Molecular cloning of a bombinin gene from *Bombina orientalis*: detection of NF-κB and NF-IL6 binding sites in its promoter

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Abstract The sequence of a gene from *Bombina orientalis* was determined which codes for antibacterial peptides. The gene comprises two exons separated by a large intron. Exon 1 codes for the signal peptide, while exon 2 contains the genetic information for two identical bombinins and one bombinin H. The promoter region of the bombinin gene contains putative recognition sites for nuclear factors, such as NFκB and NF-IL6. In vivo experiments on *B. orientalis* have shown that a short contact with bacteria is sufficient to induce a marked increase in the amount of antibacterial peptides in the skin secretion of frogs. This increase was suppressed by pretreatment with glucocorticoids. In the latter case, a significant increase of IκBα in the secretion is also detectable.

Key words: Antimicrobial peptide; Glucocorticoid; IκB; NFκB; NF-IL6; Amphibia; *Bombina orientalis*

1. Introduction

Many immunity related genes are under the dual control of the rel-containing transcription factor NFκB and different inhibitors called IκBs, all with ankyrin repeats [1]. In mammalian cell cultures, it has been demonstrated that glucocorticoids (GCs) induce a dramatic synthesis of IκBα but not related forms of this inhibitor. These increased levels of IκBα prevent the translocation of NFκB to the nucleus, thus blocking the transcription of genes with promoter sites for NFκB binding [2,3]. This observation could explain the potent immunosuppressive activity exerted by GCs.

Recently, we have demonstrated by HPLC analysis that treatment of the skin of the frog *Rana esculenta* with a cream containing a glucocorticoid inhibited all de novo synthesis of the antibacterial peptides normally present in the skin secretion [4]. Moreover, Northern blot analysis of total RNA from the skin showed that all mRNAs for the precursors of antibacterial peptides were absent. In addition, immunoblot analysis of the cytoplasmic skin proteins showed a clear rise of IκBα, detected with an antibody against human IκBα [4]. These results suggest that *Rana* frogs in vivo regulate their peptide-mediated innate immunity by an NFκB/IκBα-like mechanism. So far, all mammalian genes encoding antibacterial peptides have been found to contain promoter motifs that potentially bind NFκB-like factors [5–9], but no functional studies have yet been reported. In contrast, *Drosophila* has become a model system for both in vitro and in vivo studies of the molecular mechanisms behind the NFκB/IκBα regulation [10,11]. In insects, this type of mechanism is used for control of both immunity and development [10–13].

In order to verify the immunity related function of the NFκB/IκBα complex in another amphibian species, we have started an investigation of the frog *Bombina orientalis* to determine the structure of genes encoding bombinin-like antimicrobial peptides in this species.

In 1970, bombinin was described as an antibacterial and hemolytic peptide isolated from the skin of *Bombina variegata* [14]. More recently, isolation of peptides and cDNA clones from *B. variegata* [15] and *B. orientalis* [16] showed that the original bombinin was in fact a mixture of peptides, then referred to as bombinin-like peptides (BLP). Some of these peptides were both antibacterial and hemolytic, others had only one or the other of these functions. Some of the peptides from *B. variegata* with both functions turned out to have a D-amino acid in position 2 [17], as a result of a post-translational modification reaction, recently reviewed [18].

The goal of the present study was to understand factors that can up- or down-regulate the activity of the genes for antimicrobial peptides. Two genes from *B. orientalis* have been sequenced; the first gene contains an open reading frame coding for two copies of BLP-3 [16] and a new peptide GH-1. Interestingly, regions involved with the recognition of nuclear factors were identified upstream of this gene. The other gene is of similar design, but contains the information for a single copy of a bombinin-like peptide and another new peptide, GH-2. Experiments on the induction of the genes in live animals as well as the inhibition of induction by glucocorticoids are also reported.

2. Materials and methods

2.1. DNA preparation and genomic library construction

High molecular weight genomic DNA was isolated from the liver of *B. orientalis* (an adult male of 5 g), using a Qiagen DNA kit. The fragments produced by partial digestion of this DNA with *Sau*3a were fractionated by centrifugation on a 10–40% sucrose gradient [19]. Fractions containing the 12–23-kb fragments were collected. After ethanol precipitation, the sized DNAs were partially filled in using dATP and dGTP to create ends compatible with those of λ Fix II supplied in the λ Fix II/*Xho*I partial fill-in vector kit (Stratagene). The ligated DNAs were packaged in vitro using the protocol and the reagent supplied with Gigapack II Gold packaging system (Stratagene). Amplification of the library was performed according to the manufacturer's instructions.

2.2. Screening of genomic library

Part of the library (5×10^5 clones) was screened following the pro-

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Abbreviations: GC, glucocorticoid

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cedure of Benton and Davis [20]. The Nytran-Plus filters (Schleicher and Schuell) were probed with a full-length cDNA encoding the complete precursor of bombinin (clone 42 from *B. variegata* [15]), labelled by random priming with [α-^{32}P]dCTP (3000 Ci/mmol). Hybridization was carried out for 18 h at 42°C in 2 mM sodium phosphate buffer, pH 7.2, containing 50% formamide, 5× SSPE (150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA), 5× Denhart's solution, 0.5% SDS and 100 μg/ml herring sperm DNA. Filters were then washed at 50°C for 20 min in 0.1× SSPE, 0.1% SDS and exposed for 8 h on Kodak X-Omat SO 282 films with an intensifying screen at −80°C. Positive clones were purified by repeated plating and hybridization until 100% of the phages were positive.

2.3. Subcloning and sequencing

The restriction maps of two strongly positive clones (termed B9 and B24) were obtained with single and multiple complete digestions with *Hin*dIII, *Bam*HI and *Sac*I. The genomic DNA in B24 was digested with *Sac*I, and 8-, 6.5-, 3.5-, and 2-kb fragments were obtained. Southern analysis of these fragments showed that only the 6.5-kb fragment hybridized to the cDNA probe. The positive fragment was subcloned into pBluescript vector (Stratagene). The nucleotide sequence was determined on both strands either by the chain termination method using Sequenase kit (USB) or by automated fluorescent DNA sequencing (MediGene AG, Germany) with pBluescript universal or gene-specific primers.

The genomic DNA of clone B9 gave a 15-kb *Sac*I-*Sac*I fragment. It was digested with *Hin*dIII yielding several sub-fragments, of which only the 1.5- and 1.0-kb sub-fragments were found to hybridize to the cDNA probe. These fragments were subcloned into pBluescript and sequenced as above.

2.4. Glucocorticoid treatment of frogs and immunoblotting

Four *B. orientalis* frogs (about 10 g each) were initially given a mild electrical stimulation (8 V for 15 s at three equal intervals) to obtain a complete discharge of the cutaneous glands, and then kept in a terrarium for three weeks. After this period, two of them were injected with a 25-μg methylprednisolone solution (Solu-Medrol, Upjohn) in the dorsal sac, while two control frogs were injected with the same solution devoid of glucocorticoid. About 15 min later the four frogs were again electrically stimulated, and the secretion was washed off with 10 ml 0.1% acetic acid and freeze-dried. The protein content of the secretion was determined using a Bio-Rad protein assay kit. Total protein (100 μg) from skin secretion was separated by 12% SDS-polyacrylamide gels (Bio-Rad), electroblotted on nitrocellulose (Sartorius) and then blocked in TBST (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% Tween 20) containing 0.5% BSA. The membranes were incubated for 16 h at 4°C with 1:1000 dilution of polyclonal rabbit antibodies against a synthetic peptide corresponding to the N-terminal residues 26–39 of human IκBα (New England Biolabs Inc.). The blots were washed and incubated with 1:2000 dilution of anti-rabbit Ig-peroxidase conjugate in TBST. After several washes, antibody-reactive bands were visualized by enhanced chemiluminescence (New England Biolabs Inc.).

2.5. Bacteria-bath treatment of frogs

Nine wild frogs were divided into three groups. The first group was bathed in 200 ml LB solution for 20 min. The second group was bathed for 20 min in 200 ml of an overnight culture of *Aeromonas hydrophila* diluted with fresh LB medium to 10^6 cells/ml. The third group was injected intraperitoneally with 25 μg methylprednisolone (Solu-Medrol), left 15 min in a cage, then bathed for 20 min in the bacteria solution as above. After 1 h, the nine frogs were electrically stimulated, and the secretions were collected after washing each frog with 20 ml 0.1% acetic acid. The secretions from each group of frogs were pooled and 1 ml from each pool was fractionated by reversed-phase HPLC as reported [17].

3. Results

3.1. Structure of the bombinin gene

Six clones, isolated from 5×10^5 recombinant phages of the *B. orientalis* genomic library, were found to hybridize strongly with the cDNA coding for the complete precursor of bombinin [15]. The restriction maps of these clones, obtained by digestion with several enzymes, indicated that each clone was unique. Southern blotting analysis revealed that five of them contained the same *Sac*I-*Sac*I 6.5-kb fragment. The 6.5-kb fragment from clone B24 was subcloned in pBluescript and sequenced. The full sequence of the gene (Fig. 1) includes 3733 bp and contains 555-bp 5′- and 551-bp 3′-untranslated regions. Comparison with the published bombinin precursor cDNA [16] reveals that the coding region of the gene consists of two exons of 59 and 523 bp, respectively, interrupted by a large intron of 2045 bp. Canonical GT/AG exon-intron junction sequences are found at the extremities of the intron [21]. The first exon encodes the first 20 amino acid residues, comprising the 18-residue signal peptide. Exon 2 contains the remaining coding region (173 amino acid residues), which includes the acidic peptides 1 and 2, as reported by Gibson et al. [16], two identical copies of a bombinin-like peptide (BLP-3) and one copy of a C-terminal peptide, different from that reported by Gibson [16]. This peptide is homologous to bombinin H, antibacterial and/or hemolytic peptides found in *B. variegata* [17]. It was therefore termed GH-1 (Gene-derived bombinin H-like peptide). The 3′ flanking region of the gene includes three canonical polyadenylation sites (AATAAA) centered at 66, 77 and 133 bp downstream of the TAA stop codon, respectively. In the 5′ untranslated region, 53 bp before the presumptive transcription start site, a TATA-like box (TAAA) is located; another TATA box is centered at −124 bp. This region is preceded by at least three CAAT boxes (Fig. 1). Moreover, structural motifs for the binding of transcription factors involved in the control of immune-related molecules are found in the 5′ flanking region: an NFκB site centered at −432 bp (negative orientation) and an NF-IL6 site at −473 bp (positive orientation) from the translation initiation site.

Fig. 2 shows a schematic representation of the bombinin gene with the restriction sites used in sequencing and cloning. BLP-3 is almost identical (Leu in position 7 is substituted by Ile) to the peptide described by Gibson as inactive [16]. We have isolated BLP-3 from the skin secretion of *B. orientalis*, following reversed-phase HPLC in the conditions described previously for *B. variegata* [17]. It showed antibacterial activity towards a number of Gram-negative bacteria, the most sensitive being the human pathogen *Yersinia pseudotuberculosis* [22]. So far, peptides GH-1 and GH-2 have not been isolated from the secretion. A synthetic GH-1 showed some antibacterial and hemolytic activity. Neither the proparts 1 and 2 nor the two 8-residue spacer peptides have been isolated. They may be rapidly broken down, perhaps as part of the processing of the inactive precursor to the active effector molecules.

The sixth clone (B9) was derived from a gene encoding another bombinin precursor which was analyzed in part. From a 15-kb *Sac*I-*Sac*I fragment, several sub-fragments were obtained with digestion with *Hin*dIII (7, 3, 1.5 and 1 kb). Of these, the 1.5- and 1-kb sub-fragments strongly hybridized with the bombinin cDNA [15], while the 3-kb sub-fragment strongly hybridized with a probe containing the promoter and the signal peptide region, obtained by suitable digestion of the first gene. Only the 1.5- and 1-kb sub-fragments were sequenced, yielding an about 2500-bp stretch which includes an intron of less than 2000 bp and a coding region of about 380 bp. The exon codes for one copy of a bombinin identical to that described as clone 7 in *B. variegata* [15], and contains the information for a new C-terminal pep-

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cttaagctcagttttatgttaaaaaataaacttcaggtaatagaataaaacagtttacaaatgtcataaac      71
attatttgtgcaactgaatgattagaaatctatttcattaaaggga catgaaaccccc taagctcgtgtca    142
atacatttttaaacatctttgcaattttacttctaatatcaattttgctttattttatttgtatccttttatt    213
gaaggagtagcagtagcaatatttgcatttggagctagtttaacacatcggtaagccaatgacaacagcca      284
ttatttgtgcagccaccaatcagcagctagctccagcccctgagcctacttaggtatgcttatcaacaatgg     355
atactaagtgaacaaagcaacagcagtaaaatggaaagcagtttagaattgcatatgctctatctgaatca      426
tgatatatttaattttgactttgatgtaattgtgttttaatgtttctttaatggatagaaatcaacacattt     497
agtaaaaaatttgatatggttctaattgttcttttttttctttacttttaacaggtaaagATGAATTTTAAGT    568
                                                                  M  N  F  K
ACATAGTTGCAGTGTCCTTTTTAATAGCATCTGCATATGCACGAAGgtaaaataaattatacaaggaaaga      639
Y  I  V  A  V  S  F  L  I  A  S  A  Y  A  R  S
actatcaaaatatttattttagatataaatattttatttcagagaacctgctaaatattcaaaagagatac      710
taaattcttaaaaatgcaatggtcatatttacatatgtggtaataattgtgtgacataaagcttcccagct      781
ttcaaaaatgtgtctaatgtaaagtgctatctgcttccatttcccatccagtacaatatacattacattat      852
atgagagcacaaaatgtagtaagatgttaaaaggaagttagaaggaataaaggtggttcgaacaagggcgt     923
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gtgtgtgcacttaatcaggagagctaaacagtgagagagagagaaataacagtttttattaacttaaacta     1207
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cacaaaataaaatatatttattaaaaaccaataagtagtttaattttaaaatgttaaagtcataaaatttg     2627
tttaccaatctgatttatttatttatttttagTGAAGAGAACGACATACAGTCTCTGAGTCAGAGGGATGT     2698
                                    E  E  N  D  I  Q  S  L  S  Q  R  D  V
TTTAGAAGAAGAATCACTGAGGGAAATCAGAGGTATAGGAGCAGCCATCCTAAGTGCTGGTAAATCAGCTT     2769
  L  E  E  E  S  L  R  E  I  R  G  I  G  A  A  I  L  S  A  G  K  S  A
TAAAAGGCTTGGCTAAAGGATTGGCTGAGCATTTTGGGAAGAGAACTGCTGAAGATCATGAAGTGATGAAA     2840
L  K  G  L  A  K  G  L  A  E  H  F  G  K  R  T  A  E  D  H  E  V  M  K
AGACTGGAAGTTGCAATACAGTCTCTGAGTCAGAGGGATGTTTTAGAAGAAGAATCACTGAGGGAAATCAG     2911
 R  L  E  V  A  I  Q  S  L  S  Q  R  D  V  L  E  E  E  S  L  R  E  I  R
AGGTATAGGAGCAGCCATCCTAAGTGCTGGTAAATCAGCTTTAAAAGGCTTGGCTAAAGGATTGGCTGAGC     2982
  G  I  G  A  A  I  L  S  A  G  K  S  A  L  K  G  L  A  K  G  L  A  E
ATTTTGGGAAGAGAACAGCTGAAGAACATGAAGTGATGAAAAGACTGGAAGCCGTAATGCGTGATCTAGAT     3053
H  F  G  K  R  T  A  E  E  H  E  V  M  K  R  L  E  A  V  M  R  D  L  D
TCCTTGGATTATCCAGAGGAAGCTTCTGAAAGGGAAACCAGAGACTTCAATCAAGAGGAAAAAGAGAAACG     3124
 S  L  D  Y  P  E  E  A  S  E  R  E  T  R  D  F  N  Q  E  E  K  E  K  R
CATTATAGGGCCAGTATTAGGTTTGGTTGGTAAACCACTTGAAAGTTTACTTGAATAAttatagccagtaa     3195
  I  I  G  P  V  L  G  L  V  G  K  P  L  E  S  L  L  E  *
aactttgctttcattaatgtttgtaaaatgatgctaatcagataacatataataaagcataaataaacaca     3266
caaaaagctatttaaacaaactgcatgttctctacattctgctattaataaaaatctttgagcaaatct      3337
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gaacaaaaagcgcatcccagataaagtttatctaatgatttttctacaaaagccaacataaaatctatgga     3479
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cacacttgttcctctaatcttaatataaaaacaatgtaacaaacatgaccaaaatgtgataattaagcagc     3692
tatactaaaaaacagcaaaaaatgtacgacaggaaatatat
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Fig. 1. Sequence of the gene for BLP-3 and peptide GH-1. The two exons are given in uppercase, while non-coding regions are in lowercase. The amino acid-deduced sequence is shown under the coding DNA, and mature peptide sequences are underlined. The gt/ag exon-intron junction sequence is boldfaced. The putative binding site for NFκB is underlined and the corresponding site for NF-IL6 is double underlined. CAAT boxes in the 5′ region as well as the polyadenylation sites in the 3′ region are boldfaced. Putative TATA boxes are dot underlined and boldfaced.

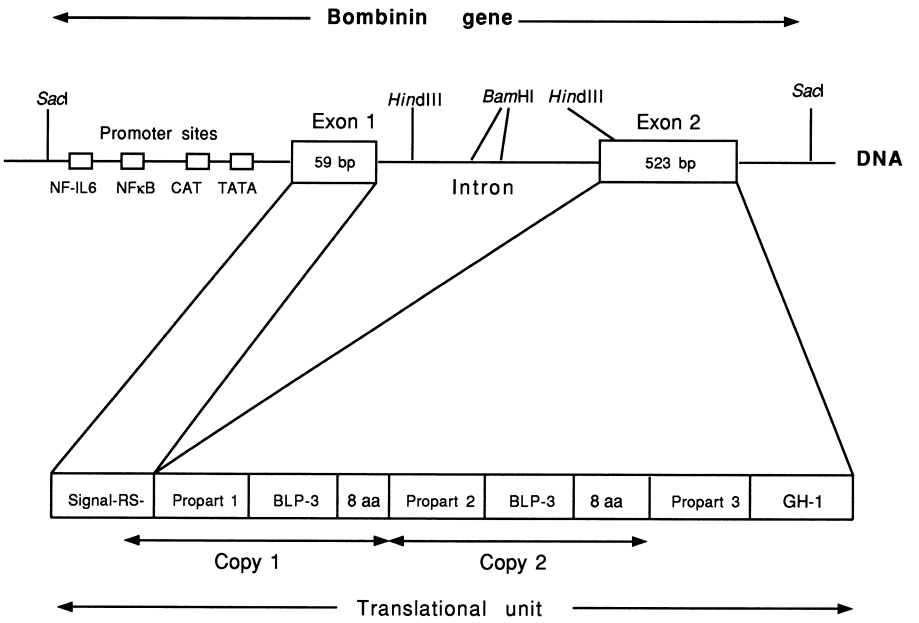

Fig. 2. Schematic representation of the bombinin gene with the indication of restriction and promoter sites. Exon 1 contains the predicted signal sequence and two residues from propart 1 (RS). Exon 2 contains proparts 1–3, two 8-residue spacer peptides, two identical copies of BLP-3 and the C-terminal peptide GH-1.

tide, GH-2, with the deduced sequence ILGPVLDLVGRALRGLLKKIG. The terminal Gly is most likely used for amidation of the penultimate Ile residue.

3.2. Gene control in vivo

In a previous paper [4], we reported on the effect of glucocorticoids on the secretion of skin antimicrobial peptides in *R. esculenta*. With that frog it was possible to treat the skin with a glucocorticoid cream, which was applied to the whole surface after having discharged the cutaneous glands by an electrical stimulation. However, in the case of *B. orientalis* such a treatment was not tolerated by the animal, which tried to remove the cream both mechanically and by an abundant secretion from the glands. Thus, GC treatment was performed by injecting in the dorsal sac a suitable amount of methylprednisolone, followed by electrical stimulation and collection of the secretion. The effect of the GC treatment was an increase in the level of IκBα, which was detected directly in the secretion by immunoblotting with anti-IκBα antibodies (Fig. 3). It is not surprising that IκBα is found in the secretion, since granular cutaneous glands in *B. orientalis* release the secretory materials through a holocrine mechanism [23].

As a test for function, we have induced the expression of the bombinin genes in the live frogs by bathing them in bac-

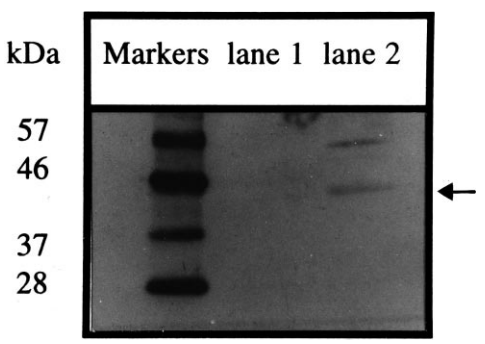

Fig. 3. Western blot analysis of proteins from frog skin secretion. Lane 1: control frog; lane 2: frog injected in the dorsal sac with a glucocorticoid solution. Proteins were probed with an antibody against human IκBα. The double band conforms to that reported in the antibody kit (New England Biolabs Inc.). The arrow indicates IκBα immunoreactivity.

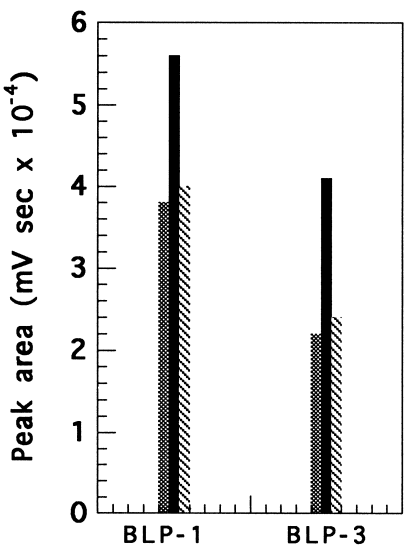

Fig. 4. Comparison of the peak areas of selected peptides (BLP-1 and BLP-3), taken as representative of antimicrobial peptides in the skin secretion of *B. orientalis* [16]. The skin secretions from frogs were lyophilized, dissolved in comparable amounts of 20% ethanol, and fractionated on a reversed-phase column (Aquapore RP-300, 4.6×250 mm, Perkin Elmer), eluted with a suitable gradient of acetonitrile/2-propanol 4:1 in 0.2% (v/v) trifluoroacetic acid. Grey bars: control frogs bathed in LB solution; black bars: frogs bathed in an *Aeromonas hydrophila* LB solution; striped bars: frogs injected with a glucocorticoid intraperitoneally and then bathed in an *A. hydrophila* LB solution.

teria-containing LB. This technique was first developed for *R. esculenta* [24]. Here we bathed the frogs for 20 min in 200 ml of an overnight culture of *Aeromonas hydrophila*. The control bath was LB medium. After this treatment, the frogs were electrically stimulated, skin secretions were collected and fractionated by HPLC. The results are shown in Fig. 4, where the peak areas of selected peptides (BLP-1 and BLP-3 [16]), taken as representative of antimicrobial peptides in the secretion, are reported for comparison. The short contact with bacteria was sufficient to induce a marked increase in peptide synthesis in the wild frogs, whereas the GC-treated frogs were unable to increase the amount of bombinins in the secretion (Fig. 4).

4. Discussion

Most antibacterial peptides are derived from precursors containing a single copy of the end-product, even though clusters of genes can be found, as is the case with human defensins [8,25] or insect cecropins [10]. However, cDNA clones for the magainins from *Xenopus laevis* [26] and the apidaecins from honeybee [27] have shown that peptide antibiotics sometimes are encoded in multiple copies in a single gene. In case of apidaecins, a comparison was made to the yeast pheromone and it was also suggested that multiple copies could be part of a regulatory device [27]. In the bombinin gene of *B. orientalis*, the precursor is encoded in two exons, the first containing the signal peptide and the second containing the information for three bioactive peptides interspersed with proparts and spacer peptides. In addition, the sequence of the exon from the second gene shows a high similarity with the cDNAs encoding the bombinins from *B. variegata*, which also contain the information for a single copy of both bombinin and bombinin H [15].

The present study was undertaken to understand factors that can up- or down-regulate the expression of the genes. As a result of several attempts to infect *R. esculenta* with *Aeromonas hydrophila* isolated from frog skin, we observed that a bath in a suspension of live bacteria was most effective [24]. This method was therefore also used in this study with *B. orientalis*. HPLC analysis of the skin secretions demonstrated that exposure to bacteria resulted in a stimulation of bombinin synthesis. This effect was not observed with frogs which had been injected with glucocorticoids (Fig. 4). The level of antibacterial peptides in a wild animal is such to maintain a balance between colonization and infection. The bacteria bath represents an extreme condition, which stimulates the production of antimicrobial peptides above the 'constitutive' level. The bacterial challenge is not able to induce peptide synthesis in glucocorticoid-treated animals, but the 'constitutive' level is not affected (Fig. 4). In addition, the Western blot analysis shows that the injection of a glucocorticoid in the dorsal sac of *B. orientalis* leads to a marked increase of IκBα (Fig. 3). Here we show that the promoter region of the bombinin gene contains recognition sites for nuclear factors (Fig. 1), such as NFκB and NF-IL6 [28–30]. Previous studies on the frog genes encoding PGLa, xenopsin, levitide and caerulein [31] were reported without focusing on the promoter region. More recently, two genes encoding the antimicrobial peptides dermaseptins from the arboreal frog *Phyllomedusa bicolor* were described [32], but, as before, emphasis was on the processing of the different precursors. Closer inspection of the published sequence of the dermaseptin *Drg1g2* gene [32] reveals the presence of both these sites, centered at −764 and −1177 bp upstream of the ATG initiation codon, respectively.

In conclusion, we have a new model system for host microbe infection. Simple exposure to bacteria results in gene activation in the skin of frogs. It will be interesting to analyze the intermediate steps in this chain of events.

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