Granular gland transcriptomes in stimulated amphibian skin secretions

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Amphibian defensive skin secretions are complex, species-specific cocktails of biologically active molecules, including many uncharacterized peptides. The study of such secretions for novel peptide discovery is time-limited, as amphibians are in rapid global decline. While secretion proteome analysis is non-lethal, transcriptome analysis has until now required killing of specimens prior to skin dissection for cDNA library construction. Here we present the discovery that polyadenylated mRNAs encoding dermal granular gland peptides are present in defensive skin secretions, stabilized by endogenous nucleic acid-binding amphipathic peptides. Thus parallel secretory proteome and transcriptome analyses can be performed without killing the specimen in this model amphibian system – a finding that has important implications in conservation of biodiversity within this threatened vertebrate taxon and whose mechanistics may have broader implications in biomolecular science.

Key words: biodiversity, cDNA cloning, conservation, functional genomics, precursor proteins.

INTRODUCTION

In recent years, drastic reductions in the populations of frogs and toads have been noted on a global scale [1]. The reasons for these population declines are unclear at present, but many factors, including climate change, habitat destruction, parasitic and microbial pathogenic disease, have been implicated [2–5]. In some instances population declines have been gradual, but in others, declines and apparent extinction events have been of dramatic short term, for example the Costa Rican golden toad (*Bufo periglenes*) and the Australian gastric brooding frogs (*Rheobatrachus vitellinus* and *R. silus*) [6,7].

In response to stress or predator attack, amphibians secrete a complex chemical cocktail from highly specialized skin structures, namely the venom or granular glands. These secretions contain a plethora of biologically active components, including alkaloids, biogenic amines, peptides and proteins [8]. In many anuran (tailless)-amphibian taxa, peptides are the predominant molecular class and are the products of endogenous gene expression within the granular gland cells of each individual species. Despite efforts directed towards the structural and functional analysis of frog skin peptides for several decades, the vast majority of species remain unstudied. The extraordinary diversity of frog skin peptides and the fact that many are structural analogues of endogenous vertebrate neuropeptides [8], renders these an intriguing resource for the understanding of human neurochemical complexity and for potential novel drug lead discovery – the latter being a factor of particular current relevance as interest in peptide therapeutics undergoes a renaissance in the pharmaceutical industry [9].

In the past, acquisition of frog skin molecular libraries necessitated the killing of the frog and extraction of the dissected skin in organic solvents. This procedure was biodestructive, often requiring several hundreds of specimens, and choice of extraction medium was highly selective in terms of component solubility [10,11]. The introduction of the non-invasive mild transdermal electrical stimulation technique [12] revolutionized sample acquisition, by removing the need for specimen death and by producing a more defined and molecularly complete secretion for granular-gland secretory proteomic analyses. However, molecular-biological studies related to cloning of cDNAs encoding granular-gland peptides, still required killing of specimens and library construction from excised skin [13,14]. In common with the original peptide-extraction technology, this molecular approach suffers from the two inherent disadvantages of specimen death and non-selective cDNA library construction, as the granular glands in most species represent a minor cellular component of total skin.

Consistent with evolving ethics in contemporary biological science, we describe a technique that facilitates concomitant transcriptomic and proteomic study of granular-gland peptides and proteins in a manner that is non-lethal and non-invasive. In addition, as the technique is readily performed under field conditions, ecological considerations and biodiversity conservation have been completely addressed, as specimens can be sampled and released at their site of capture.

MATERIALS AND METHODS

Experimental species biodata

Species of anuran amphibians employed were representative of taxa previously studied by conventional methods and known to produce significant defensive skin secretions. These included the African clawed frog (*Xenopus laevis*) (family Pipidae), the North American leopard frog (*Rana pipiens*) (family Ranidae), the Australasian White's tree frog (*Litoria caerulea*) (family Hylidae, subfamily Pelodryadinae), the Chinese large-webbed bell toad (*Bombina maxima*) (family Bombinatoridae), the African running frog (*Kassina maculata*) (family Hyperoliidae) and two species of Central American leaf frog (family Hylidae, subfamily Phyllo-

Abbreviations used: Bm8, *Bombina maxima* (Chinese large-webbed bell toad) 8 kDa polypeptide; Bv8, *Bombina variegata* (yellow-bellied toad) 8 kDa polypeptide; DC, direct current; MAP, mitogen-activated protein; msec, milliseconds; NMU, neuromedin U; poly(A)$^+$, polyadenylated; RACE, rapid amplification of cDNA ends; RT-, reverse transcription.

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medusinae), the red-eyed leaf frog (*Agalychnis callidryas*) and the Mexican leaf frog (*Pachymedusa dacnicolor*). The latter two species represent a taxon (Phyllomedusinae) renowned for it's copious and chemically complex defensive skin secretions [15]. Frogs were obtained from a variety of commercial sources and housed within a purpose-designed tropical frog facility for at least 2 years prior to performing the described experiments. All were housed in single-species terraria under at 12 h/12 h light/dark cycle. Diurnal temperature variation ranged from 21 to 27 °C, humidity between 60 and 80 % and specimens were fed multivitamin-enriched crickets three times per week.

In vitro cDNA library construction from skin secretions

Defensive skin secretion was obtained from captive adult specimens of each species by mild transdermal electrical stimulation [2–7 V DC (direct current) (dependent on frog size), 4 ms pulsewidth, 50 Hz] applied to areas of concentrated venom glands via platinum electrodes for three periods of 10 s duration [12]. Samples of extruded defensive secretion (10–20 μl) were collected using RNase-free filtered pipette tips and placed in Cell Lysis/mRNA Stabilization Solution (Dynal Biotech UK, Bromborough, Wirral, Merseyside, U.K.). Polyadenylated [poly(A)$^+$] mRNA was isolated using magnetized oligo(dT) beads as described by the manufacturer (Dynal Biotech UK). mRNA was eluted in 20 μl of RNase-free water and first-strand cDNA synthesis for 3′- and 5′-cDNA rapid-amplification-of-cDNA-ends (RACE) reactions were performed using a ClonTech SMART RACE kit. The remainder of the skin secretion was washed from the dorsal skin surface of frogs using deionized water and immediately snap-frozen in liquid nitrogen. Following freeze-drying, the skin secretion was weighed and stored in glass vials at −20 °C. After stimulation, frogs exhibited no observable ill effects, and captive specimens were returned to their respective terraria. In other studies, field-caught specimens were released at the site of capture. The whole procedure described above takes less than 1 min to perform. Using a slight modification, cDNA libraries could be constructed as described above from freeze-dried skin secretion that had been stored for 2 years at −20 °C. A portion (2–10 mg) of freeze-dried skin secretion was dissolved in 1 ml of cell lysis/mRNA stabilization solution prior to poly(A)$^+$ mRNA capture on the magnetized oligo(dT) beads.

Peptide precursor cDNAs targeted for cloning

Magainin, a 23-amino-acid residue antimicrobial peptide from the African clawed frog (*X. laevis*), is generally regarded as the archetypal frog skin peptide [16]. This peptide was chosen as a 'control' for the technique insofar as the nucleotide sequence of the precursor has been long established. A 5 mg portion of freeze-dried skin secretion was employed to construct a venom-gland transcriptome library. Specific sense (5′-ATGTTCAAAGGATTATTTATCTGT-3′) and antisense (5′-TGTTGCTGAAACTATTCAACCCATAT-3′) primers were designed that flanked the open reading frame of the magainin precursor, and the transcript was amplified by conventional PCR.

To effect cloning of novel peptide precursors from the remaining species, degenerate primers were designed to appropriate regions of primary structures either generated within our research programme or derived from the recent literature. The peptides chosen for the present study were the antimicrobial peptides ranatuerin 2P, ranatuerin 2Pa and brevinin 1Pb from *R. pipiens* [17], the skin neuropeptide analogue neuromedin-U-23 (NMU-23) from *L. caerulea* [18] and the black-mamba (*Dendroaspis polylepis polylepis*) protein A homologue Bm8, [*B. maxima* (Chinese large-webbed bell toad) 8 kDa polypeptide]. Primers designed for each of these peptides were:

Ranatuerins/brevinin

Sense 5′-ATGTCACCTGAAGAAATCCCTC-3′

Antisense 5′-GACATCTGGTGTGCAATTAGCT-3′

NMU

Sense 5′-GAYGARGARGTICARGTICC-3′

Antisense 5′-AAACCCGCTGATCTCCTTCCATT-3′

Bm8

Sense 5′-GCNGTNATHACNGGNGTNTGYGAY-3′

Antisense 5′-GGCTCATGTTTTATTGTCCT-3′.

Gel-retardation experiments

To test if nucleic acid interaction with skin secretion components was a possible explanation for intact mRNA recovery, we designed two *in vitro* experiments involving assessment of gel retardation of DNA by incubation with either synthetic amphipathic peptides (the major skin secretion components) or crude skin secretion. In the first experiment, three different synthetic replicates of amphipathic peptides (maximins) derived from *B. maxima* skin secretion were incubated with a 200 bp PCR product from a frog skin peptide 3′-RACE reaction. Peptides were added in concentrations mimicking those found in skin secretion. After 20 min of incubation, samples were subjected to gel electrophoresis. In a second experiment, designed in a similar format, genomic DNA, isolated from leg muscle of a frozen *L. caerulea* cadaver, was incubated for 20 min with ten-fold decreasing dilutions of freeze-dried skin secretion from the same species. Samples were likewise subsequently subjected to gel electrophoresis.

RESULTS

All of the nucleotide sequences of the novel cloned precursor protein cDNAs obtained have been submitted to the EMBL nucleotide database and the accession numbers are given in Table 1. Conserved 'housekeeping' protein precursor partial

Table 1 EMBL accession numbers of novel amphibian venom protein/peptide precursors cloned from stimulated skin secretions

	EMBL	
Species	Venom protein/peptide	accession no.
R. pipiens	Ranatuerin 2P	AJ427747
	Ranatuerin 2Pa	AJ427748
	Brevinin 1Pb	AJ427746
L. caerulea	NMU-23A	AJ457825
	NMU-23B	AJ457826
B. maxima	Bm8-a	AJ440230
	Bm8-b	AJ440231
	Bm8-c	AJ440232
	Bm8-d	AJ440233
	Bm8-e	AJ440234
	Bm8-f	AJ440235
K. maculata	α-Tubulin	AJ488156
A. callidryas	67 kDa laminin receptor/ribosomal protein	AJ488157
P. dacnicolor	40 S ribosomal protein S16	AJ488155

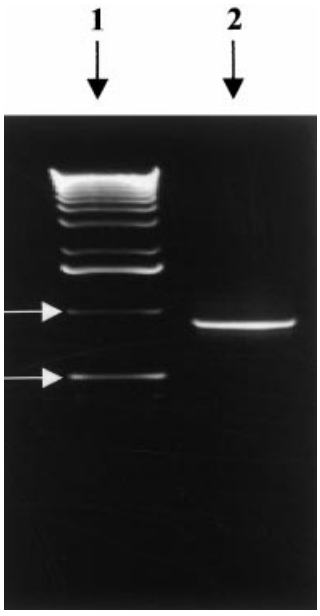

Figure 1 Reverse-transcription (RT-) PCR of magainin precursor cDNA

Gel electropherogram of RT-PCR product (923 bp) (right lane) from magainin precursor cDNA produced by capture and transcription of corresponding poly(A)$^+$ mRNA contained in *X. laevis* skin secretion. The left lane shows the calibration ladder with the upper arrow indicating band of 1018 bp, the lower arrow indicating band of 509 bp.

sequences were identified during transcriptome sequencing of the skin secretion libraries of *K. maculata*, *A. callidryas* and *P. dacnicolor* and have been included to illustrate the applications of the technique to the wider bioscience community, in particular to molecular systematists.

African clawed frog (*X. laevis*)

Direct sequencing of the prepromagainin-directed PCR product (923 bp) (Figure 1) confirmed the presence of authentic magainin precursor mRNA template in stimulated *X. laevis* skin secretion.

Leopard frog (*R. pipiens*)

The primer employed to facilitate cloning of the *R. pipiens* antimicrobials was designed to the signal peptide consensus sequence from European *Rana* frog antimicrobials. This successfully identified several antimicrobial peptide clones, including the target sequences (Figure 2). The organization of the open-reading frames of these novel precursors was similar to other *Rana* frog antimicrobials, consisting as it does of a hydrophobic signal peptide, an intervening region rich in acidic amino acid residues and a -Lys-Arg- propeptide convertase processing site followed by a single copy of mature peptide at the C-terminus. Antimicrobial peptide precursor mRNAs are thus well-represented in skin secretion, reflecting the relatively high abundance of their mature peptides.

White's tree frog (*L. caerulea*)

The degenerate primer pool designed for the N terminal region of *L. caerulea* skin NMU generated a single 3′-RACE reaction product. Following cloning and sequencing, which confirmed the authenticity of this product, a single 5′-RACE primer was manufactured to exact sequence in the 3′-non-translated region. 5′-RACE reactions produced two distinct cDNA products that were separately cloned and sequenced. While the larger product was highly homologous with both human and rat NMU precursors, the smaller was a splice variant lacking the coding sequence for the highly conserved amphipathic peptide immediately proximal to the NMU-encoding sequence (Figure 3). Thus the mRNA of a novel splice variant of the NMU precursor is present in the skin-secretion-derived cDNA library and interestingly, such variants have not previously been reported from other vertebrates.

Large-webbed bell toad (*B. maxima*)

Bm8 represents the *B. maxima* homologue of Bv8 (78 % sequence identity), originally isolated from *B. variegata* skin secretion [19]. Bv8 was found to exhibit a high degree of structural similarity to protein A from the venom of the black mamba (*D. p. polylepis*). Although black-mamba protein A was of unknown function, bioactivity studies with Bv8 established that both polypeptides induced hyperalgaesia (exaggerrated pain sensitivity) in mouse models of pain induction. More recent research has identified the structure and neural distribution of the mammalian counterpart of these polypeptides and has established that they are neurotrophic, act via a specific mitogen-activated protein (MAP) kinase activating receptor and are encoded near synteny breakpoints on mouse chromosome 6 and human 3p21 [20,21]. The primary structure of Bm8 and the nucleotide sequence of its precursor were found to be highly homologous with those of Bv8. However, six different cDNAs were isolated from *B. maxima* defensive skin secretion encoding a family of structural isomers (Bm8a–Bm8f) (Figure 4). This heterogeneity does not reflect individual variation, as all were present in material from a single individual. Peptides having computed molecular masses corresponding to individual mature peptides were identified by

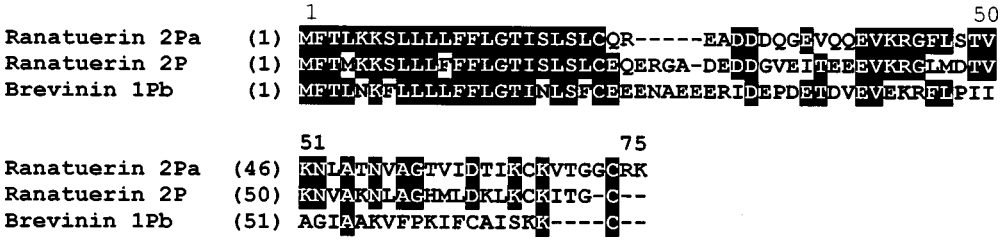

Figure 2 Antimicrobial peptide precursors from *R. pipiens*

Alignment of translated open-reading-frame amino acid sequences of *R. pipiens* antimicrobial peptides ranatuerin 2Pa, ranatuerin 2P and brevinin 1Pb. Note the highest degree of conservation of the putative signal sequence (residues 1–22). Identical residues are boxed in black.

```
        1                                                          50
NMU-B   MQKGSEDTTQ NRCHQHSIGG HSTCGLLLLI ILVSWTSICE GAPFSSPVLG
NMU-A   MQKGSEDTTQ NRCHQHSIGG HSTCGLLLLI ILVSWTSICE GAPFSSPVLG

        51                                                         100
NMU-B   AEDELPLWNG IDDACSAVLP DPQLAVSSTL RELCFMVMRM QQKSQGEEKD
NMU-A   AEDELPLWNG IDDACSAVLP DPQLAVSSTL RELCFMVMRM QQKSQGEEKD

        101                                                        150
NMU-B   DFKR..... .......... .......... .......... ......EEVQ
NMU-A   DFKRFLFHYS KSHDSGNSDI TSSVLHPLLQ LLPQLHDRRM KRLTSDEEVQ

        151                     175
NMU-B   VPGGVISNGY FLFRPRNGRR SAGFR
NMU-A   VPGGVISNGY FLFRPRNGRR SAGFR
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Figure 3 NMU precursors from *L. caerulea*

Alignment of translated open-reading-frame amino acid sequences of *L. caerulea* skin NMU transcripts. The NMU-B transcript clearly displays a deletion of residues 101–146 of the open reading frame, indicating the presence of one or more exons in the corresponding region of the frog NMU gene.

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              1                                                    50
Bm8a    (1)   MKCFAQIVVLLLVIAFSHGAVITGVCDRDAQCGSGTCCAASAFSRNIRFC
Bm8b    (1)   MKCFAQIVVLLLVIAFSHGAVITGVRDRDAQCGSGTCCAASAFSRNIRFC
Bm8c    (1)   MKCFAQIVVLLLVIAFSHGAVITGVCDRDAQCGSGTCCAASAFSRNVRFC
Bm8d    (1)   MKCFAQIVVLLLVIAFSHGAVITGVCDRDAQCGSGTCCAASAFSRNIRFC
Bm8e    (1)   MKCFAQIVVLLLVIAFSHGAVITGVCDRDAQCGSGTCCAASAFSRNIRFC
Bm8f    (1)   MKCFAQIVVLLLVIAFSHGAVITGVCDRDAQCGSGTCCAASAFSRNIRFC

              51                                                96
Bm8a    (51)  VPLGNNGEECHPASHKVPYNGKRLSSLCPCNTGLTCSKSGEKFQCS
Bm8b    (51)  VPLGNNGEECHPASHKVPYNGKRLSSLCPCNTGLTCSKSGEKYQCS
Bm8c    (51)  VPLGNNGEECHPASHKVPYNGKRLSSLCPCNTGLTCSKSGEKFQCS
Bm8d    (51)  VPLGNNGEECHPASHKVPYNGKRLSSLCPCNTGLTCSKSGEKSQCS
Bm8e    (51)  VPLGNNGEECHPASHKVPYNGKRLSSLCPCNTGLTCPKSGEKFQCS
Bm8f    (51)  VPLGNNGEECHPASHKVPSDGKRLSSLCPCNTGLTCSKSGEKYQCS
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Figure 4 Bm8 precursors from *B. maxima*

Multiple alignment of the open reading frames of isomers Bm8a–Bm8f. Identical residues are boxed in black. Putative signal peptide (residues 1–19) and mature peptides (residues 20–96) account for the entire precursor. Sites of microheterogeneity are restricted to residues 6, 27, 49–50, 67 and 73 of the mature peptide.

either liquid chromatography/MS or conventional Edman degradation. The precursors for each peptide were highly condensed, consisting of a putative signal peptide and a single copy of Bm8. The sequencing electropherograms from each clone were found to be unequivocal in base identification. The granular-gland library of this species is thus sufficiently robust and representative of transcripts to permit identification of multiple microheterogeneities in nucleotide sequences present in highly homologous peptide-encoding cDNA populations.

Gel-retardation experiments

All three synthetic amphipathic peptides (maximins) completely inhibited the 200-base PCR product mobility under the standard gel-electrophoresis conditions employed (Figure 5). This was indicative of a molecular interaction either resulting in an increase in molecular mass or in charge neutralization. The latter is probably more likely, as the number of amphipathic peptide molecules which could bind to this size of nucleic acid fragment theoretically could not increase molecular mass sufficiently to explain lack of entry into the gel. The crude defensive skin secretion likewise inhibited the mobility of genomic DNA and in a dose-dependent fashion (Figure 6). This effect is most likely due to amphipathic peptide interactions, as this secretion is a rich source of such (caerins) and degradation would have been certain to produce lower-molecular-mass fragments, which were not observed.

DISCUSSION

The nucleotide sequences of full-length cloned peptide precursor cDNAs presented here have unequivocally established the presence of representative granular-gland transcripts in stimulated amphibian defensive skin secretions. Their presence in both fresh and freeze-dried samples from representative members of the majority of amphibian taxa known to produce bioactive peptide-rich secretions, likewise attributes a high degree of scientific

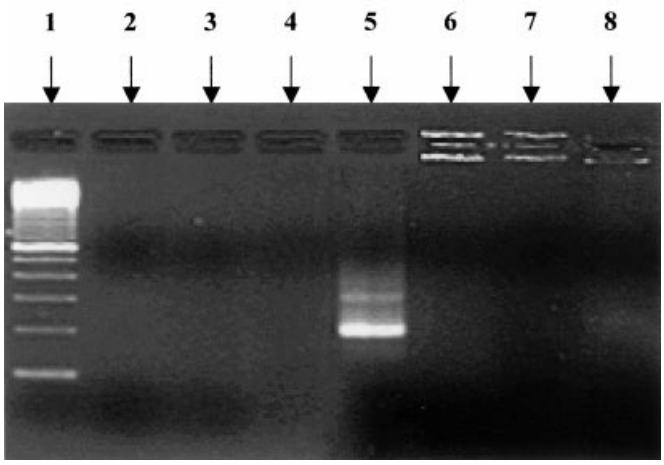

Figure 5 Gel-retardation experiment with synthetic amphipathic peptides

Gel electropherogram illustrating retardation of a 200 bp RT-PCR product following incubation with synthetic frog skin amphipathic peptides. Lane 1, standard DNA ladder, each band representing 100 bp increments; lanes 2–4, 3 μg each of maximins 1–3; lane 5, 200 bp RT-PCR product; lanes 6–8, 200 bp RT-PCR product containing 3 μg of maximins 1-3 respectively. The polynucleotide has clearly been retarded on the gel by each peptide – in fact it has not migrated from the loading wells.

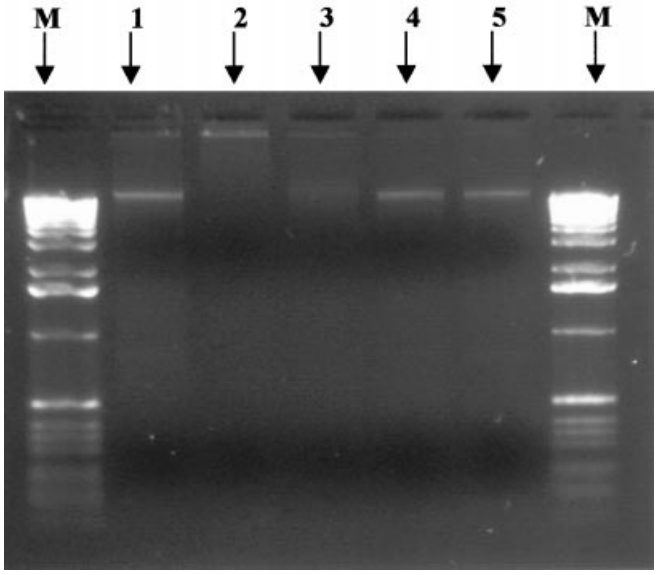

Figure 6 Gel-retardation experiment with freeze-dried skin secretion

Gel electropherogram illustrating retardation of *L. caerulea* somatic DNA following incubation with skin secretion from the same species. Lane 1, DNA; lane 2, DNA + 140 μg of skin secretion; lane 3, DNA + 14 μg of skin secretion; lane 4, DNA + 1.4 μg of secretion; lane 5, DNA + 0.14 μg of secretion. Lanes marked 'M' represent DNA standard ladders.

robustness to the finding and the techniques employed. This discovery has wide implications in the conservation and warehousing of genetic biodiversity for a group of tetrapod vertebrates experiencing considerable survival pressures in the biosphere and additionally addresses current ethical issues in the use of live animals for research (reduction, refinement and replacement).

We searched for a molecular mechanism whereby the structural integrity of labile poly(A)$^+$ mRNA could be afforded protection and began by determining if any known secretion components would be likely to fulfil this role. One of the major component classes in the defensive skin secretions of anuran amphibians is the amphipathic peptides [8]. These display broad spectrum antibacterial activity, but have also been shown to possess anti-fungal, anti-protozoan and anti-(cancer cell) activities [8,16]. Although the killing mechanism of these peptides has been assumed to be via membrane lysis of target organisms [22], some amphipathic defensive peptides, notably tachyplesin from horse-shoe-crab (*Tachypleus tridentatus*) haemolymph, have been shown to have a mode of action involving a nucleic acid interaction [23]. The amphipathic peptides melittin and cecropin, from honeybee (*Apis mellifera*) venom and wax-moth (*Golleria mellonclia*) haemolymph respectively, can effectively inhibit cell-associated production of HIV-1 by suppressing viral gene expression through nucleic acid binding [24]. Perhaps most convincingly, synthetic amphipathic peptides can bind to nucleic acids and effect condensation of DNA for transfection purposes [25]. The skin secretions of all amphibians used in the present study are rich in multiple molecular forms of several different families of amphipathic peptides. In order to determine if interaction of these peptides with nucleic acid was a possible explanation for intact mRNA recovery, we designed *in vitro* gel-retardation experiments that involved incubation of DNA with synthetic replicates of amphipathic peptides (maximins) derived from *B. maxima* and with crude *L. caerulea* skin secretion. The mobility-inhibition data obtained were consistent with those described previously for nucleic acid/natural amphipathic peptide interaction and with those obtained using an entirely contrived synthetic amphipathic peptide [25].

To seek the origin of the mRNA present in the defensive skin secretion, it was necessary to focus on the microarchitecture of the venom glands. The cellular structure of the amphibian dermal granular gland has been extensively studied, and two cellular components are essential to function. The neck of the gland consists of myoepithelial cells that are innervated by sympathetic, probably noradrenergic, nerves. Upon injection of adrenaline into dorsal lymph sacs, skin secretion will be effectively stimulated by induced contraction of myoepithelial elements [26]. This invasive procedure can be circumvented by direct contraction induction by transdermal electrical stimulation as used in the present study [12]. The base of the granular glands contains the cells that synthesize the defensive skin secretion peptides/proteins and are typified, using transmission electron microscopy, by the presence of large, pleiomorphic, electron-dense secretory granules that occupy much of the apical cytoplasm [26]. However, studies have shown that towards the end of the active synthesis cycle, such cells fuse to form a single secretory syncytium [26,27]. Similar secretory syncytia have also been observed in *Xenopus* stomach, where they express the mRNA for the antimicrobial skin peptide magainin [28]. The combination of a granular-gland contraction stimulus applied to a full secretory syncytium will produce rupture and extrusion of contents. Since all granular-gland syncytium intracellular components will be extruded in this process, proteome and transcriptome should be co-secreted – a hypothesis substantiated by the acquired experimental data.

The present study unequivocally demonstrates that the killing of frogs for skin cDNA library construction, hitherto regarded as essential for the cloning of granular-gland secretion components, is unnecessary and that the robustness of data derived from subsequent scientific procedures is not compromised. In fact, the technique described paves the way for construction

of an international repository for the purpose of functional genomic studies on venomous amphibians. Species can be sampled using this simple, non-invasive procedure without disturbance of ecological parameters, such as local population dynamics. The granular-gland transcriptome and proteome are both accessible from a sample of freeze-dried skin secretion that, under standard laboratory freezer conditions, remains unaltered for at least 6 years for proteome analysis and at least 2 years for transcriptome analysis. An additional finding within the study programme is that mRNAs representing 'housekeeping' gene transcripts are, as one would expect from rupture and extrusion of a syncytial cytoplasm, represented in the skin secretion. From cDNA libraries of freeze-dried skin secretion of *K. maculata*, *A. callidryas and P. dacnicolor*, we have sequenced cDNAs of 'housekeeping' proteins such as α-tubulin, laminin receptor and various ribosomal proteins. Of course we cannot be absolutely certain that these transcripts originate from the granular-gland syncytium, owing to their cellular ubiquity, but nonetheless their identified presence subserves their subsequent applications. These fundamental ubiquitous proteins are often of most interest to molecular phylogeneticists and systematic biologists as they are for the most part highly conserved, with structural differences in either nucleic acid or amino acid sequence being of taxonomic significance. The requirements of such scientists can thus also be afforded by employment of this non-invasive sampling technology. Consequently, the drug lead discovery potential through systematic proteomics and the warehousing of pre-extinction anuran amphibian genetic information can be secured virtually indefinitely.

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