acid sequences differing from each other and also from those published by Laure (1975) and Dos Santos et al. (1993). The basis of this discrepancy is not clear but possible causes include the fact that Crotalus specimens were obtained from a commercial serpentarium and the exact local of origin of the snakes used is not known, and/or the cDNA library was

prepared from three specimens and it is not clear whether they were all crotamine-positive.

Because of regional variation in venom composition, the microheterogeneity of myotoxins in a single specimen and in the snake population as a whole, we report here the nucleotide sequence of crotamine isoform precursors in an individual specimen of *C. durissus terrificus*. The cDNA sequences described in this paper from clones KA19, MK9 and MK41 were submitted to GenBank under Access No. AF044674, AF053075 and AF055988.

2. Materials and methods

2.1. Venom glands

The venom glands were obtained from single adult specimens of *Crotalus durissus terrificus* crotamine-plus from Martinópolis (São Paulo, Brazil) and crotamine-minus from São Luis do Paraitinga (São Paulo, Brazil), provided by the Instituto Butantan Serpentarium. The glands were removed three days after venom milking, when a maximum level of RNA synthesis is achieved (Rottenberg et al., 1971), quickly frozen in liquid nitrogen and finely powdered in a mortar and pestle under liquid nitrogen.

2.2. Poly(A^+) RNA isolation

Poly(A^+) RNA from the powdered crotamine-plus glands was obtained using the Fast Track mRNA Isolation Kit (Invitrogen). Total RNA from the powdered crotamine-minus glands was isolated by the single-step method extraction using guanidine thiocyanate–phenol–chloroform mixture (Chomczynski and Sacchi, 1987) and poly(A^+) was prepared using the polyATract mRNA Isolation Systems (Promega) following the manufactured's instructions.

2.3. cDNA synthesis and cDNA library construction

cDNA synthesis was accomplished using the UniZAP XR Kit (Stratagene) from 6.9 μg of crotamine-plus poly(A^+) RNA. This cDNA was then ligated into the UniZAP XR vector – a λ phage derivative (Stratagene) and packaged using a Ready-To-Go Packing Extract Kit (Pharmacia Biotech). The packaged cDNA was then titered, the primary library amplified and stored at −70°C in 7% DMSO.

2.4. cDNA library screening

A PCR-based screening protocol was used (Israel, 1993, 1995) as follows: the cDNA phage library was subdivided into 64 aliquots of 1000 plaque forming units (pfu) per well in a microplate, and propagated for approximately 6 h. Wells that

contained a crotamine cDNA clone were identified by the synthesis of a PCR product of 100 bp size that hybridized to an internal crotamine oligonucleotide probe. Phages of the positive well were diluted to 25 pfu per well, reamplified and rescreened in the same way. By the third screening, randomly collected plaques proved positive for crotamine cDNA.

2.5. PCR reaction

The specific oligunucleotides primers used for PCR-based cDNA library screening were sense 5′crot (5′-CAGTGTCATTAAGAAAGGAGG-3′) and anti-sense 3′crot163 (5′-ATGGACTGTCGATGGAGATG-3′) (Gibco BRL). Each PCR reaction mixture (30 μl final volume) contained 30 pmol of each specific oligonucleotide primer, 0.58 U *Taq* polymerase (Gibco BRL, 5000 U/ml), 200 μM each of dATP, dCTP, dGTP and dTTP, 3.5 mM $MgCl_2$, 1×PCR buffer (Gibco BRL), and 1 μl DNA template (recombinant phage stock). Each round of screening contained a tube without template (negative control), and a tube containing an aliquot of a myotoxin-*a* cDNA phage library (positive control), a gift from Dr. A.A. Tu, Colorado State University. PCR was performed in a thermal cycler (Perkin Elmer 9600) for 30 cycles, each consisting of denaturation at 94°C for 1 min, annealing at 43°C for 45 s, polymerization at 72°C for 1 min, after the initial cycle of 94°C for 4 min. At the end of all cycles, samples were maintained a 72°C for 7 min, then kept at 4°C until gel analyses.

2.6. Analysis of PCR reaction products

PCR reaction products were analyzed by electrophoresis in 2% agarasoe/TAE gel, visualized with ethidium bromide, and transferred to a positively charged nylon membrane (GenScreen Plus, Du Pont) by downward alkaline elution (Zhou et al., 1994). After 2.5 h of transfer, the membrane was neutralized with 2×SSC and pre-hybridized at 42°C for 2 h with 10 ml of a 6×SSC solution containing 0.5% SDS, 5×Denhardt's, 0.05% PP_i, 100 μg/ml of boiled herring sperm DNA. Hybridization was done at 42°C, overnight, in a 6×SSC solution containing 0.5% SDS, 1×Denhardt's solution, 0.05% PP_i, 100 μg/ml yeast tRNA, and the internal oligonucleotide HO7 (5′-GGCCGCTCTAGAACTAG-3′) (Gibco BRL) labeled with DIG-dUTP (Boehringer-Mannheim) using a TdT enzyme (Terminal deoxydinucleotidyl transferase, Gibco BRL) (Vincent et al., 1982; Ausubel et al., 1995). Hybridized bands which corresponded to a specific PCR product were visualized by immunodetection with anti-DIG/alkaline phosphatase conjugate (Boehringer-Mannheim).

2.7. DNA sequencing

Phage inserts were excised from UniZap XR vector and recircularized in the presence of ExAssist Helper phage (Stratagene) to form pBluescript phagemid.

DNA sequencing was performed on both strands of the excised insert by the dideoxy chain termination (Sanger et al., 1977) using ^{T7}Sequencing Kit (Pharmacia Biotech) and external (SK, M13) and internal (5′crot, 5crot 163) primers. Simultaneously, DNA sequencing was performed by the detection of fluorescent labeled DNA (ABI Prism dye terminator cycle sequencing, Perkin Elmer) in an automated sequencer (ABI Prism 377, Perkin Elmer). The cDNA sequences were translated using the CLONE program (Scientific and Educational Software, USA), and aligned using MACAW (Multiple Alignment Construction and Analysis Workbench) (Karlin and Altschul, 1990; Schuller et al., 1991; Lawrence et al., 1993). After translation into amino acid sequence, both protein and cDNA were compared in OWL Composite Protein Sequence Database (University of Leeds, Leeds, UK) using the BLAST program (Altschul et al., 1990, 1997).

2.8. Northern blot analysis

Northern blot analysis was conducted on total and poly(A^+) RNA from glands of a single crotamine-minus *C. durissus terrificus* specimen and on a sample of poly(A^+) RNA from crotamine-plus *C. durissus terrificus* glands which have been used to construct the cDNA library. The RNAs (10 μg of total RNA and 0.5 μg each of crotamine-minus and crotamine-plus mRNA) were submitted to electrophoresis in a guanidine thiocyanate denaturing gel (Goda and Minton, 1995) and transferred to a nylon membrane (GenScreen Plus, Du Pont) by alkaline transfer (Zhou et al., 1994). The RNAs were fixed by baking for 30 min at 80°C under vacuum. The membrane was pre-hybridized at 42°C for 2 h in 50% formamide, 5×SSC, 5×Denhardt's solution, 0.1% *n*-lauroylsarcosine, 0.02% SDS, containing 100 μg/ml boiled herring sperm DNA. Hybridization was performed at 42°C, overnight, by addition of 250 ng of 350 bp crotamine cDNA labeled with DIG-dUTP by random primer (DIG DNA labeling Kit, Boehringer, Mannheim) to 5 ml of pre-hybridization solution. The membrane was washed using high stringency conditions and the hybridized bands were revealed with anti-DIG/alkaline phosphatase conjugate (Boehringer-Mannheim).

3. Results

3.1. Nucleotide sequence of crotamine precursors

To study the polymorphism of the crotamine gene, a cDNA phage library was constructed from the venom glands of a single specimen *C. durissus terrificus* from a known geographical site.

For the library screening, a method based on a high stringency of PCR reaction was used. The specificity of PCR reaction was checked by hybridization of PCR

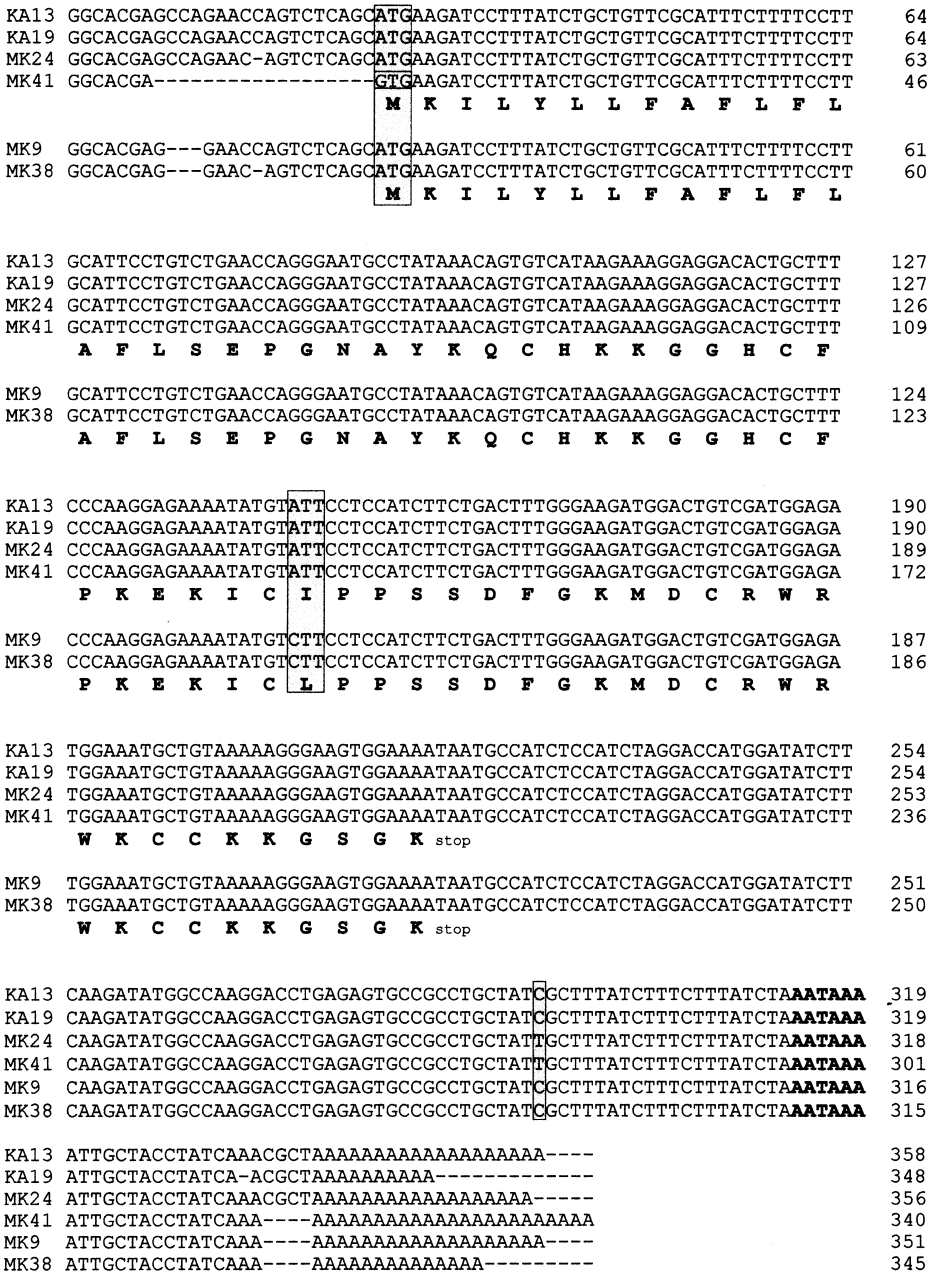

Fig. 1. Nucleotide and deduced amino acid sequences of crotamine isoforms precursors from six distinct full-length cDNAs clones (KA13, KA19, MK24, MK41, MK9, and MK38). The cDNAs, 340–360 nucleotides, are numbered at the right side of the sequences, encoding two crotamine isoforms precursors. Significant nucleotide changes are boxed and the putative poly(A) signal is accented. Corresponded amino acids are shown under the nucleotide sequences and are denoted by one-letter symbols. They include an 22 amino acids signal peptide, and 42 amino acids constituting mature crotamine isoforms and a terminal lysine, which is post-translationally removed.

products with an internal labeled primer (results not shown). Fifteen positive crotamine clones were isolated and sequenced.

All sequenced cDNA clones, ranging from 340 to 360 nucleotides, have open reading frames (ORFs) of the same size of 198 nucleotides. Alignment of these 15 sequences, using the MACAW program results in groupings of six types of sequences (Fig. 1). All of them predict precursors of crotamine, when compared to sequences deposited in the OWL database using BLAST, with conserved cysteines involved in disulfide bonds and conserved basic residues characteristic of small basic myotoxins (Fig. 2). These crotamine precursors have 63 amino acids, the first 22 correspond to signal peptide, the following amino acids (from 23 to 62) include a mature crotamine isoform and lysine, the last amino acid which presumably is removed in a post-translational process. Of these six distinct cDNAs, two encode crotamine with amino acid sequence identical to the one published by Laure (1975), and four code for a crotamine isoform with isoleucine at position 19 (crotamine-Ile 19).

All crotamine cDNAs present some base substitution or deletion at 5′ and 3′ untranslated regions (UTRs). One of them (clone MK41) shows a gross 18 base pair deletion at the 5′ end including the first base adenine of the initiation codon. In this way, the universal ATG start codon is changed to a rare GUG as the initiation codon.

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Signature                          KxCHxxxxHCxxxxxxCxxxxxxxxKxxCxx+xKCCKK
2895610     MKILYLLFAFLFLAFLSEPGNAYKQCHKKGGHCFPKEKICIPPSSDFGKMDCRWRWKCCKKGSGK--  65
2981144     MKILYLLFAFLFLAFLSEPGNAYKQCHKKGGHCFPKEKICLPPSSDFGKMDCRWRWKCCKKGSGK--  65
MYX1_CRODU MKILYLLFAFLFLAFLSEPGNAYKRCHIKGGHCFPKEKICIPPSSDFGKMDCPWRRKCCKKGSGK--  65
MYX2_CRODU -KILYLLFAFLFLAFLSEPGNAYKRCHIKGGHCFPKEKICIPPSSDFGKMDCPWRRKSLKKGSGK--  64
MYX3_CRODU MKILYLLFAFLFLAFLSEPGNAYKRCHIKGGHCFPKGKICIPPSSDFGKMDCPWRRKCCKKGSGK--  65
MYX4_CRODU --------------FLSEPGNAYKQCHKKGGHCFPKEKICIPPSSDFGKMDCRWRWKCCKKRSGK--  51
MYXC_CRODU ----------------------YKQCHKKGGHCFPKEKICLPPSSDFGKMDCRWRWKCCKKGSG---  42
MYX1_CROVV ----------------------YKQCHKKGGHCFPKEKICIPPSSDLGKMDCRWKWKCCKKGSG---  42
MYX_CROAD  ----------------------YKRCHKKGGHCFPKTVICLPPSSDFGKMDCRWRWKCCKKGSVNNA  45
MYXC_CROVH ----------------------YKRCHKKGGHCFPKTVICLPPSSDFGKMDCRWKWKCCKKGSVN--  43
MYX2_CROVV ----------------------YKRCHKKEGHCFPKTVICLPPSSDFGKMDCRWKWKCCKKGSVNNA  45
MYX2_CROVC ----------------------YKRCHKKGGHCFPKEKICTPPSSDFGKMDCRWKWKCCKKGSVN--  43
MYX1_CROVC ----------------------YKRCHKKEGHCFPKTVICLPPSSDFGKMDCRWKWKCCKKGSVN--  43
CRO_Ile-19 ----------------------YKQCHKKGGHCFPKEKICLPPSSDFGKMDCRWRWKCCKKGSG---  42
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Fig. 2. Alignment of crotamine and myotoxins sequences from several *Crotalus* snakes. Shown above the sequences is the myotoxins consensus pattern (signature), with alternative basic residues (R and K) denoted as a '+' signal. Regions of amino acids variability are enclosed by rectangles. Sequences 2895610 and 2981144 – crotamine isoform precursors from *C. durissus terrificus* (this work); MYX1_CRODU, MYX2_CRODU, MYX3_CRODU and MYX4_CRODU crotamine sequences from *C. durissus terrificus* (Smith and Schmidt, 1990); MYXC_CRODU – crotamine from *C. durissus terrificus* (Laure, 1975); CRO_Ile-19 – crotamine Ile-19 from *C. durissus ruruima* (Dos Santos et al., 1993); MYX1_CROVV – myotoxin-*a* from *C. viridis virids* (Fox et al., 1979); MYX_CROAD CAM-toxin from *C. adamanteus* (Samejima et al., 1987); MYXC_CROVH – peptide C from *C. v. helleri* (Maeda et al., 1978); MYX1_CROVC and MYX2_CROVC – myotoxin I and II from *C. viridis concolor* (Bieber et al., 1987).

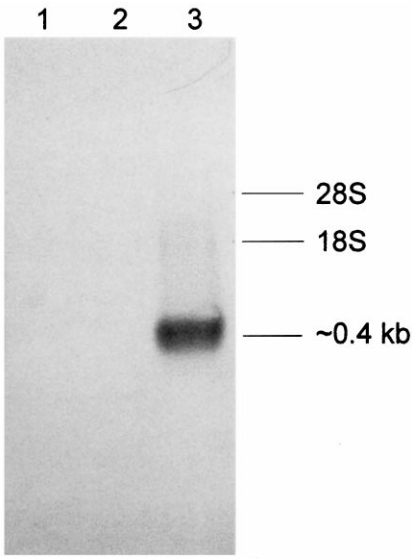

Fig. 3. Northern blot analysis of total and poly(A^+) RNA isolated from crotamine-minus and plus *C. durissus terrificus* venom glands. Total RNA (10 μg) and poly(A^+) RNA (0.5 μg) of crotamine-minus *C. d. terrificus* (lanes 1 and 2) and poly(A^+) RNA (0.5 μg) of crotamine-plus *C. d. terrificus* (lane 3) were hybridized to DIG-dUTP labeled crotamine cDNA. The migration of 28S and 18S ribosomal RNAs is indicated as well as the relative size of crotamine mRNA.

3.2. Northern blot analysis

Since there are geographical variations in the composition of snake venom, Northen blot analysis of total and poly(A^+) RNA from a crotamine-minus South American rattlesnake (*C. durissus terrificus*) and poly(A^+) RNA of crotamine-plus animal was performed. As shown in Fig. 3, a band of approximately 0.4 kb corresponding to crotamine transcript is observed in the lane loaded with a crotamine-plus poly(A^+) RNA sample. No band is present in lanes with crotamine-minus total RNA and poly(A^+) RNA samples, although a large amount of total and poly(A^+) RNA were used.

4. Discussion

In this work, we report the presence of crotamine isoform precursors, based on nucleotide sequence analysis of cDNAs obtained from glands of a single specimen of *C. durissus terrificus*, captured in a known geographical region.

These cDNAs, of 340–360 nucleotides, grouped for maximal alignment, include ORFs with 198 nucleotides, variable size 5′ UTRs, two complete isoform precursor sequences, putative polyA signals, and variable size 3′ UTRs. After being translated to the amino acid sequence and scanned in the OWL database

using the BLAST program, sequenced cDNAs and predicted amino acids sequences indicate that all code for a functional crotamine isoform, since they present the entire crotamine precursor sequence. Furthermore, they present the consensus pattern KxCHxKx(2)HCx(2)Kx(3)Cx(8)Kx(2)Cx(2)[RK]xKCCKK of other myotoxins with conserved cysteines involved in disulfide bonds and conserved basic amino acid residues (Griffin and Aird, 1990). Although the 5′ and 3′ UTRs of crotamine cDNAs show some nucleotide variation, one observes here a certain degree of similarity with 5′ and 3′ UTRs of myotoxin-*a* from *C. viridis viridis* (Norris et al., 1997).

One crotamine isoform described here has an amino acid sequence identical to that described by Laure (1975), while the other predicted isoform has the isoleucine residue at position 19 (crotamine Ile-19). Interestingly, the existence of the crotamine Ile-19 has been reported only in the venom of *C. durissus ruruima* (Dos Santos et al., 1993) – a subspecies of *C. durissus* that inhabits the northen region of Brazil, while the subspecies *terrificus* is found in the central and southern parts of the country.

The precise locality of capture is very important since the venom composition shows regional variation and it is not correlated with the snake's philogeny (Daltry et al., 1996). It is known that the South American rattlesnake *C. d. terrificus* can be additionally classified as crotamine-plus and crotamine-minus depending on the presence or absence of crotamine in the venom (Gonçalves, 1956; Schenberg, 1959a,b). The reason for this difference is not yet clear but, basically, it should be at either the mRNA or DNA level.

To examine if the absence of crotamine in the venom of crotamine-minus snakes is due to the lack of crotamine mRNA in the venom glands, a northern blot analysis of total and poly(A^+) RNA from a crotamine-minus, and of poly(A^+) RNA from a crotamine-plus *C. d. terrificus* glands was performed, using the entire crotamine cDNA precursor as the probe. Preliminary results indicate the presence of crotamine transcripts of approximately 0.4 kb in crotamine-plus *C. d. terrificus* venom glands, but absence in crotamine-minus *C. d. terrificus* snakes. These results suggest that the lack of crotamine expression in the venom of a crotamine-minus specimen operates at the DNA level.

Given our present data, some comments should be made on the work of Smith and Schmidt (1990). (1) They analyzed four clones and although the nucleotide sequence analysis predicted the existence of multiple variants of the crotamine, only two of these four had an intact ATG, thus coding for functional precursors. (2) These multiple variants showed 5–6 amino acid differences with published data from Laure (1975) and Dos Santos et al. (1993), a rather high substitution rate for a peptide of 42 amino acids, although this can happen. (3) Since their cDNA library was constructed from three *C. d. terrificus* specimens of unknown origin, it is difficult to discuss the number of isoforms in one animal. (4) It is not clear whether those three snake samples were all crotamine positive.

Interestingly, in the present work a critical change was observed in the crotamine cDNA precursor involving the initiation codon. Deletion of 18 nucleotides of the 5′ UTR end of the clone MK41 results in juxtaposition of a G

to the remaining UG of initiation codon. A similar phenomenon has been observed in the gene of human hypoxanthine-guanine phosphoribosyltransferase (HRPT), where a 13 base pair deletion in exon 1 of HRPT forms a functional GUG initiation codon (Davidson et al., 1994). Initiation of protein synthesis at the non-AUG start codon is not rare and several examples have been found recently in eukaryotes cellular genes. Thus proto-oncogenes and gene encoding fibroblast growth factor initiate translation at CUG (Hann et al., 1988; Prats et al., 1989), while *Drosophila* choline acetyltransferase (Sugihara et al., 1990) and human parainfluenza virus type 1 (hPIV1 *P*/*C*) gene (Boeck et al., 1992) initiate at GUG. There are several more examples, but the majority of these genes are involved in highly regulated metabolic pathways. This kind of regulation is related to the low efficiency of translation at non-AUG codons (Peabody, 1989; Sugihara et al., 1990). Although initiation at non-AUG codons is accomplished at low efficiency, according to Kozak (1990, 1997), it seems to show the same strong dependence on the mRNA features that benefits AUG recognition.

Venom composition shows a regional variation and there are *C. durissus terrificus* with a low concentration of crotamine. Whether crotamine genes with GUG start codon play any special role in these cases is under investigation.

Acknowledgements

This work was supported by FAPESP, Fundação de Amparo à Pesquisa do Estado de São Paulo, and CNPq, Conselho Nacional de Desenvolvimento Científico e Tecnológico. We are thankful to Marilyn Kozak and to Alvaro Pietro da Silva for helpful discussions.

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