Genomic structure of a potassium channel toxin from *Heteractis magnifica*

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Abstract We provide information on the gene encoding the K^+ channel toxin, HmK, of the sea anemone *Heteractis magnifica*. A series of DNA amplifications by PCR, which included the amplification of the 5′-untranslated region of the gene, showed that an intron of 402 nucleotides separated the sequence that encodes the matured toxin from the signal peptide sequence. A second 264 nucleotide intron interrupted the 5′-untranslated region of the previously reported HmK cDNA. Two possible transcription-initiation sites were identified by primer extension analysis. Corresponding TATA-box consensus sequences, characteristic of a promoter region, were also located from PCR products of uncloned libraries of adaptor-ligated genomic DNA fragments. The coding region for matured HmK is intronless. The same is also true for other sea anemone toxins reported thus far. More notably, a similar intron-exon organization is present in other ion channel-blocking toxins from scorpions implying that molecules having similar functions share a similar organization at the genomic level suggesting a common path of evolution.

Key words: Potassium channel toxin; *Heteractis magnifica*

1. Introduction

A new class of K^+ channel-blocking peptides has been isolated from three species of sea anemone: *Bunodosoma granulifera* (toxin named BgK) [1], *Stichodactyla helianthus* (ShK) [2,3], and *Anemonia sulcata* (AsKC1, AsKC2, AsKC3, and AsKS) [4]. The shorter toxins, BgK, ShK and AsKS, and the longer AsKCs consist of 35–37 and 58–59 amino acid residues, respectively. Nevertheless, these toxins contain three disulfide bonds [5]. They selectively block Kv1.2 [5–7] and Kv1.3 [8,9] channels. To date, studies have focused exclusively on their structure-function relationships, with elucidation of the complete three-dimensional structures of two of these toxins, ShK and BgK, by NMR and modeling [9,7] and delineation of important residues [6,10,9,7].

In contrast, little is known about the regulation of biosynthesis and processing of these molecules. We have recently cloned and expressed a cDNA encoding a new K^+ channel toxin (HmK) from the tropical sea anemone, *Heteractis magnifica* [11]. This 35 amino acid toxin has an amino acid sequence homology of 60% and 40% to ShK and both BgK and AsKS, respectively, while sharing the same disulfide pairing motif. The coding region (222 bp) of HmK cDNA encodes a 74 amino acid polypeptide precursor. Of these, the first 39 amino acids constitute the signal peptide. This long signal peptide, however, is not required for the proper folding of the toxin, as demonstrated by us previously [11]. Here, we provide the first report on the structure of the sea anemone K^+ channel toxin gene and its regulating elements.

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The genomic DNA sequence reported in this paper has been submitted to GenBank with accession number AF020047.

2. Materials and methods

2.1. Materials

Taq DNA polymerase and [α-^{33}P]dATP were obtained from Amersham International Inc. (UK). PromoterFinder Construction kit and Advantage Genomic PCR kit were purchased from Clontech Laboratories, Inc. (USA). Reverse transcriptase, T4 DNA ligase and pT7 Blue vector were from Promega, New England Biolabs Inc. and Novogen (USA), respectively. *Escherichia coli* strain used was JM109 [F′ *tra*D36 *lac*I^q Δ(*lac*Z)M15 *pro*A$^-$B$^-$I e14$^-$ (McrA$^-$) Δ(*lac-pro*AB) *thi gyr*A96 (Nal^r) *end*AI *hsd*R17 ($r_k^-m_k^+$) *rel*AI *sup*E44 *rec*A1] [12]. DNA sequencing reagents were from Perkin Elmer-Applied Biosystems Inc. (Foster City, CA). Custom oligonucleotides were supplied by the Bioprocessing Technology Center (BTC), National University of Singapore. All other chemicals (of analytical grades or ultra pure) were purchased either from Boehringer Mannheim, Pharmacia Biotech, Sigma, BDH, or Aldrich.

2.2. Heteractis magnifica

Heteractis magnifica specimen was obtained from a local aquarium. It was identified using the taxonomic guide by Fautin and Allen [13].

2.3. Preparation of genomic DNA

The method of Blin and Stafford [14] was used to isolate high molecular weight genomic DNA from the tentacles of *H. magnifica*. Freshly excised tentacles were ground into a fine powder using a mortar containing liquid nitrogen. Ten volumes of extraction buffer (0.5% SDS, 0.1 M EDTA and 20 μg/ml pancreatic RNase in 10 mM Tris-HCl buffer, pH 8) were added and the solution incubated for 1 h at 37°C. Proteinase K was then added to a final concentration of 100 μg/ml and the suspension of lysed cells was incubated at 50°C for 3 h. The solution was extracted twice with phenol, once with phenol: chloroform (1:1) and once with chloroform. In all the extractions, the organic and aqueous phases were mixed by slow inversion for 10 min. Genomic DNA was precipitated with ethanol, spooled and dissolved in a final volume of 100 μl Tris-EDTA buffer.

2.4. DNA walking by PCR

This was used to map intron/exon junctions in the HmK toxin gene. First, the sequence coding the matured toxin was amplified from the genomic DNA. Primers used were designed based on the HmK cDNA reported earlier [11] and had the sequence: G1 (sense), 5′-AGG ACG TGC AAG GAC TTG ATG CC-3′ and G2 (anti-sense), 5′-GCA GGA GCC ACA GGT CTT GCG GC-3′. The cycling parameters were: 30 cycles of 95°C, 1 min; 50°C, 1 min; 72°C, 2 min; 72°C for an additional 10 min after the final cycle. Next, the complete coding region of HmK precursor was amplified. Sense primer G3, 5′-ATG AAG TCC CAG ATG ATT GCT GC-3′, binds to the beginning of the signal peptide sequence while the antisense primer used was G2. Cycling parameters were as above. A PromoterFinder DNA Walking Kit (Clontech laboratories) was then used to amplify the 5′-untranslated region of the HmK toxin gene. The protocol used was based on

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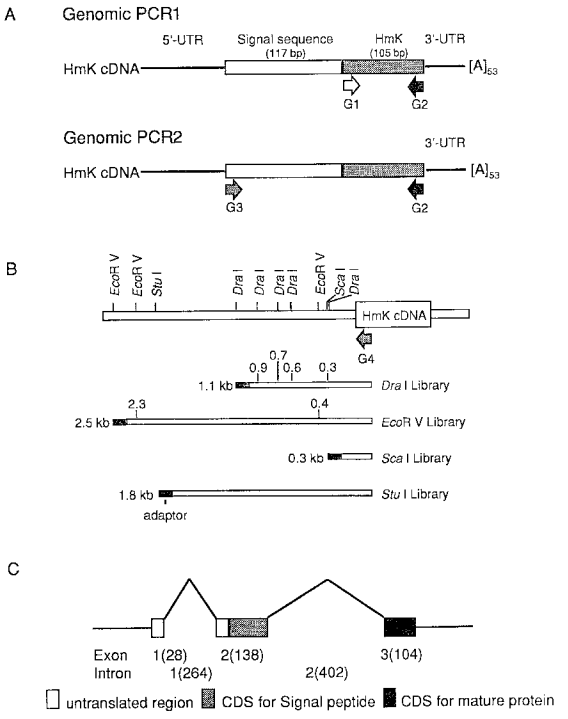

Fig. 1. Strategy of analysis, partial restriction map, and organization of the HmK toxin gene. A: The coding region of HmK toxin gene was amplified in parts. Primers were designed based on the cDNA reported earlier and the locations are indicated in the figure. B: DNA walking in the *H. magnifica* HmK toxin gene. Five adaptor-ligated libraries were constructed as described in the text. Simple restriction map was generated from the data shown in Fig. 2C. G4 is a gene-specific primer designed for walking upstream from the known mRNA sequence. C: Organization of the gene. The exons are boxed.

the manufacturer's specification. In brief, five separate aliquots of DNA, with a high average molecular size, were completely digested with five different enzymes; *Dra*I, *Eco*RV, *Pvu*II, *Sca*I and *Stu*I, that left blunt ends. Each batch of digested genomic DNA was then ligated separately to special adaptors provided in the kit. For DNA walking, the adaptor-ligated libraries were used as templates in long-distance PCR (LD-PCR) amplifications. PCR amplifications were performed using a long distance thermostable DNA polymerase mixture of *Tth* polymerase and Tth antibody to provide automatic 'hot-start' (Clontech). Primers used in the LD-PCR were the adaptor primer (AP1; 5'-GTA ATA CGA CTC ACT ATA GGG C-3') provided in the kit, and a 30-mer gene-specific primer, G4 (5'-GAG AAC AGC AGC AAT CAT CTG GGA CTT CAT-3'). Cycling parameters for LD-PCR were: 7 cycles of 94°C, 25 s; 72°C, 3 min; followed by 32 cycles of 94°C, 25 s; 67°C, 3 min; and 67°C for an additional 7 min after the final cycle. The PCR products were purified after gel electrophoresis using the Geneclean procedure (Bio-101), and subcloned into pT7 Blue vectors (Novagen). The inserts were sequenced on both strands with the forward and reverse M13 primers using the method of Sanger et al. [15] on an automated DNA sequencer (Applied Biosystems Inc., model 373A).

2.5. Primer extension analysis

Total RNA was isolated from the tentacles of *H. magnifica* using a modified guanidinium isothiocyanate extraction method [16,17]. The primer extension analysis was carried out by hybridizing 10 μg total RNA with 100 ng primer G4 in 10 μl 1×hybridization buffer (150 mM KCl, 10 mM Tris-HCl, pH 8.3, 1 mM EDTA) in a microfuge tube. 10 μg of total yeast RNA was used in the control experiment and carried out under the same experimental conditions. The hybridization was performed by incubating the tubes for 90 min in a 65°C water bath after which the tubes were allowed to cool slowly to room temperature. The RNA-primer hybrid was ethanol precipitated and redissolved in 10 μl of DEPC-treated water and made up to a final volume of 20 μl with 10 μl of reverse transcriptase (RT) mix (1 mM dNTP, 20 U rRNasin, 100 U MuLV reverse transcriptase, 2×Promega RT buffer) supplemented with [α-^{33}P]dATP having a specific activity of 1000–3000 Ci/mmol (Amersham). Reverse transcriptase reactions were carried out at 42°C for 1 h. The reactions were terminated by adding 130 μl RNase reaction mix (100 μg/ml salmon sperm DNA, 20 μg/ml DNAse-free RNase A) to each tube, incubating for 15 min at 37°C, followed by the addition of 15 μl of 3 M sodium acetate. The samples were extracted with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with ethanol and resuspended in 5 μl of sequencing gel loading buffer for analysis on a 6% acrylamide/7 M urea DNA sequencing gel. A set of dideoxy nucleotide DNA sequencing reactions was generated using the Perkin-Elmer AmpliCycle sequencing kit and used as size markers. The primer used was typically G4 and pT7 Blue clone harboring the 5'-flanking region of the gene as template.

3. Results

3.1. Intron-exon organization of HmK toxin gene

PCR amplifications were used to map the intron/exon junctions in the K^+ channel toxin gene. The strategy used in the mapping is outlined schematically in Fig. 1. In the first amplification, using G1 and G2 as sense and antisense primers, respectively, to the sequence coding matured HmK, two DNA fragments of the same length (105 bp) and sequence were obtained from PCR of genomic DNA, and that of control

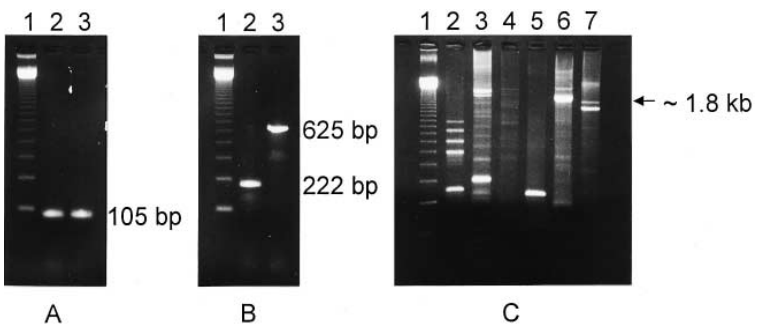

Fig. 2. Amplification products generated from the coding region and walking upstream from the coding region of HmK toxin gene. A: Amplification of HmK genome coding the matured toxin. Lane 1: 123 bp ladder of DNA size markers; lanes 2 and 3: RT-PCR (control) and genomic PCR respectively using primers G1 and G2 (PCR1 in Fig. 1A). B: Amplification of the complete coding region of HmK gene. Lane 1: 123 bp ladder of DNA size markers; lanes 2 and 3: cDNA amplification (control) genomic PCR, respectively, both amplifications used primers G3 and G2 (PCR2 in Fig. 1A). C: Amplification products generated from walking upstream from the coding region of the HmK toxin gene. LD-PCR products in lanes 2–6 were obtained from the *Dra*I, *Eco*RV, *Pvu*II, *Sca*I and *Stu*I adaptor-ligated DNA libraries, respectively. Primers were typically AP1 and G4. Lane 7 shows 1.5 kbp amplification product of human tissue-type plasminogen activator (tPA) locus using tPA primers, carried out under the same conditions and represents the positive control. Lane 1: 123 bp ladder of DNA size markers.

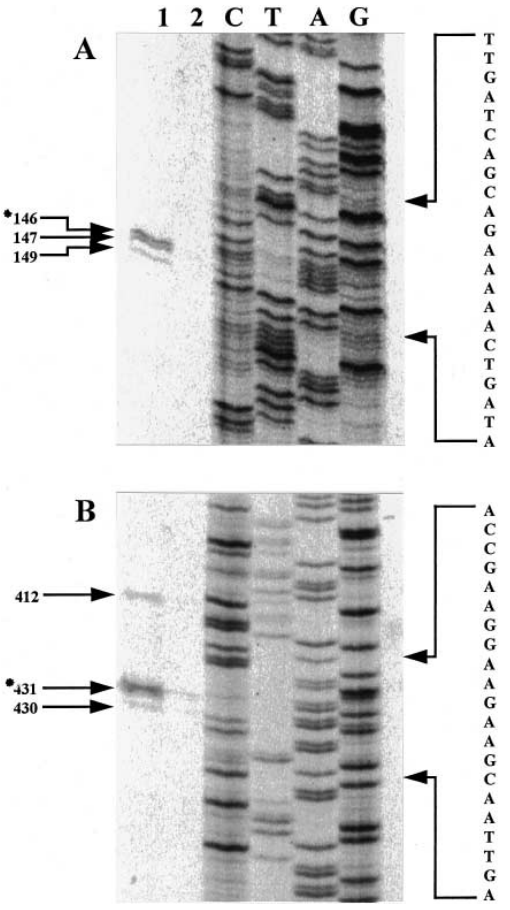

Fig. 3. Primer extension analysis for determination of the transcription-initiation site(s) of the HmK toxin gene. Primer G4 was hybridized with either 10 μg total *H. magnifica* RNA (lane 1) or 10 μg total yeast RNA (control experiment, lane 2) and extended with reverse transcriptase. Lanes C, T, A, G represent a set of dideoxy nucleotide DNA sequencing reactions generated with primer G4 and TA vector pT7 Blue harboring the 5′-flanking region of the gene as template and were used as markers. Part of the nucleotide sequence determined is given on the right. The relative sizes of the extension products and the most probable transcription-initiation sites are indicated by asterisks.

RT-PCR reaction performed on total *H. magnifica* RNA (Fig. 2A). This implied that the coding region of the matured HmK toxin is *intronless*. A second PCR, using G3 as the sense primer that annealed to the beginning of the leader sequence and G2 as the antisense gene-specific primer, amplified a single band of about 625 bp from the genomic DNA (lane 3, Fig. 2B). The control cDNA is 222 bp in length (lane 2, Fig. 2B), indicating that the amplified fragment contains about 400 bp of intron sequence(s). Subsequent sequence analysis of the 625 bp fragment revealed that a single intron of 402 bp separated the leader sequence from the structural sequence (Fig. 4). The intron, interestingly, neither disrupted the leader signal sequence nor the sequence coding the matured toxin except for the first base of the latter. The donor and acceptor splice site of the intron-exon junction was consistent with the rule that introns begin with GT and end with AG [18].

3.2. The 5′-untranslated region of the HmK toxin gene

Fig. 2C shows the results of amplifications from the 5′ region of the leader sequence of the HmK toxin gene to an upstream site restricted by one of the five restriction enzymes used (Fig. 1B). *Dra*I and *Eco*RV libraries generated multiple PCR products ranging in size from 300 bp to 1.1 kbp (lane 2, Fig. 2C) and 400 bp to 2 kbp (lane 3, Fig. 2C), respectively. In the case of *Sca*II and *Stu*II libraries, single major PCR products were generated with sizes 300 bp (lane 5, Fig. 2C) and 1.8 kbp (lane 6, Fig. 2C), respectively. The absence of a major product in the *Pvu*II library (lane 4, Fig. 2C) is probably because the distance between the primer and the upstream restriction site is greater than the capability of the system. We chose to sequence the larger 1.8 kbp PCR fragment from the *Stu*II library for scanning regulatory elements. Two walks upstream from the coding region of the toxin precursor generated about 800 bp sequence information. The first walk used primer G4 while the primer for the second walk, G5, was synthesized based on the first walk and had the sequence 5′-CTA TGA GTT ACG GGC CTT TAG AGA ATT ACC-3′. The nucleotide sequence of the coding region of HmK toxin gene and its 5′-untranslated region is given in Fig. 4. The sequence identical to the HmK cDNA reported earlier [11] is capitalized and boxed. Filled-in box represents the coding region of the matured toxin. Shaded-in box typically exemplifies the signal peptide sequence, while the 5′-untranslated region of HmK cDNA is denoted by open box. This 5′-untranslated sequence is interrupted by an intron of 264 bp. This second exon-intron border is also consistent with the GT/AG rule.

3.3. Determination of transcription-initiation site of HmK gene

The transcription initiation site was assigned by primer extension using primer G4, which is complementary to the 5′-end of the toxin precursor DNA sequence. Two major primer extension products were observed (Fig. 3). An alignment of these products with the gene sequence showed that the smaller product corresponds to an A at position 430 (transcription-initiation site 1, TIS1) and the larger product to another A (285 bp upstream of TIS1) at position 145 (TIS2). A putative CAAT-element and a TATA box motif are located 32 bp and 20 bp, respectively, upstream from TIS1, while the same elements are also present 134 bp and 41 bp, respectively, upstream from TIS2.

4. Discussion

4.1. Gene structure of HmK toxin

Analysis of the genomic DNA sequence of HmK toxin indicated that the gene was interrupted by two introns. The first intron disrupted the 5′-non-coding region of the previously reported cDNA [11] while the second intron separated the leader sequence from the coding region of the matured toxin in a remarkably precise manner, leaving the two regions practically intact. Fig. 1C shows a schematic of HmK gene organization. There are two possible sites for transcription initiation (TIS1 and TIS2; Fig. 4), with their respective putative CAAT-elements and TATA-box motifs. This is the first report on the existence of multiple transcription-initiation sites in animal toxin genes although this phenomenon is not unusual in eukaryotic gene expression which are highly regulated at the level of transcription initiation [19]. Of the two possible sites, TIS1 appears to be the more likely candidate as transcription-initiation site for the HmK cDNA [11], since an identical sequence to the 5′-untranslated HmK cDNA sequence is

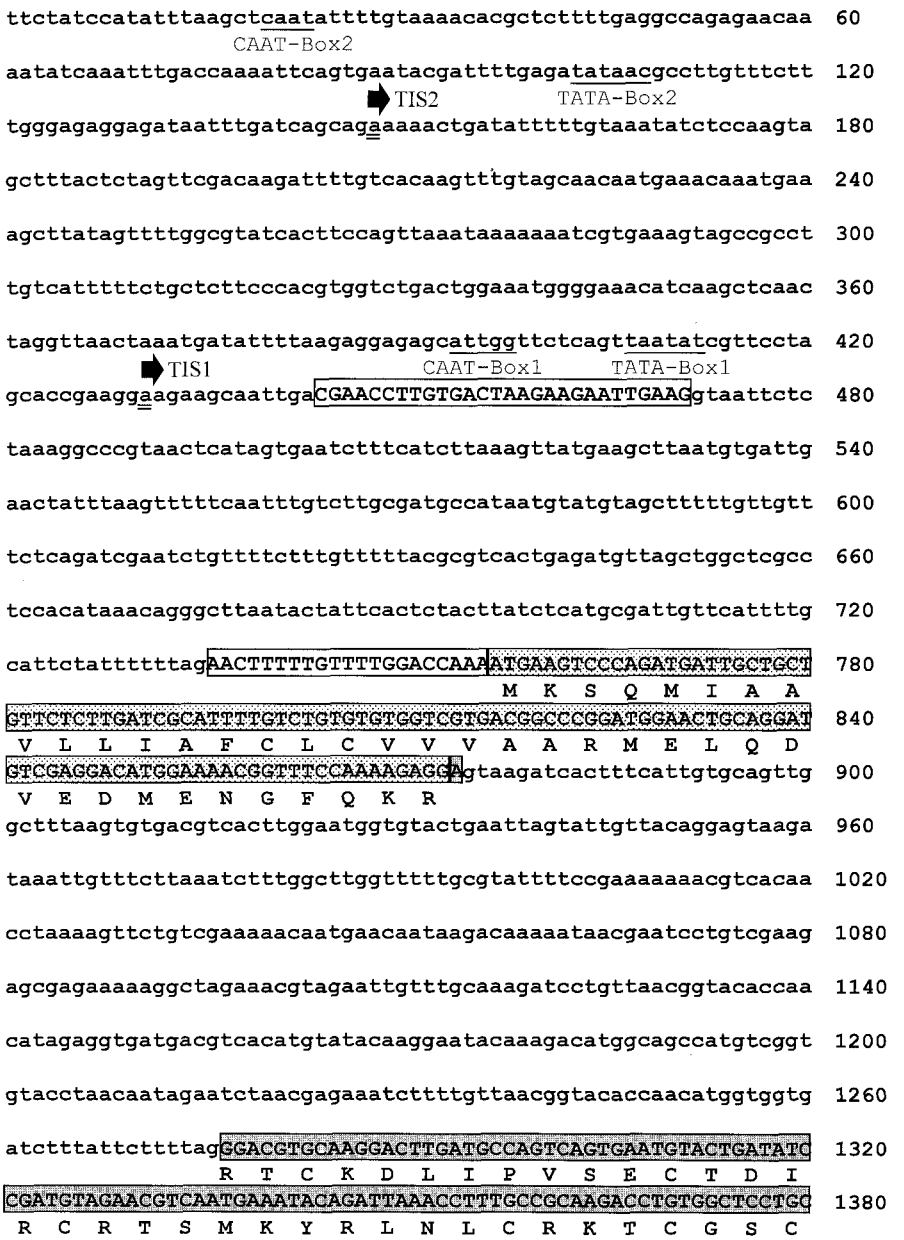

Fig. 4. Nucleotide sequence of the HmK toxin gene including the introns and the 5′-flanking region. The sequence identical to the previously reported HmK cDNA is in capital letters and boxed. The filled-in box represent the matured toxin sequence, the signal peptide sequence box is shaded while the 5′-untranslated sequence is in the open box. The deduced amino acid sequence of the coding region is presented in the one-letter code below the codons. Transcription-initiation sites (TIS1 and TIS2), assigned by primer extension analysis, are indicated by arrows and the corresponding nucleotide double-underlined. Their corresponding TATA-boxes and CAAT-boxes that may be involved in gene regulation are underlined. The intron-exon organization of the gene is shown schematically in Fig. 1C.

present 13 bp downstream to the TIS1 (capitalized and boxed, Fig. 4). This sequence, however, is interrupted by an intron of 264 bp. Further, from the fact that the cDNA sequence begins 13 bp downstream from the TIS1 indicates that the cDNA reported earlier was not of full length. The possible role of alternative transcription-initiation site(s) (e.g. TIS2) is not clearly understood but they may mediate the regulation of expression of these genes. In vivo studies have shown that a longer 5′-untranslated region increases the efficiency of translation over that of a shorter one and may be necessary for the efficient initiation of translation in some genes [20]. The intensity of the signals obtained through our primer extension analysis for both transcription-initiation sites, TIS1 and TIS2, was similar. Precisely which transcription initiation site is used predominantly by the toxin producing cells may depend on the level of stress the sea anemone was experiencing. It may opt for the faster, but less efficient TIS1 (as evident from the size of the cDNA we obtained), when the animal was removed from its natural environment and the tentacles were cut.

4.2. *Comparison of HmK intron-exon organization to other known toxin gene structures*

The data on K^+ channel toxin gene presented here is the first for a sea anemone, although two other groups of toxins have been studied at genomic level from these animals. These are neurotoxic peptides (calitoxins) from *Calliactis parasitica*

[21] and the cytolysin equinatoxin II from *Actinia equina* [22]. Of particular interest is the equinatoxin II gene which lacks introns within its coding region. The presence of such an intronless gene was correlated to the relative low position of cnidarians in the evolutionary lineage. In spite of the fact that there are similar uninterrupted genes from different classes of the phylum cnidaria [23–25], it is not a general rule to cnidarian gene organization (see later). It may, nevertheless, reflect on the ability of these predatory organisms to evolve a selected set of genes for quick response to external stimuli (as illustrated by Anderluh et al. [22]). In contrast to the intronless genes, the calitoxins *clx*-1 and *clx*-2 genes are interrupted by two introns. The distribution of the introns is very similar to the HmK gene. In all three cases, the first intron is located either at the end (in the case of HmK), or almost to end (*clx*-1 and *clx*-2) of the signal peptide sequence. The second intron is present in the 5′-untranslated region of all the three genes.

An interesting common feature of all the sea anemone genes reported so far is that the coding region for the matured proteins is always uninterrupted by introns. In this respect, the earlier correlation between the absence of introns in the genes of cnidarians to the low positioning of this phylum in the evolution tree by Anderluh et al. [22] is more appropriate if it is restricted to the coding regions of the matured proteins. Such an arrangement reflects a common origin of cnidarian genes [22] and may describe a novel genome evolution mechanism in which primitive organisms acquire new genes through either one or a combination of the known processes (multiplication, re-arrangement etc.) while sharing common signal peptide and 5-untranslated sequences [26].

On the other hand, the genomic organization of HmK differs from those of apamin and mast cell-degranulating peptide (MCD peptide), the two K^+ channel toxins from the venom of honey bee, *Apis mellifera* [27]. The honey bee venom toxins, interestingly, are encoded by two genes in tandem which share the same 3′-exon. Together, the genes contain six exons separated by introns, with the bulk of the apamin gene residing in the third intron of the MCD peptide gene. The structural organization of the coding region of HmK gene, however, resembled the scorpion voltage-sensitive sodium channel toxin genes. The gene encoding the toxin AaH I′ from the scorpion *Androctonus australis* has a single intron of 425 bp located near the end of the signal peptide of the toxin precursor [28]. An identical intron-exon organization is also present in the genomic region encoding toxins IV-5, from the scorpion *Tityus serrulatus* Lutz and Mello [29], and toxin gamma (also called toxin VII) from the scorpion *Tityus serrulatus* [30]. A single intron of 347 bp and 475 bp interrupted the C-terminal of the signal peptide of these toxins respectively. Interestingly, like the sea anemone genes, the coding regions of the matured toxins are always intact. The similarity in organization of the HmK coding region to the scorpion voltage-sensitive sodium channel toxin genes suggests that these toxins probably have had evolved from the same ancestral gene. Ignoring the species difference barrier, this hypothesis is not far fetched as it is common knowledge today that the ion channels are believed to have evolved from a common ancestral molecule, of which the potassium channels make the best candidate, being the most ancient of the channels [31]. It is therefore not unreasonable to speculate a similar path of evolution for molecules that have the capabilities to block these ion channels.

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