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Research Article

Identification and analysis of venom gland-specific genes from the coastal taipan (*Oxyuranus scutellatus*) and related species

L. St Pierre[a,b], R. Woods[a], S. Earl[a], P. P. Masci[c] and M. F. Lavin[a,*]

[a] The Queensland Cancer Fund Research Unit, The Queensland Institute of Medical Research, PO Box Royal Brisbane Hospital, Herston, Queensland, 4029 (Australia), Fax: +61 7 3362 0106, e-mail: martinL@qimr.edu.au
[b] School of Life Sciences, Queensland University of Technology, Brisbane (Australia)
[c] Faculty of Health Sciences, University of Queensland, Brisbane (Australia)

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Abstract. Australian terrestrial elapid snakes contain amongst the most potently toxic venoms known. However, despite the well-documented clinical effects of snake bite, little research has focussed on individual venom components at the molecular level. To further characterise the components of Australian elapid venoms, a complementary (cDNA) microarray was produced from the venom gland of the coastal taipan (*Oxyuranus scutellatus*) and subsequently screened for venom gland-specific transcripts. A number of putative toxin genes were identified, including neurotoxins, phospholipases, a pseudechetoxin-like gene, a venom natriuretic peptide and a nerve growth factor together with other genes involved in cellular maintenance. Venom gland-specific components also included a calglandulin-like protein implicated in the secretion of toxins from the gland into the venom. These toxin transcripts were subsequently identified in seven other related snake species, producing a detailed comparative analysis at the cDNA and protein levels. This study represents the most detailed description to date of the cloning and characterisation of different genes associated with envenomation from Australian snakes.

Keywords. Gene cloning; Australian elapid; *Oxyuranus scutellatus*; pseudechetoxin; calglandulin, phospholipase A_2; L-amino acid oxidase.

Introduction

Snake venoms are complex mixtures of pharmacologically active protein and polypeptide toxins that have specifically evolved to alter the physiological mechanisms of their prey. Their components are typically small in size, cysteine rich and often exert a multitude of activities with specific molecular targets, including ion channels and a range of enzymes [1]. Of the thousands of species of snake known worldwide, the Australian venomous snakes, which belong almost exclusively to the elapid family, are recognised as having amongst the most potently toxic venom in the world [2]. Given this relatively high degree of toxicity, Australian elapid venoms have been the target for much research into their biochemical and physiological properties, particularly in relation to their effects on mammalian homeostatic mechanisms. Surprisingly, there has been relatively little research interest into the individual components of the venom at the molecular/genomic level.

* Corresponding author.

The exact clinical features observed within mammalian prey as a result of a bite from an Australian elapid vary depending on the species of snake involved. However, venom injection generally results in significant neurological effects, including disorientation, flaccid paralysis and respiratory failure in association with multiple coagulopathic effects [3]. Typically the local effects of the venom at the site of injury are minor, however, once in the circulation, the systemic effects are of major clinical significance. An exception is envenomation by the mulga (*Pseudechis australis*), whose bite often results in local necrosis [4]. The neurotoxic, haemotoxic and myotoxic effects of Australian elapid envenomation are largely the result of the activity of a myriad of neurotoxins and phospholipase A_2 (PLA_2) enzymes within the venom [5, 6]. Similarly, the procoagulant effects observed in Australian snake venoms are predominantly due to the presence of prothrombin activators that are functionally and structurally similar to mammalian coagulation factor X [7]. Although some activities have been assigned to other less predominant components within the venom, their protein sequences and overall role in the venom remain largely unknown.

The venom from a single Australian elapid may contain upwards of a hundred different proteins. Characterisation of the venom components of Australian elapids to date has primarily focussed on the PLA_2 enzymes, due to their abundance, ease of purification and relative stability [8]. PLA_2 enzymes are found within most snake venoms regardless of genera and display similar primary sequence and catalytic function to their mammalian counterparts. However, in contrast to mammalian enzymes, venom PLA_2s may be highly toxic with a plethora of physiological effects [9]. These include haemorrhagic, myotoxic, haemolytic, hypotensive, oedema forming, platelet aggregating, convulsant, cardiotoxic and pre- and postsynaptic activities. This variation in activity (even within multiple isoforms from an individual snake) is observed despite what appears to be a high degree of conservation in their primary sequence, and is often independent of the phospholipase catalytic activity of the protein [8].

Other than the phospholipases, neurotoxins and prothrombin-activating enzymes commonly described from the venom of the Australian elapids, a number of other components of potential pharmacological importance have been identified within the venom of these snakes. These include venom natriuretic peptides which display structural and functional similarities to their mammalian counterparts and have been implicated in the stimulation of natriuresis, diuresis and vasorelaxation [10, 11]. Pseudechetoxin, and its related homolog pseudecin, are peptidic toxins that target cyclic nucleotide-gated (CNG) ion channels isolated from the venom of the mulga (*P. australis*) and red-bellied black snake (*Pseudechis porphyriacus*), respectively [12]. Pseudechetoxin binds to the pore turret of CNG ion channels and inhibits the flow of current, the first peptide toxin known with this function, and has been used to study the role of these channels in humans. This is a prime example of a novel toxin used to study the pharmacology of a mammalian target [13]. Interestingly, the study of snake venoms is not just limited to the components within the venom themselves, but also includes research into proteins within the venom gland responsible for the manufacturing and transport of toxins. Calglandulin, a putative calcium binding protein identified from the venom gland of the island jararaca, *Bothrops insularis*, displays structural homology to the calmodulin family of proteins. This protein has been implicated in the export of toxins out of the venom gland cells and into the venom itself [14].

Given the diversity of proteins and polypeptides known to exist within the venoms of Australian snakes, and given the relative potency of these components in altering mammalian functional systems, this study was aimed at identifying and further characterising those components within the venom of the coastal taipan (*O. scutellatus*) and related Australian elapids at a molecular level which may be of diagnostic or therapeutic benefit. In this paper we describe the construction and subsequent screening of a complementary (cDNA) microarray chip from the venom gland of the coastal taipan as a means of gene discovery in this elapid and also in related species.

Materials and methods

RNA isolation and cDNA synthesis. Venom glands were excised from Australian elapids including the coastal taipan (*Oxyuranus scutellatus*), inland taipan (*Oxyuranus microlepidotus*), common brown snake (*Pseudonaja textilis*), tiger snake (*Notechis scutatus*), rough-scaled snake (*Tropidechis carinatus*), Stephen's banded snake (*Hoplocephalus stephensii*), red-bellied black snake (*Pseudechis porphyriacus*) and mulga (*Pseudechis australis*) collected under National Parks and Wildlife's permit number WISP031229905. Snap frozen glands were homogenised with a polytron and RNA isolated using Tri Reagent (Sigma-Aldrich, St Louis, USA). First strand cDNA was synthesised from 1 μg of total RNA with an oligo$(dT)_{12–18}$ primer via reverse transcription with 200 units of Superscript II RNAse H^- Reverse Transcriptase (Invitrogen, Mt Waverly, Australia). Total RNA was also isolated from the liver of *O. scutellatus* and *O. microlepidotus*.

cDNA library construction and microarray chip production. A full-length cDNA library was produced from RNA isolated from the venom gland of a coastal taipan (*O. scutellatus*) using the SMART cDNA Library Construction Kit (Clontech, Palo Alto, USA). Briefly,

first strand cDNA was synthesised using 1 μg of total RNA with 1 μM SMART II and 1 μM CDS III/3' primers. The cDNA was polymerase chain reaction (PCR) amplified, digested with proteinase K and *Sfi* 1 restriction enzymes, size fractioned to obtain full-length transcripts and ligated into the λTripleEx2 vector according to the manufacturer's protocol. The total ligation mixture was inserted into a bacteriophage using the Packagene Lambda DNA Packaging System, then transfected into *Escherichia coli* XL1 Blue (Promega, Madison, USA). A total of 4800 clones were then isolated from the packaged λphage cDNA library and PCR products amplified from each clone, with the incorporation of a 5'amino C6 group so as to allow fixation of the PCR product to the slide when arraying the chip. The reaction mixture contained 1 unit of taq buffered in 10 mM Tris pH8.8, 50 mM KCl, 0.1% Triton X-100, 2.5 mM MgCl2, 200 nM dNTPs and 0.1 ϱmol of each of the 5' LTD forward (5'-C_6-CTC GGG AAG CGC GCC ATT GTG TTG GT-3') and 3' LTD reverse (5'-C_6-ACG ACT CAC TAT AGG GCG AAT TGG CC-3') primers. The PCR reaction mixture was thermocycled at 95 °C for 5 min, then 35 cycles of 95 °C for 20 s, 67 °C for 20 s, 72 °C for 3 min with a final extension of 72 °C for 7 min and precipitated with isopropanol. The 4800 *O. scutellatus* venom gland PCR transcripts were then arrayed onto a polylysine-coated slide in duplicate using an automated GMS 417 arrayer and ultraviolet (UV) cross-linked to the chip with a Biorad cross-linker set to 65 mJ, blocked, washed and used in subsequent RNA hybridisations.

RNA amplification. Venom gland and liver RNA samples to be hybridised to the microarray chip were first amplified linearly to produce large quantities of sample. Approximately 1 μg of total RNA was amplified with 1 μg of T7amp primer (5'-AAA CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG CGC $T_{(15)}$-3'), 200 units Superscript III RNAse H^- Reverse Transcriptase and 500 nM dNTPs. The reaction was thermocycled at 37 °C for 5 min, 45 °C for 5 min then 10 cycles alternating between 60 °C for 2 min and 55 °C for 2 min. Second strand synthesis was performed with 40 units of *E. coli* DNA Polymerase 1, 2 units of RNAse H and 200 μM dNTPs at 16 °C for 2 h (Invitrogen, Mt Waverly, Australia). *In vitro* transcription was performed off the second strand template with a T7 MEGAscript kit according to the manufacturer's instructions (Ambion, Austin, USA). The amplified RNA (aRNA) was then treated with 50 units of DNAse 1 and further purified via phenol:chloroform extraction prior to labelling and hybridisation to the *O. scutellatus* microarray chip.

Microarray hybridisation, analysis and screening. Hybridisation to the chip was performed using the indirect labelling method for microarrays, whereby Cy3 monofunctional dye-labelled sample cDNA was hybridised to the chip in conjunction with Cy5 dye-labelled *O. scutellatus* venom gland cDNA as a reference, in duplicate runs. Samples analysed on the microarray chip include those from the venom glands of *O. microlepidotus*, *P. textilis*, *P. porphyriacus*, *P. australis*, *N. scutatus* and *T. carinatus*. aRNA isolated from the livers of *O. scutellatus* and *O. microlepidotus* was also used to produce labelled cDNA which was then hybridised to the array to detect genes whose expression levels were increased in the venom gland. Comparisons between RNA and aRNA from *O. scutellatus* were also performed to ensure no inherent bias in the RNA amplification process. For each hybridisation, a total of 2.5 μg of aRNA was incubated at 70 °C for 10 min with 2.5 μg of oligo-dT primer and cDNA then synthesised with the incorporation of amino allyl labelled dUTP (aa-dUTPs) in a 4:1 ratio with dTTP (Sigma-Aldrich, St Louis, USA). Remaining RNA was hydrolysed with 1 M NaOH and 0.5 M EDTA at 65 °C for 15 min and the cDNA purified to remove unincorporated aa-dUTPs with a Millipore (Bedford, USA) PCR cleanup kit. The cDNA samples were then labelled with monofunctional Cy3 and Cy5 dyes (Amersham Biosciences, Cardiff, UK), combined and incubated with 10 μg of Cot1 DNA and 20 μg of poly dA to prevent non-specific hybridisation to the array. Samples were hybridised to a printed *O. scutellatus* venom gland microarray chip at 45 °C overnight and then washed. The microarray chips were then scanned with a GMS Array Scanner (Genetic Microsystems Inc., Woburn, USA), and comparable images on both Cy3 and Cy5 channels were imported into Imagene analysis software (Biodiscovery Inc., El Segundo, USA) and images overlayed. Gene expression data relative to the *O. scutellatus* venom gland aRNA population were then analysed with Genespring 6 software (Silicone Genetics, Forest Hill, Australia) as previously described [15]. In this manner it was possible to select a suite of genes of interest that could then be isolated from the original library and sequenced for further characterisation.

The conversion of λTripleEx2 clone with a desired insert to a pTripleEx2 plasmid involves the excision and circularisation of a complete plasmid from the recombinant phage by the Cre recombinase-mediated site-specific recombination at the *loxP* sites present within the clone by a bacterial host in which Cre recombinase is being expressed. Selected clones of interest from the results of the microarray hybridisations were isolated from the original phage preparations of the 4800 recombinant cDNA library for sequence determination. Converted clones were purified with a QIAGEN (Hilden, Germany) miniprep kit according to the manufacturer's instructions and sequencing performed in both forward and reverse directions where necessary. A total of 621 clones were selected from the taipan cDNA library as a result of the

microarray analysis, converted to plasmid and subsequently sequenced.

Identification of pseudechetoxin-like cDNAs. A dominant clone observed from sequence results was that of a pseudechetoxin-like transcript previously identified in *P. australis*. Primers were designed from the published 5'-UTR and 3'-UTR sequences of pseudechetoxin and used in the amplification of a similar product in related species. PCR amplification of the 717 bp coding sequence of the gene was performed with 500 ng of cDNA template, 1 unit of AmpliTaq gold buffered in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.25 mM $MgCl_2$ and 200 μM dNTPs with 50 ρmol of each of the forward (5'-GGA GTT ACA CTG GGG CTC-3') and reverse (5'-ACT GAA TGG GAG ATC AGC-3') primers (Applied Biosystems, Foster City, USA). The reaction mixture was then thermocycled at 95 °C for 10 min, followed by 35 cycles of 95 °C for 30 s, 54 °C for 45 s and 72 °C for 1 min, with a final extension of 72 °C for 7 min. The resulting PCR products were analysed on a 1% TAE agarose gel and bands of interest excised, purified with a QIAex II gel extraction kit and cloned via the pGEM-T vector system (Promega, Madison, USA). The ligation mix was transformed into competent dH5α *E. coli*, with recombinant clones selected on LB-Amp plates (50 μg/mL) supplemented with IPTG and X-Gal. Multiple clones were then sequenced from each snake with a Big Dye version 3.1 Terminator sequence ready reaction kit (Perkin-Elmer, Norwalk, USA). Alignments were performed with BioEdit software (Isis Pharmaceuticals Inc., Carlsbad, USA) to determine the consensus sequence from each elapid. For phylogenetic analyses, the predicted protein sequences were aligned using ClustalW, subjected to Maximum Likelihood analysis using MolPhy version 2.3 via the Dayhoff model in local rearrangement of NJ trees mode (1000 bootstrap replicates) [16]. In these analyses a cysteine-rich venom protein from the Chinese cobra, *Naja atra* (AAP20603), was defined as an outgroup.

Identification of calglandulin-like cDNAs. A clone demonstrating increased expression in the *O. scutellatus* venom gland originally proved unidentifiable by BLAST searches. Therefore this sequence was further characterised by 5'-RACE with a SMART RACE cDNA Amplification kit (Clontech, Palo Alto, USA). 5'-RACE products were amplified from *O. scutellatus* and *P. textilis* 5'-RACE cDNA with a gene-specific primer designed from the clone identified from the library (5'-CTC TTA CCC CCT TCT TCT TGG TAT CGC CG-3'). Products were run on a 1% TAE agarose gel, purified for both snakes and subsequently cloned and sequenced. Sequence alignment results identified the protein as being closely related to a calglandulin-like transcript previously cloned from Bothrops insularis. The full-length coding sequence of the calglandulin-like gene was subsequently identified in the other Australian elapids by PCR. Amplification of an approximately 480 bp PCR product was performed with forward (5'-CGA GGA AAT GGC AGCAAC ACT AAC-3') and reverse (5'-GTC TTA CTG AGT CAG TTT GAA GG-3') primers in a manner similar to that of the pseudechetoxin-like cDNA. The PCR reaction was thermocycled at 95 °C for 10 min followed by 30 cycles of 95 °C for 30 s, 56 °C for 40 s and 72 °C for 1 min, with a final extension at 72 °C for 7 min and gel purified, cloned, sequenced and aligned as previously described.

The quantity of calglandulin-like transcripts in the coastal taipan and related species were then analysed by quantitative PCR (qPCR) to confirm the microarray expression results. Accordingly, cDNA was produced from 1.5 μg of venom gland total RNA, as well as liver RNA purified from *O. scutellatus* and *O. microlepidotus*. Prior to cDNA synthesis, total RNA was digested with 2 units of RQ1 DNAse polymerase to remove any contaminating genomic DNA (Promega, Madison, USA). In all cases 7.5 μl of a 2× Sybr Green PCR master mix was added to 5 ρmol of a forward (5'-GAT CGG CAT CAA CCC CAC CAA-3') and reverse (5'-GTC CCA CTC AAT GTA GCC CTT G-3') primer mix and 20 ng of cDNA template, in a total volume of 15 μl (Applied Biosystems, Foster City, USA). A 190 bp fragment of the calglandulin-like gene was amplified using primers based on sequence 100% identical to all snakes. Each template was run in triplicate including the amplification of a 60S ribosomal protein house-keeping gene (which demonstrated equivalent expression in all tissue samples identified on the microarray) that was amplified as a 195 bp product with forward (5'-GCA AGC GTA TGA ACA CCA ACC C-3') and reverse (5'-AGA GCA GCT GGG ACG ACC ATT C-3') primers. Reaction mixtures were thermocycled at 95°C for 10 min, followed by 35 cycles of 95°C for 15 s, 64°C for 15 s and 72°C for 30 s on a Rotor-Gene 3000 (Corbett Research, Mortlake, Australia). A standard curve of cycle time using a range of known quantities of plasmid as template was also performed and the results, including cycle time for product formation and calculated transcript concentration, analysed in Microsoft Excel. Averages of the triplicate samples in duplicate runs were then taken, and the results normalised to that of the house keeping gene.

Identification of novel PLA_2 toxins. A full-length PLA_2 transcript coding for the *O. scutellatus* venom protein β-taipoxin was identified from screens of the microarray. Using primers designed from this sequence, other PLA_2 genes were identified from the venom glands of related snake species via PCR and cloning. The PCR amplification of the coding sequence of these genes was performed as described above with forward (5'-TGC TTG CAG CTT CAC CAC TGA C-3) and reverse (5'-TCC TCG

CGC TGA AGC CTC TCA AA-3) primers. All reactions were then thermocycled at 95 °C for 10 min, 12 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, then another 13 cycles of 95 °C for 30, 64 °C for 30 and 72 °C for 1 min with a final extension of 72 °C for 7 min. PCR products were then cloned and sequenced, and an alignment was performed for a minimum of 12 clones identified from each snake.

Identification of L-amino acid oxidases. A putative toxin, L-amino acid oxidase (LAAO) was previously identified in a number of vipers [17, 18]. To investigate the presence of this gene in the venom glands of Australian elapids, PCR was performed using primers designed from an alignment of a number of these snakes. Amplification of a 1550-bp product was performed with forward (5'-GAT GAA TGT CTT CTT TAT GTT CTC-3') and reverse (5'-TTA AAG TTC ATT GTC ATT GCT CA-3') primers which corresponded to the 5'- and 3'-coding sequences of the LAAO gene in *Trimeresurus stejnegeri* (AY338966), *Crotalus adamanteus* (AF071564), *Calloselasma rhodostoma* (AJ271725) and *Crotalus atrox* (AF093248). A PCR reaction mixture was prepared as described above, and the reaction thermocycled for 8 min at 95 °C, followed by 20 cycles of 95 °C for 30 s, 53 °C for 30 s and 72 °C for 2 min 30 s, and an additional 20 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min 30 s with a final extension of 72 °C for 7 min. PCR products were examined on a 1% TAE agarose gel, purified, cloned and sequenced in both directions. Multiple clones from *O. scutellatus*, *P. australis* and *N. scutatus* were examined, and alignments of the deduced amino acid sequence were performed.

Results

Generation of a cDNA microarray from the venom gland of *O. scutellatus*. A cDNA microarray from the venom gland of the coastal taipan (*O. scutellatus*) was generated using the SMART cDNA library construction kit. The titre of the amplified library was calculated to be approximately 2.5×10^6 pfu/ml with an insert size varying from 200 bp to 2 kb. Sequence analysis of 136 clones randomly selected from the library revealed a redundancy of approximately 52%. A cDNA microarray chip was then established with 4800 PCR products of inserts amplified from random clones selected from this library.

Identification of venom gland-specific transcripts. In order to identify specific genes that may be of functional importance in the venom gland of the coastal taipan, a cross comparison of gene expression levels between the venom gland and the liver was performed, which would be expected to only overlap in the expression of housekeeping genes or those involved in cellular regulatory functions. Total RNA isolated and amplified from both the venom gland and the liver of *O. scutellatus* was used to make labelled cDNA, which was then cross-hybridised to the microarray chip to identify genes that were differentially expressed between these two tissues. All results were confirmed by performing comparisons with RNA isolated from the closely related species of snake, *O. microlepidotus*. Following normalisation and filtering, an average of 57% of transcripts demonstrated an increase in expression in the venom gland compared with the liver (fig. 1). To establish whether these genes were specific to the venom gland, 582 transcripts were selected from those overexpressed in this tissue for further analysis. Clones were identified by their position on the microarray grid, the original bacteriophage was isolated and DNA fragments of interest were recombined into a plasmid vector via a Cre-recombinase system for DNA sequencing. Thirty-nine clones that demonstrated increased expression in the liver were also selected for sequencing, bringing the total number of sequenced transcripts to 621.

Using this approach, a total of 58 separate transcripts were identified from the *O. scutellatus* cDNA microarray chip (GenBank accession numbers DQ084027–DQ084065 and DQ085813–DQ085855). The results in table 1 identify 8 transcripts (including various isoforms) corresponding to genes coding for known toxin families previously described from the venom of elapid snakes. These include a pseudechetoxin-like protein, PLA_2 enzymes, α-neurotoxins, a venom natriuretic peptide and a venom nerve growth factor. In addition to these known toxin genes 18 transcripts were identified that were also overexpressed in the venom gland but coded for proteins predicted to have a non-secretory/regulatory function within the venom gland, including a calglandulin-like transcript (table 1). A number of other transcripts corresponding to non-secretory proteins that were downregulated in the venom gland compared with the liver were also identified, again predicted to be important for regular cellular maintenance (for example a polyadenylate binding protein). While many of the clones demonstrated homology to known genes, a total of 24 of the 58 different transcripts displayed no known identity via BLAST search analysis. To confirm the specificity of expression of the toxin genes to the venom gland, qPCR was performed from venom gland and liver RNA of both the inland and coastal taipan with primers specific for these genes. In all instances it was not possible to detect expression of these proteins within the liver of these snakes, confirming their venom gland-specificity (results not shown).

Characterisation of pseudechetoxin-like cDNAs. A clone present in abundance on the microarray chip demonstrated significant similarity to pseudechetoxin, a

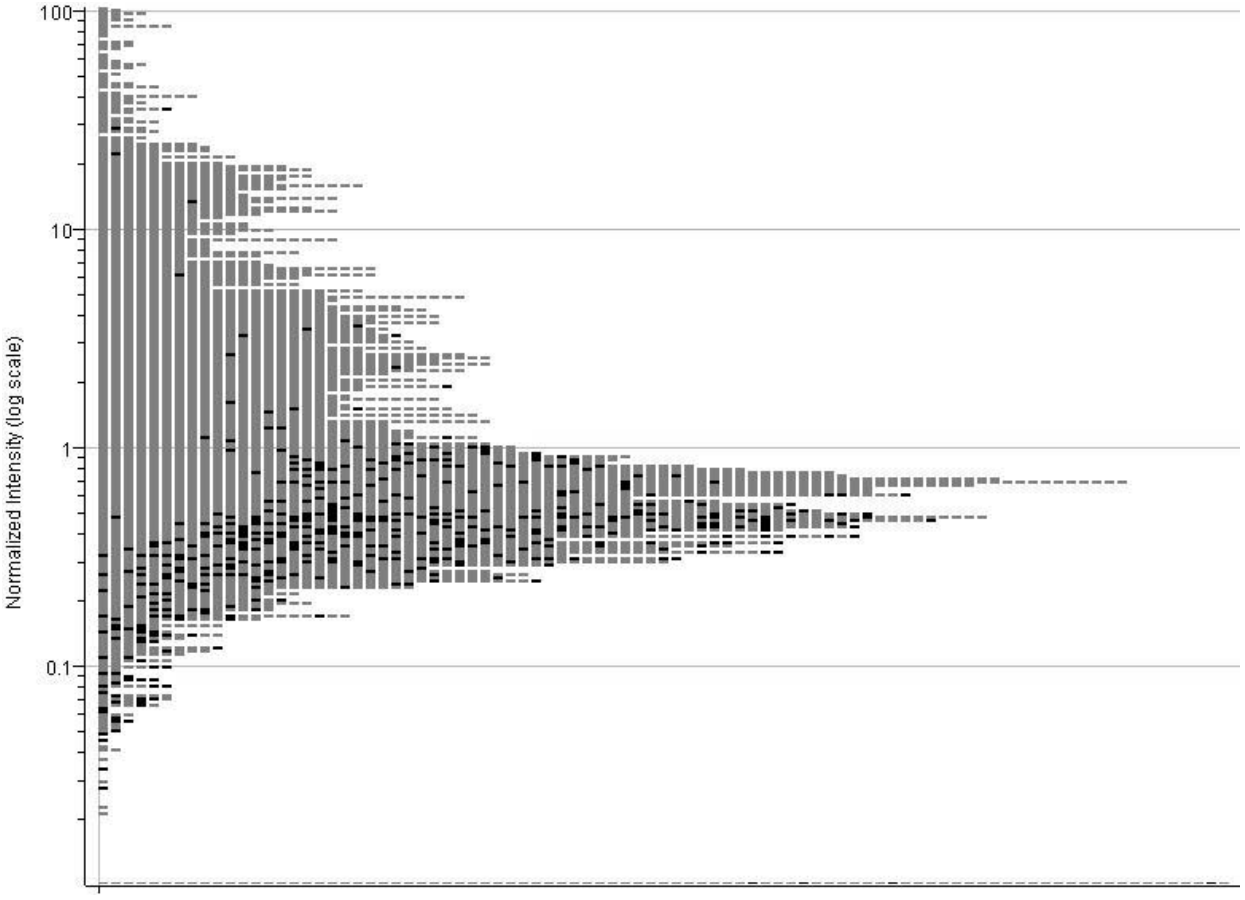

Figure 1. Histogram representing the normalised expression (in log scale) of all 4800 transcripts present on the *O. scutellatus* venom gland microarray chip. The Y-axis denotes fold change between the two RNA populations: those above 1 demonstrate an increased expression in the liver, whilst those below 1 are more highly expressed in the venom gland. All known toxin and venom gland-specific genes identified in screens of the library are highlighted in black. The skew below the axis is due to the presence of a greater number of venom-specific genes on the chip as the venom gland was the original source tissue for its production.

peptidic toxin previously isolated from the venom of *P. australis* that targets cyclic nucleotide-gated (CNG) ion channels [12]. Using primers based on the published *P. australis* sequence, the full-length coding sequence of the pseudechetoxin-like cDNA was amplified from a total of seven Australian elapids, including *P. australis* and *P. porphyriacus*. The results in figure 2 depict a protein alignment for the precursor toxin for all seven snakes. No product was identified in the rough-scaled snake (*T. carinatus*). The unprocessed protein was 238 amino acids in length with an overall identity of 83% between the seven snakes, and even higher between any two species. The sequences observed for *P. australis* and *P. porphyriacus* were identical to those reported by Yamazaki et al. [19]. Pseudechetoxin is a member of the cysteine-rich secretory protein (CRISP) family of snake toxins, as is evidenced by a number of conserved cysteine residues, particularly within the C-terminal region of the protein [20]. Phylogenetic analysis of the deduced amino acid sequence reveals a distinct clustering pattern, with high bootstrap support (fig. 3). The two *Oxyuranus* genera associate closely with *P. textilis* with an overall identity of 96%, and likewise, *N. scutatus* and *H. stephensii* are most closely related to each other (97%). It is not surprising to observe the clustering of the *P. porphyriacus* and *P. australis* sequences, as they arise from members of the same genus.

Characterisation of calglandulin-like cDNAs. The most abundant clone identified in screens of the microarray represented a transcript corresponding to a calglandulin-like protein, an EF-hand protein with conserved Ca^{2+} binding motifs first identified from the venom gland of the island jararcaca, *B. insularis*. The original clone isolated from the *O. scutellatus* cDNA library was a 465-bp fragment that represented the entirety of the 3'-UTR region of the gene. Subsequent 5'RACE analysis identified a 471-bp open reading frame with 93.4% identity to the coding sequence of calglandulin from *B. insularis*. This gene was designated CAGLP (calglandulin-like protein).

Table 1. Transcripts identified from the *Oxyuranus scutellatus* venom gland microarray chip.

Name	No. of clones	Insert size (bp)	Putative function	Expression ratio	GenBank accession no.
Transcripts with increased venom gland expression					
Secreted/venom proteins					
Pseudechetoxin-like protein	191	1279	cyclic nucleotide-gated ion channel blocker	+ 5.6-fold	DQ084035
PLA_2 enzymes (3 isoforms)	53	760	haemostatic, myotoxic and neurotoxic activities	+ 5.2-fold	AY691657
-Neurotoxins (2 isoforms)	22	536	inhibitors of neuromuscular transmission	+12.4-fold	DQ085855
Venom natriuretic peptide	1	498	natriuresis and vasoactivity	+ 5.1-fold	DQ084065
Venom nerve growth factor	1	324	undefined role in the venom	+ 3.4-fold	DQ084064
Non-secreted/regulatory proteins					
Calglandulin-like protein	217	469	exportation of toxins into the venom	+ 2.4-fold	DQ084027
Ribosomal proteins* (8 different forms)	70	665	ribosomal protein function	+ 1.8-fold	DQ084040–DQ084047
Creatine kinase (2 isoforms)	5	688	muscle-derived enzyme	+ 5.5-fold	DQ084048
Other proteins with known homology (7 different proteins)	13	381–927	proteins with known homology involved in regular cellular maintenance and activity	+ 2.2- to + 99-fold	DQ084054–DQ084058, DQ084062, DQ084063
Unknown transcripts (8 different transcripts)	9	167–1022	transcripts with no known or predicted homology	+ 1.2- to + 44-fold	
Transcripts with increased liver expression					
Proteasome homolog	4	619	involved in regulated intracellular proteolytic cleavage	– 2.9-fold	DQ084049
Polyadenylate binding protein homolog	1	1037	required for translation initiation	– 2.8-fold	DQ084050
Translocon-associated protein (TRAP)-delta homolog	4	608	transmembrane protein where nascent secretory proteins enter the endoplasmic reticulum	– 7.2-fold	DQ084051
Other proteins with known homology (5 different proteins)	7	150–900	proteins with known homology involved in regular cellular maintenance and activity	– 3.7- to –11.7-fold	DQ084052, DQ084053, DQ084059–DQ084061
Unknown transcripts (16 different transcripts)	23	225–1050	transcripts with no known or predicted homology	–1.2- to –53.5-fold	

The average fold difference in the expression levels of each gene between the venom gland and liver of *O. scutellatus* is also shown (+ values indicate increased venom gland expression, – values indicate increased expression in the liver, a value of 1 would denote equal expression).

*Values shown for ribosomal proteins are for ribosomal protein L13a only. GenBank accession numbers are included where appropriate and total number of clones sequenced, average insert length and putative function as predicted by homology also shown.

Failure to identify the original cDNA library clone via a BLAST homology search (prior to 5'RACE) was due to the low identity (54.9%) between the 3'-UTR sequences of the *B. insularis* and *O. scutellatus* transcripts. PCR was employed to isolate full-length cDNAs from eight related Australian elapids. An alignment of the deduced amino acid sequences revealed complete conservation of this protein between all snakes with the exception of a glutamic acid in place of an aspartic acid at position 67 of *T. carinatus* (fig. 4). A single nucleotide change at the third position explains this variance. This high degree of conservation was also maintained within calglandulin from *B. insularis* where there were only three amino acid differences across the 156-residue protein [14].

To determine the relative expression of CAGLP within the venom glands of Australian elapids, the *O. scutellatus* microarray was hybridised with labelled cDNA from the related species. Calglandulin was selected for such a cross-species comparison due to its presence (and significant sequence identity) in all species involved in this study. The average expression of this transcript compared with that of *O. scutellatus* was determined in duplicate labelled cDNA hybridisations to the microarray chip and confirmed by qPCR analysis using 60S ribosomal RNA

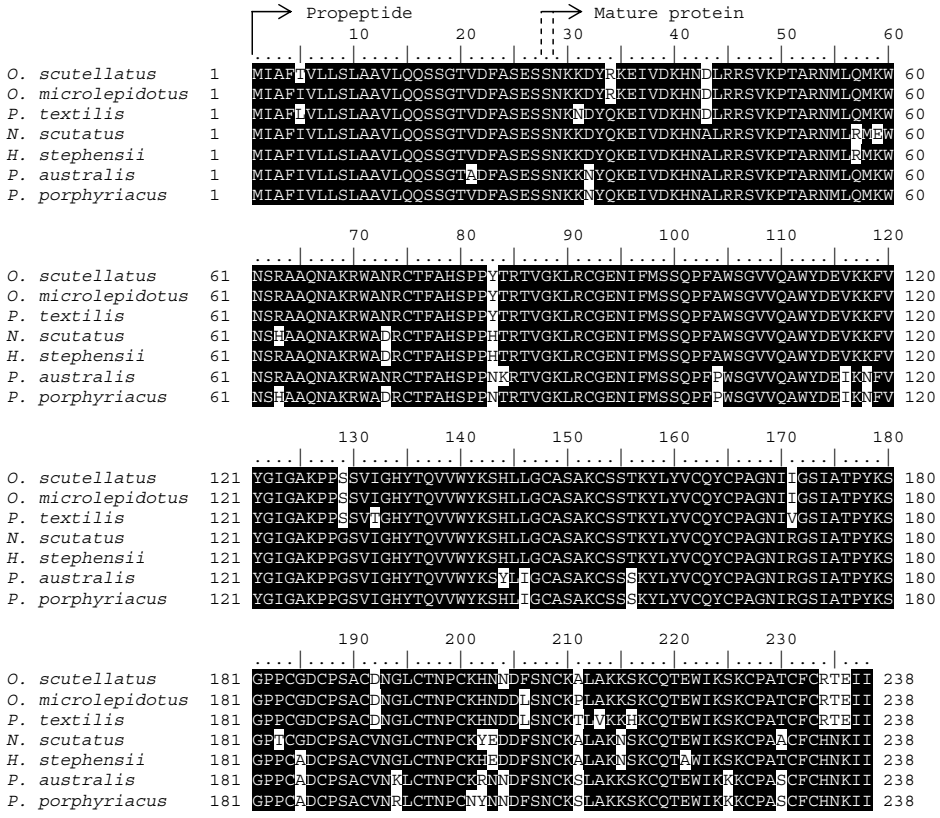

Figure 2. Alignment of the deduced amino acid sequences of pseudechetoxin-like proteins determined from multiple cDNA clones from seven Australian elapid species. Putative propeptide and mature protein sequences are also shown with arrows. GenBank accession numbers are as follows: *O. scutellatus* DQ084035, *O. microlepidotus* DQ084036, *P. textilis* DQ084037, *N. scutatus* DQ084038 and *H. stephensii* DQ084039.

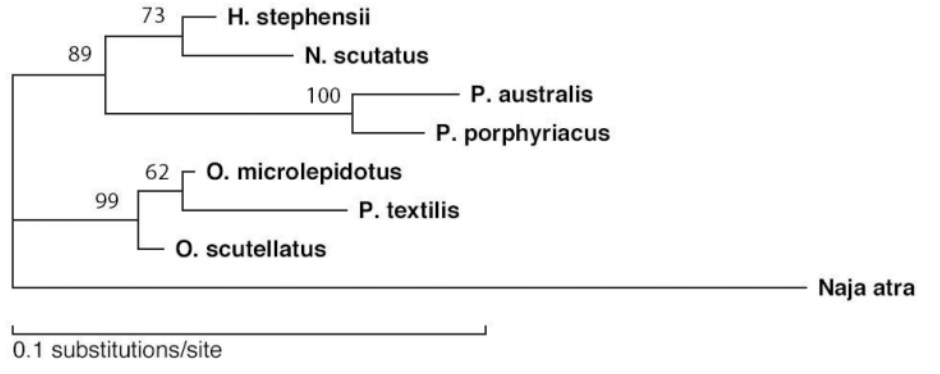

Figure 3. Phylogenetic relationship of the pseudechetoxin-like proteins identified in Australian elapids as determined by the maximum likelihood method using deduced protein sequences. Numbers above branches indicate the percentage of 1000 bootstrap replicates supporting the topology shown.

for standardisation (fig. 5). It is evident from both the array and qPCR results that CAGLP is not expressed in the liver of either *Oxyuranus* species, confirming observations by Junqueira-de-Azevedo et al. [14] that calglandulin is a venom gland-specific protein. Expression of the CAGLP gene within the venom gland varied between Australian elapids, with the greatest amount present in *P. textilis*, whilst *N. scutatus* had the lowest level of transcription. No significant bias in experimental results was observed as a consequence of amplifying the RNA used in hybridisations (fig. 5, last column).

Characterisation of PAL_2 enzymes. The third most abundant clone detected from screens of the *O. scutellatus* microarray were PLA_2 cDNAs. One isoform, corresponding to the full-length mesenger RNA (mRNA) transcript of the β-chain of taipoxin comprising a 438-bp open reading frame, 92-bp 5'-UTR and 230-bp 3'UTR, represented the majority of the PLA_2 clones identified on the microarray. Taipoxin is a potent toxin within the venom of the coastal taipan and is composed of three PLA_2 chains: α, β and γ [21]. Evidence from other Australian elapids, including *P. australis*, *N. scutatus* and the lowland copperhead, *Austrelaps superbus*, indicates that there are multiple PLA_2 variants present within the venom of each of these snakes [22–24]. In order to investigate the presence of other PLA_2 enzymes within the venom of *O. scutellatus*, primers were designed within the 5'- and 3'-UTRs of the β-taipoxin gene to amplify potential isoforms from venom gland cDNA. DNA se-

```
                                10        20        30        40        50        60
                       ....|....|....|....|....|....|....|....|....|....|....|....|
Calglandulin        1  MAATLTPEQITEYKGIFEMFDEEGNGLVKTDDLESLMSLVGINPTKRDLANMAKDVDKDK  60
O. scutellatus      1  MAATLTPEQITEYKGIFEMFDEEGNGLVKTDDLESLMSLIGINPTKRDLANMAKDVDKDK  60
O. microlepidotus   1  MAATLTPEQITEYKGIFEMFDEEGNGLVKTDDLESLMSLIGINPTKRDLANMAKDVDKDK  60
P. textilis         1  MAATLTPEQITEYKGIFEMFDEEGNGLVKTDDLESLMSLIGINPTKRDLANMAKDVDKDK  60
N. scutatus         1  MAATLTPEQITEYKGIFEMFDEEGNGLVKTDDLESLMSLIGINPTKRDLANMAKDVDKDK  60
T. carinatus        1  MAATLTPEQITEYKGIFEMFDEEGNGLVKTDDLESLMSLIGINPTKRDLANMAKDVDKDK  60
H. stephensii       1  MAATLTPEQITEYKGIFEMFDEEGNGLVKTDDLESLMSLIGINPTKRDLANMAKDVDKDK  60
P. australis        1  MAATLTPEQITEYKGIFEMFDEEGNGLVKTDDLESLMSLIGINPTKRDLANMAKDVDKDK  60
P. porphyriacus     1  MAATLTPEQITEYKGIFEMFDEEGNGLVKTDDLESLMSLIGINPTKRDLANMAKDVDKDK  60

                               70        80        90       100       110       120
                       ....|....|....|....|....|....|....|....|....|....|....|....|
Calglandulin       61  KGTFNCDGFLALMGIYHEKSKNQDEELRAAFKVFDKEHKGYIEWDTLKYVLMNAGEPLNE  120
O. scutellatus     61  KGTFNCDGFLVLMGIYHEKSKNQDEELRAAFKVFDKEHKGYIEWDTLKYVLMNAGEPLNE  120
O. microlepidotus  61  KGTFNCDGFLVLMGIYHEKSKNQDEELRAAFKVFDKEHKGYIEWDTLKYVLMNAGEPLNE  120
P. textilis        61  KGTFNCDGFLVLMGIYHEKSKNQDEELRAAFKVFDKEHKGYIEWDTLKYVLMNAGEPLNE  120
N. scutatus        61  KGTFNCDGFLVLMGIYHEKSKNQDEELRAAFKVFDKEHKGYIEWDTLKYVLMNAGEPLNE  120
T. carinatus       61  KGTFNCEGFLVLMGIYHEKSKNQDEELRAAFKVFDKEHKGYIEWDTLKYVLMNAGEPLNE  120
H. stephensii      61  KGTFNCDGFLVLMGIYHEKSKNQDEELRAAFKVFDKEHKGYIEWDTLKYVLMNAGEPLNE  120
P. australis       61  KGTFNCDGFLVLMGIYHEKSKNQDEELRAAFKVFDKEHKGYIEWDTLKYVLMNAGEPLNE  120
P. porphyriacus    61  KGTFNCDGFLVLMGIYHEKSKNQDEELRAAFKVFDKEHKGYIEWDTLKYVLMNAGEPLNE  120

                              130       140       150
                       ....|....|....|....|....|....|....|.
Calglandulin       121 QEAELMMKEADKDGDGTIDYEEFVAMMTGESFKLTQ  156
O. scutellatus     121 HEAELMMKEADKDGDGTIDYEEFVAMMTGESFKLTQ  156 DQ084027
O. microlepidotus  121 HEAELMMKEADKDGDGTIDYEEFVAMMTGESFKLTQ  156 DQ084028
P. textilis        121 HEAELMMKEADKDGDGTIDYEEFVAMMTGESFKLTQ  156 DQ084029
N. scutatus        121 HEAELMMKEADKDGDGTIDYEEFVAMMTGESFKLTQ  156 DQ084030
T. carinatus       121 HEAELMMKEADKDGDGTIDYEEFVAMMTGESFKLTQ  156 DQ084031
H. stephensii      121 HEAELMMKEADKDGDGTIDYEEFVAMMTGESFKLTQ  156 DQ084032
P. australis       121 HEAELMMKEADKDGDGTIDYEEFVAMMTGESFKLTQ  156 DQ084033
P. porphyriacus    121 HEAELMMKEADKDGDGTIDYEEFVAMMTGESFKLTQ  156 DQ084034
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Figure 4. Alignment of the deduced amino acid sequences from multiple cDNA clones of the calglandulin-like protein identified in all Australian elapid species involved in this study, compared with calglandulin from *B. jararaca*. GenBank accession numbers are provided at the end of each sequence.

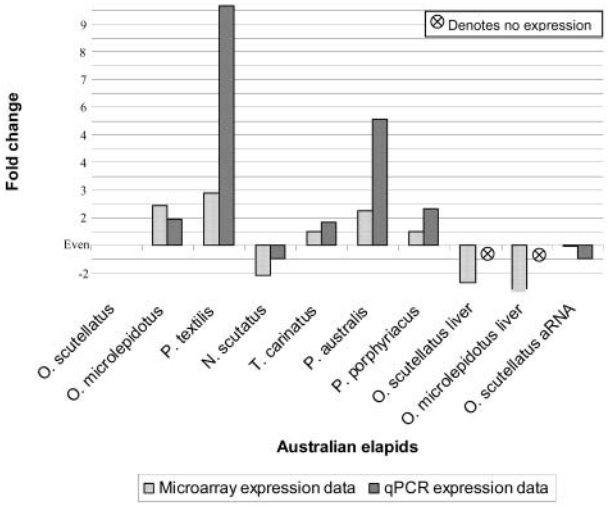

Figure 5. Graph comparing expression of the calglandulin-like genes in all Australian elapids relative to that of the coastal taipan (*O. scutellatus*) as determined by hybridisation of labelled cDNA prepared from aRNA to the *O. scutellatus* microarray chip and by qPCR. Note the absence of expression of this gene within the liver of the *Oxyuranus* genera, and the relative equal expression when comparing total RNA with amplified RNA. Positive values denote a relative increase in expression in that species.

quencing of isolated clones provided evidence for at least eight separate isoforms, including the original β-chain of taipoxin. The results in figure 6 demonstrate an alignment of the deduced amino acid sequence of these variants. It is likely that there are additional PLA_2s present within the venom of *O. scutellatus* not detected by these primer pairs.

Overall there was a significant degree of identity between the PLA_2 precursor proteins from the venom gland of the coastal taipan (46.1%) with complete conservation of cysteine residues involved in putative disulfide bond formation (fig. 6). Two of the clones corresponded to the full-length coding sequence of β-taipoxin with the exception of a single amino acid change within a highly conserved 27-residue signal peptide. Two additional clones (designated OS-1 and OS-3) equated to the mature protein sequence of the previously characterised PLA_2 OS-1 described by Lambeau et al. [25]. The identified clones OS-6 and OS-7 demonstrated significant similarity to the previously described α-chain of taipoxin and the mature protein sequence of OS-2, respectively, differing by only a few amino acids. The N-terminal region (amino acids 1–33 of the mature protein sequence without signal peptide) of the final two clones, OS-4 and OS-5, were identical to each other and 88% identical to the β-chain of taipoxin in this region. However for the remaining 86–94 amino acids, there was little sequence similarity between these two clones. Interestingly OS-4 differed by only a single amino acid compared with OS-1 in this region, while OS-5 was identical to OS-2 over the C-terminal portion of the protein. The multitude of PLA_2 genes described here for *O. scutellatus*, and demonstrated previously for other snake species, are likely to have arisen by gene duplication and recombination events [26]. It is also likely that such a recombination event was responsible for the generation of the PLA_2 transcripts OS-4 and OS-5.

When a similar approach was employed to identify related PLA_2 cDNAs in other Australian elapids, multiple

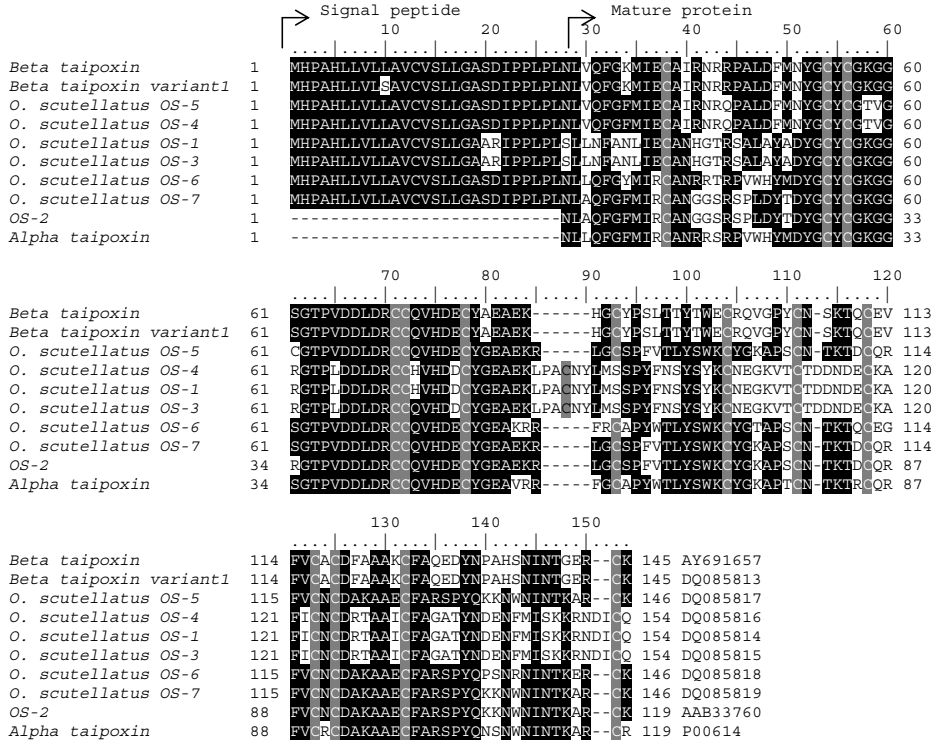

Figure 6. Alignment of the deduced amino acid sequences from all PLA_2 clones identified from the venom gland of the coastal taipan (*Oxyuranus scutellatus*) compared with the previously published sequences OS-2 and alpha taipoxin. Note that secretory phospholipases OS-6 and OS-7 demonstrate significant identity to alpha taipoxin and OS-2, respectively. Conserved cysteine residues predicted to be involved in disulfide bond formation are shaded gray. GenBank accession numbers are included at the end of the sequence.

transcripts were identified. The results in figure 7 demonstrate an alignment of these phospholipase proteins deduced from cDNA sequences. All clones identified, with the exception of PT-PLA1 (AF082983) from *P. textilis* and NS-1 (X14043) from *N. scutatus*, represent the first description of these sequences at the cDNA level. The predicted amino acid sequences for most of these clones have not been described previously at the protein level. The clones listed for each snake in figure 7 are a representative group of distinct isoforms identified from each snake; in many cases there were several other clones documented that differed by only a small number of amino acids from the variants shown. These are too numerous to list but are identifiable by their accession numbers via the Genbank database (DQ085813–DQ085854).

While there is considerable variation between the 25 sequences listed in figure 7, it is evident that the 27-amino acid signal peptide is highly conserved, as well as a block of sequence between residues 52 and 84 of the precursor protein. Interestingly, this conserved sequence contains both the Ca^{2+} binding loop (residues 55–59 of the precursor protein, which has a conserved motif W/YCGxG in PLA_2s) and phospholipase active site (residues 71–78 of the precursor protein, which was previously shown to contain the conserved motif CCxxHDxC in other PLA_2s) [8]. Not surprisingly, many of the physiological sites associated with various activities observed between different PLA_2s (for example neurotoxicity, myotoxicity and anticoagulant effects) have been mapped to the less conserved C-terminal region of the protein [9]. Fourteen cysteine residues previously shown to be involved in disulfide linkages within PLA_2s are also completely conserved amongst all species [3].

Characterisation of LAAO cDNAs. The LAAO family convert L-amino acids into keto acids, ammonia and hydrogen peroxide, the latter product proving to be a potent bactericide. It is speculated that LAAOs present within the venom assist in preventing putrification of the prey during the long period of digestion [27]. With the knowledge that LAAOs exist at least in the mulga, *P. australis*, and that there appears to be significant sequence identity between Australian elapids and other snake genera, primers were designed from a number of viper LAAOs to identify homologous proteins [28]. A full-length 1554-bp LAAO coding sequence was amplified from the venom gland cDNA of *O. scutellatus*, *N. scutatus* and *P. australis*. Alignment of the deduced amino acid sequence from multiple cDNA clones reveals 89.6% identity across the three precursor proteins (fig. 8). There was a high degree of identity within the signal peptide, a common feature of all Australian elapid toxins identified thus far, even between different species.

Discussion

In this study we employed a cDNA microarray chip established from the venom gland of the coastal taipan (*O. scutellatus*) for identification and cross-species compari-

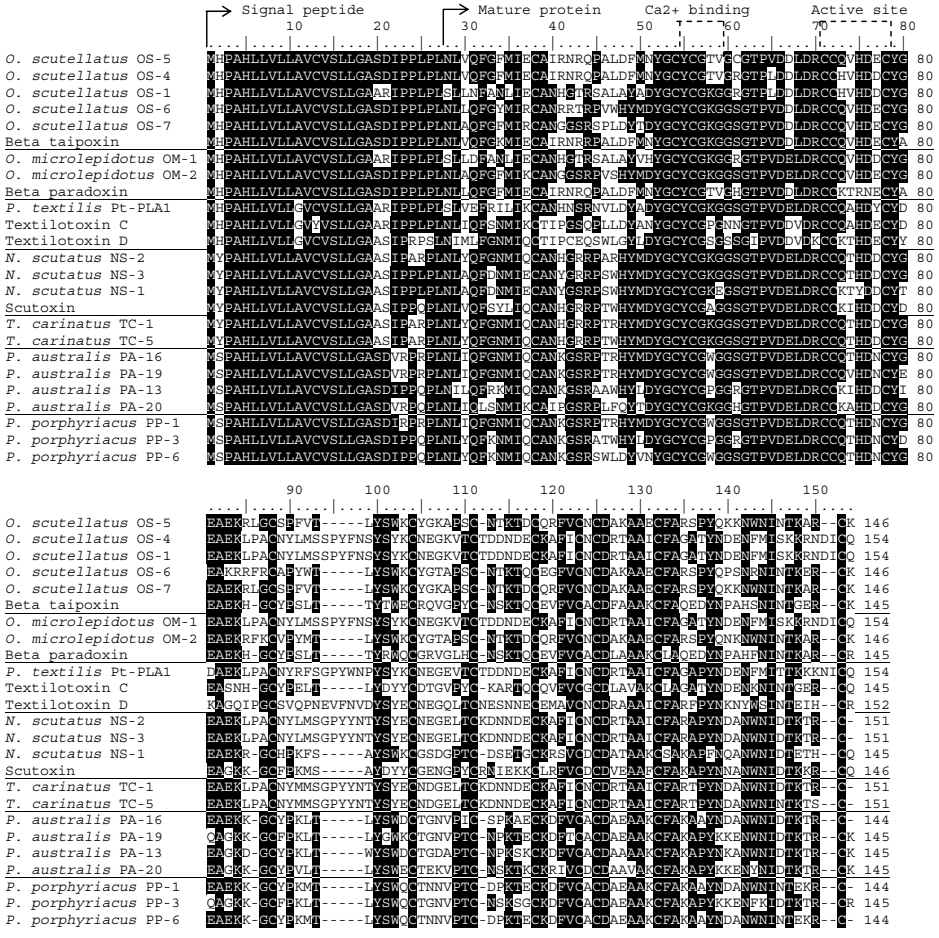

Figure 7. Alignment of the protein sequences of phospholipase PLA_2 enzymes deduced from cDNA clones identified from the venom glands of seven Australian elapids (proteins from individual species are separated by lines). All sequences with the exception of *P. textilis* Pt-PLA1 and *N. scutatus* NS-1 represent novel clones at the level of cDNA. The signal peptide sequence, Ca^{2+} binding site and active site are shown. Where proteins have previously been published, prior nomenclature is applied; otherwise novel PLA_2s are identified via a numbering system. All unique sequences and their variants (not shown) identified in this study are accessible via the Genbank database (DQ085813–DQ085854).

sons of venom gland-specific transcripts. By hybridising labelled cDNA from *O. scutellatus* a suite of genes that demonstrated increased expression in the venom gland compared with the liver were identified. Of the 58 unique transcripts identified from screens of the microarray chip, 34 demonstrated increased expression in the venom gland compared with the liver. These clones included all genes coding for proteins whose putative functions (as determined by BLAST homology searches) corresponded to venom toxins, as well as those involved in the processing and secretion of toxins into the venom. It was possible to further verify these results by screening the microarray chip with cDNA isolated from the venom gland of the closely related taipan, *O. microlepidotus*, in association with qPCR analysis, which confirmed their venom gland-specific nature. Toxin-related genes represented only 14% of all different transcripts identified in this study, compared with 30% previously reported by Junqueira-de-Azevedo and Ho [29], who performed a random survey of gene expression and diversity in *B. insularis*.

Of the toxin genes identified, three families of secreted proteins predominated. The great majority of these, 191 of 268 clones (71%), were represented by an *O. scutellatus* pseudechetoxin-like gene. Pseudechetoxin, and its related homolog pseudecin, are peptidic toxins that target CNG ion channels isolated from the venom of the mulga (*P. australis*) and red-bellied black snake (*P. porphyriacus*), respectively [12]. Full-length cDNAs coding for both of these toxins have recently been reported and have been shown to bind the pore turret of CNG ion cannels and inhibit the flow of current [13, 19]. CNG ion channels, which have been identified in a number of body tissues, including the brain, heart and kidneys, play a central role in signal transduction in retinal photoreceptors and olfactory neurons. They modulate the membrane potential of the cell and intracellular calcium levels in response to stimulus-induced changes in cyclic nucleotide concentration, but their role in the envenomation process is not yet fully defined [30, 31]. Overall the pseudechetoxin-like proteins identified in this study exhibit a significant degree of identity (83%) across the entire precursor molecule. However, even though only 7 amino acid differences were observed between the *P. australis* and *P. porphyriacus* mature protein sequences, a 30-fold difference in affinity for CNG ion channels was previously observed between the two [19]. Comparison of the *P. australis* sequences with that of the *O. scutel-*

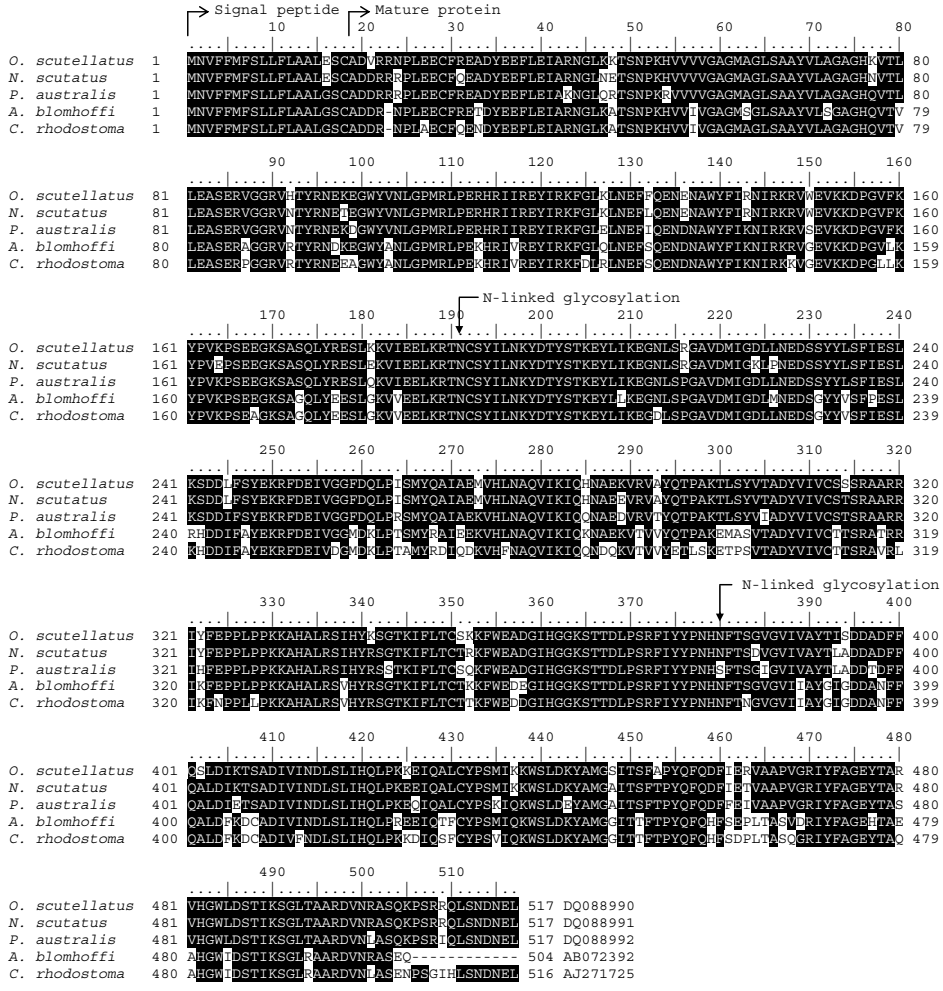

Figure 8. Protein alignment of the deduced amino acid sequences from multiple cDNA clones of LAAOs identified from the venom gland of Australian elapids compared with the previously characterized proteins from the Mamushi snake (*Agkistrodon blomhoffi*) and Malayan pit viper (*Calloselasma rhodostoma*). N-linked glycoslyation sites previously identified in *C. rhodostoma* are also shown, and GenBank accession numbers are provided at the end of each sequence.

latus sequence reveals a 23-amino acid difference in the mature toxin sequence, suggesting that there may be large differences in capacity to bind to ion channels for the pseudechetoxin-like proteins from different elapids. Hence, the data described here represent a valuable tool for further probing the structure and function of CNG ion channels.

The phylogenetic relationship described for pseudechetoxin-like proteins (which is based on deduced amino acid sequence for a specific toxin gene) is in good agreement with the evolutionary relationship of elapid snakes which was previously based on a range of other parameters that included internal and external morphology, immunological distances, ecological and biochemical means [32]. Advances in DNA and protein sequencing has allowed for molecular approaches to establish phylogenetic relationships between snakes, that is, the inference of species trees from gene trees [33–36]. Molecular sequence data will prove to be a powerful tool since not only can it indicate the order of divergence between species, but it also provides a measure of the timing of that divergence [37]. The phylogenetic relationship described here for pseudechetoxin resembles that of the factor X-like protease family recently identified in Australian elapids [38]. Yamazaki et al. [19] reported the processing of 211- and 210-amino acid mature proteins for pseudechetoxin and pseudecin, respectively. This difference in size was due to the presence of an extra serine residue at the N-terminus of pseudechetoxin as determined by Edman degradation. This is of interest because the recognition sequence between the signal peptide and mature protein is 100% conserved within these two species. This suggests that in the case of pseudechetoxin, a proteolytic cleavage occurs between the two serine residues (SES↓SNK), while for pseudecin the cleavage site would be SESS↓NK. The data in figure 2 demonstrate that the signal peptide is highly conserved in all seven Australian elapids and that the region surrounding the proposed cleavage site (SESSNK) is identical. This suggests that processing of the precursor protein occurs by cleavage at a single site and that the single amino acid difference reported in *P. porphyriacus* might be explained by cleavage of the N-terminal serine from pseudecin during the purification procedure.

Identification of venom gland-specific transcripts from the *O. scutellatus* microarray was not limited only to toxins, indeed, the most abundant clone identified (approximately 35% of all clones screened) corresponded to a calglandulin-like protein. Calglandulin is an EF-handed protein with conserved Ca^{2+} motifs, first identified from the venom gland of the island jararaca, *B. insularis*, and has been implicated in the process of exporting toxins out of the cell and into the venom [14]. Members of the EF-handed protein family contain conserved Ca^{2+} binding motifs involved in intracellular communication, vesicular transport and membrane fusion, the most widely studied of these being calmodulin [39, 40]. Calglandulin displays significant structural homology to calmodulin, both consisting of four EF-hands (the helix-loop-helix motif that coordinates Ca^{2+} binding) and hence has been implicated in the secretion of toxins from the venom gland. This is supported by the localisation of the protein specifically to the venom gland. Given the significant identity observed between calglandulin and the homolog identified in Australian elapids in this study, in conjunction with their absence in the liver, it is suggested that these proteins play a similar role within the venom. Such a high degree of conservation between two different families of snakes (vipers and elapids) may be indicative of the highly specific function of this protein. Interestingly, a novel EF-hand protein with 80% similarity to calglandulin has been isolated and cloned from the human skeletal muscle, CAGLP [41]. The homologs identified in Australian elapids along with calglandulin and human CAGLP represent a distinct group within the EF-handed Ca^{2+} binding protein family.

PLA_2 enzymes are a ubiquitous family of proteins present within many venomous animals, including Australian snakes. Of the three PLA_2 isoforms identified from screens of the microarray chip, the most abundant corresponded to a full-length mRNA transcript of the β-chain of taipoxin. Taipoxin, composed of three chains (α, β and γ), is a potently active toxin within the venom of the coastal taipan [21]. The neurotoxic and myotoxic effects of taipoxin can be attributed to the α-chain, with the presence of the γ-chain enhancing these effects [42]. Interestingly, the β-chain is neither toxic nor demonstrates phospholipase activity on its own, and somewhat surprisingly has been shown to have mitogenic activity [43, 44]. A number of other PLA_2 enzymes have been identified at the protein level from the venom of *O. scutellatus*, including secretory phospholipases OS-1 and OS-2, as well as a PLA_2 chain present within the multimeric protein taicatoxin. However, no full-length cDNAs have been published from this snake [25, 45, 46]. This study represents the first description of the cloning of a PLA_2 from the venom gland of *O. scutellatus*. PLA_2s demonstrate extensive variation in physiological effects (which may be independent of phospholipase catalytic activity) despite a high degree of conservation in their primary structure. It is likely that the large number of PLA_2s described here from seven Australian elapids may also possess a variety of activities; however, based on primary sequence alone, it is difficult to assign specific activities to any one isoform. For example, despite displaying approximately 65% overall identity at the mature protein level, the PLA_2s scutoxin from *N. scutatus* and PA-9C from *P. australis* display potent presynaptic neurotoxic effects and myotoxic activity, respectively, with variable phospholipase enzymatic activity [24, 47]. Similarly, while the α-chain of taipoxin is neurotoxic, the β-chain displays no toxicity or phospholipase activity (and indeed demonstrates mitogenic properties) despite being in complex with the α-chain in native venom and displaying 64% identity to each other at the mature protein level [43, 44, 48]. The PLA_2 variants identified in this study therefore represent an excellent platform for examining how these sequence variations effect activity and how they may be applied in a therapeutic setting.

Of the six PLA_2 isoforms identified from the closely related species *O. microlepidotus*, one demonstrated 85% identity to the β-chain of taipoxin (fig. 7) [43]. This clone has been designated β-paradoxin-like because of its likely identity with a β-taipoxin homolog known to be present within the venom of this snake [49]. Even though the protein sequence of β-paradoxin has not been determined, a purified form has demonstrated presynaptic neurotoxic activity similar to that of taipoxin [50]. It is of interest that the other PLA_2 isoforms from *O. microlepidotus*, as a group, are most closely related to those of *O. scutellatus*. Two clones identified from *P. textilis* had deduced amino sequences identical to the previously reported protein sequence for the C and D chains of textilotoxin [51]. Textilotoxin is a noncovalently bound, multimeric protein complex composed of five PLA_2 chains, A, B, C and two D chains, that is a highly potent presynaptic blocker of neuromuscular transmission [52]. Other novel PLA_2 cDNA sequences identified in this study that have previously been characterised at the protein level include scutoxin from *N. scutatus*; however in most instances the other PLA_2 clones represent their first description at either the protein or nucleotide level [53]. Indeed, for *T. carinatus* and *O. microlepidotus*, the above sequences represent the first description of any phospholipase from the venom gland of these snakes.

LAAOs catalyse the oxidative deamination of L-amino acids, and are widely distributed in a number of organisms, including their presence within snake venoms where they are postulated to be toxins [54]. A protein with LAAO activity was previously isolated and partially characterised from the venom of *P. australis* [28]. However, no protein or gene sequence was described for this snake, or from any other Australian elapid; primary research focus on snake venom LAAOs has been their

characterisation from vipers [17, 18]. A full-length product was identified in at least three Australian snakes, *O. scutellatus*, *P. australis* and *N. scutatus*, with a significant degree of identity in deduced protein sequences among the three species. Previous investigations of LAAO from the crude venom of Australian elapids demonstrated *P. australis* to have relatively high levels of activity, *N. scutatus* to have moderate activity and *O. scutellatus* to have very low activity rates [7]. Given the significant degree of identity in the deduced primary structure of the LAAOs identified in this study, it would suggest that the varying rates of activity observed by Tan and Ponnudurai [7] may be attributed to the relative quantity of LAAO in the venom. However, one notable difference between the Australian elapid sequences is the absence of a second N-linked glycosylation site in *P. australis* that was previously observed in *C. rhodostoma*, although it is difficult to draw conclusions about the effect of this variation on activity [18]. The LAAO previously isolated from the venom of *P. australis,* while non-toxic and non-proteolytic, demonstrated potent antibacterial effects, possibly as a result of the catalytic release of hydrogen peroxide. It has been postulated that these antibacterial effects protect the snake from putrification of prey during the digestion process [28]. The native LAAO identified by Stiles et al. [28] had a molecular size of 56 kDa, which corresponds well with the predicted molecular size of 56.7kDa for the LAAO identified in this study. Given the well-described antibacterial effects, as well as recently identified platelet aggregating and apoptotic effects of LAAO, these molecules are of significant pharmacological interest. This study represents the first description of the cloning of a full-length LAAO transcript from the venom gland of an Australian elapid.

The present study of the *O. scutellatus* venom gland transcripts identified a significant number of the components associated with envenomation process from Australian snakes. Many of these sequences represent the first description for these products at the cDNA and/or protein level, and is the most comprehensive study of the venom gland transcriptome for the eight elapids involved in this study. This approach provides a useful means for the characterisation of multiple isoforms of a toxin family not readably identifiable at the protein level (for example the phospholipases), as well as a method for the identification of novel venom gland transcripts. Despite demonstration of the relatively high degree of toxicity of Australian snake venoms, they have remained largely understudied at the molecular level. This is epitomised by the fact that just a handful of nucleotide and protein entries are present within the GenBank database for the inland taipan (*O. microlepidotus*) despite this snake having one of the most toxic venoms known [2]. The data described here address this deficiency, acting as a significant resource for identifying and characterising venom toxin genes, as well as providing important phylogenetic information for clarifying the evolutionary relationships of all snake toxins.

Acknowledgments. We are grateful to QRxPharma and the Australian Research Council for support, and wish to thank J. Sambono for the provision of venom glands. We also express our gratitude to Dr. D. J. Miller for assistance with phylogenetic analyses.

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