MOLECULAR CLONING AND ANTIBACTERIAL ACTIVITY OF A LEBOCIN-LIKE PROTEIN FROM *MANDUCA SEXTA*

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MOLECULAR CLONING AND ANTIBACTERIAL

ACTIVITY OF A LEBOCIN-LIKE PROTEIN FROM

MANDUCA SEXTA

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DEDICATION

I dedicate my thesis to Shri Bikkavolu Lakshmi Ganapathi swamy.

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CHAPTER I

INTRODUCTION

Immunity is the ability of an organism to defend against invading pathogens. There are two kinds of immunity, innate and acquired. Innate immunity is fast and less specific whereas acquired immunity, which involves the production of antibodies, is slow and more specific.

One of the successful species in evolution are insects. Apart from seas, they colonize all ecological niches and account for 70% of all animal species. Insects are continuously exposed to pathogenic microorganisms and are able to defend against infection (Hoffmann, 1995). They have an efficient defense system considered as innate immunity, which includes both humoral and cellular responses (Tzou et al., 2000). The cellular reactions of insects in response to invading microorganisms include phagocytosis, nodulation and encapsulation, whereas humoral immune reactions include activation of proteolytic pathways and the synthesis of antimicrobial peptides (Gillespie and Kanost, 1997; Hoffmann, 1995; Hultmark, 1993; Meister et al., 1997).

Microbial cell wall components like peptidoglycans, lipopolysaccharides and β-1,3-glucan which are absent in host cells are recognized by pattern recognition proteins such as peptigoglycan recognition protein and Gram-negative bacterial binding protein. These recognition proteins are present in the hemolymph or on the surface of immune

cells, bind to pathogens-associated molecular patterns and trigger both cellular and humoral immune response in insects (Gillespie et al., 1997; Lavine et al., 2002).

A battery of potent antibacterial and antifungal peptides, which are mainly produced in the fat body, are released into the hemolymph as a result of wounding or injection of bacteria, fungi, or their cell wall components (Hoffmann et al., 1993; Hultmark, 1993; Yamakawa et al., 1999). Many antimicrobial peptides have been isolated from the hemolymph of immune challenged insects (Cociancich et al., 1994). They are categorized into five groups: cecropins, cysteine-containing peptides, proline-rich peptides, glycine-rich peptides, and lysozymes (Meister et al., 1997). Most of them are less than 10 kDa, hydrophobic, membrane active, and carry net positive charges (Hultmark, 2003; Reddy et al., 2004).

Synthesis of antimicrobial peptides and other immune proteins in *Drosophila* is regulated by Toll and Imd pathways. In response to Gram-positive or fungal infection the Toll pathway is activated whereas in response to Gram-negative bacterial infection, Imd pathway is activated. Identification of orthologous genes in the genomes of *Anopheles gambiae*, *Apis mellifera*, and *Tribolium castaneum* suggests the existence of similar signaling pathways for induced synthesis of immune-responsive proteins in other insect species (Christophides et al., 2002; Evans et al., 2006; Zou et al., 2007). Recently it was found that, in lepidopteran insects proteolytic activation of pro-spatzle is required for the induced transcription of antimicrobial peptide genes (Wang et al., 2007).

Our laboratory works on the immune responses of *Manduca sexta* which is an excellent model organism for insect biochemical research. In the tobacco hornworm

M. sexta, transcription of immunity-related genes is up-regulated in response to bacterial infection (Zhu et al., 2003; Kanost et al., 2004). At least five different types of antimicrobial peptides have been identified in *M. sexta* which include attacins, cecropins, moricin, gloverin and lebocin (Kanost et al., 2004; Zhu et al., 2003). Among them lebocins are proline-rich antimicrobial peptides, which are weakly active against Gram-negative bacteria. Lebocin primarily serve to reduce the minimum inhibitory concentrations (MIC) of other antimicrobial peptides.

Specific objectives of my research include 1) Isolation and sequencing of lebocin cDNA clones, 2) screening, sequencing and identification of upstream regulatory regions of Lebocin genomic clones, 3) testing the antimicrobial activity of lebocin.

CHAPTER II

LITERATURE REVIEW

Insect Immunity:

Insects have an efficient immune system similar to innate immunity of vertebrates. The immune system of insects involves physical barriers (cuticle, gut and trachea), cellular responses (phagocytosis, nodulation and encapsulation) and humoral responses (phenoloxidase cascade and synthesis of antimicrobial peptides) (Gillespie and Kanost, 1997).

Physical barrier

Cuticle is the outermost layer secreted by epidermis of insects. Sclerotized cuticle, gut and trachea serve as a physical barrier against wounds and invading microorganisms (Tzou et al., 2000). Microorganisms that surpass this barrier are encountered in the hemocoel by both cellular and humoral responses (Engstrom, 1999; Tzou et al., 2002).

Recognition

Pattern recognition receptors (PRRs) in the hemolymph mediate the activation of innate immunity by pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway, 2002). Innate immune system of both vertebrates and arthropods contain

specific PRRs which recognize non-self molecules. These include peptidoglycan recognition proteins (PGRPs), Gram-negative bacteria-binding proteins (GNBPs), β-1,3-glucan recognition proteins (βGRPs), lipolysaccharide-binding proteins, hemolin, and C-type lectins. These proteins may be constitutively expressed or strongly induced in response to microbial infection (Gillespie et al., 1997).

PRRs bind to these conserved molecular patterns like peptidoglycans, lipoteichoic acids, lipopolysaccharides and β-1,3-glucans which are specifically found in bacteria or fungi but are absent in host cells (Hoffmann et al., 1999; Yu et al., 2002). Binding of PRRs to PAMPs triggers both cellular and humoral immune response in insects (Gillespie et al., 1997; Bulet et al., 1999; Lavine et al., 2002). Synthisis of hemolymph proteins with antibacterial activities is stimulated by peptidoglycan or lipopolysaccharides injected into *Manduca sexta* or *Bombyx mori* (Kanost et al., 1988; Morishima et al; 1995; Gillespie et al., 1997)

Cellular responses

Circulating hemocytes in the hemolymph perform cellular responses like phagocytosis, encapsulation, and nodule formation. Insect hemocytes can be classified into several groups on the basis of their morphology and function (Lavine and strand, 2002). Bacterial or fungal cells, which are small in size, are engulfed by plasmatocytes. Nematode or parasitoid eggs which are too big to be engulfed, are encapsulated by plasmatocytes and granulocytes. Prophenoloxidase (proPO), whose active form is responsible for melanin synthesis, is produced by oenocytoids (Ashida and Brey, 1998).

Humoral responses

The humoral reactions involve both primary and secondary. The primary responses include recognition of microbial cell wall components and initiation of serine protease cascades such as, proPO activation. The secondary response includes transcriptional activation of antimicrobial peptides (AMPs) (Engstrom, 1999).

Signaling pathways and transcriptional activation

Synthesis of AMPs and other immune proteins in *Drosophila* is regulated by the Toll and Imd pathways. In response to Gram-positive or fungal infection the Toll pathway (Michel et al., 2001) is activated whereas in response to Gram-negative bacterial infection, Imd pathway (Lemaitre., 1996) is activated. Toll-like receptor (TLR) and tumor necrosis factor (TNF) pathways in mammals, which cause activation of transcription factor NF-κB share many similarities with these pathways (Hoffmann and Reichhart, 2002).

The Toll pathway in *Drosophila* is initiated by Gram-positive bacterial or fungal infection. This pathway is activated by a serine protease that cleaves spatzle which then binds and activates the receptor Toll. A receptor-mediated signal transduction then leads to phosphorylation and dissociation of IκB-like inhibitor Cactus from DIF which is an NF-κB like transcription factor, which then translocates to the nucleus and activates synthesis of antimicrobial genes (Belvin and Anderson, 1996; Hoffmann and Reichhart, 2002).

Likewise, in response to Gram-negative bacterial infection IMD pathway is initiated. A pattern recognition receptor PGRP-LC activates kinase-based signal

transduction pathway resulting in the phosphorylation and translocation of another NF-κB like transcription factor Relish into the nucleus. This then activates the transcription of immunity-related genes (Hoffmann and Reichhart, 2002; Hancock et al., 2006).

Insect antimicrobial peptides

Antimicrobial peptides (AMPs) are a critical component of the natural defense system in various organisms ranging from prokaryotes to plants, arthropods, and vertebrates (Bulet et al., 2004). Most of them are less than 10 kDa, hydrophobic, membrane active, and have net positive charges (Hultmark, 2003; Reddy et al., 2004). Since the isolation of cecropin from bacteria-challenged diapausing pupae of *Hyalophora cecropia* (Steiner et al, 1981), over 200 AMPs have been identified in arthropods. Upon microbial infection, expression of *Drosophila* AMP genes is up-regulated by transcription factors from the Rel family (i.e. Dif, Relish, and Dorsal) through the Toll and Imd pathways (Lemaitre and Hoffmann, 2007). Identification of orthologous genes in the genomes of *Anopheles gambiae, Apis mellifera,* and *Tribolium castaneum* suggests the existence of similar signaling pathways for induced synthesis of immune-responsive proteins in other insect species (Christophides et al., 2002; Evans et al., 2006; Zou et al., 2007). Fat body, a tissue analogous to human liver, synthesizes AMPs and secretes these heat-stable molecules into the plasma to kill a broad spectrum of microorganisms.

Insect AMPs are categorized into five groups: cecropins, cysteine-containing peptides, proline-rich peptides, glycine-rich peptides, and lysozymes (Meister et al., 1997). Structurally, they include peptides with α-helical conformation (e.g. cecropins, sarcotoxins, moricins), with β-sheet or α-helix/β-sheet mixed structures (e.g. defensins,

thanatin, drosomycin), and with certain residues overrepresented in the sequences (e.g. lebocins, apidaecins, attacins, diptericin, and gloverins). Expression of antimicrobial peptides can be either constitutive, as in the hemocytes of shrimp, oysters or horseshoe crab (Bache`re et al., 2004; Iwanaga and Kawabata, 1998), or induced upon microbial infection, such as in *Drosophila* (Lemaitre et al., 1996; Imler and Hoffmann, 2000; Hancock et al., 2006).

In the tobacco hornworm, *Manduca sexta*, transcription of AMPs and other immunity-related genes is up-regulated in response to bacterial infection (Zhu et al., 2003; Kanost et al., 2004). At least five different types of antimicrobial peptides are identified in *M. sexta* which include attacins, cecropins, moricin, gloverin and lebocin (Kanost et al., 2004; Zhu et al., 2003).

Attacins which are glycine-rich antimicrobial peptides are found in dipteran and lepidopteran insects. They block the sysnthesis of outer membrane protein of Gram negative bacteria (Hetru et al., 1998).

Cecropins are found only in lepidopteran and dipteran insects. They are ~3 kDa amphipathic proteins (Steiner et al., 1981). They are active against both Gram-positive and Gram-negative bacteria. They penetrate the plasma membrane of the pathogens, disturb the electrochemical gradient, and kill the bacteria (Durell et al., 1992).

Exclusively found in lepidopteran insects so far, moricin-like AMPs are active against both Gram-positive and Gram-negative bacteria (Hara and Yamakawa, 1995). They affect structural integrity of bacterial plasma membrane. Moricin synthesis is induced by microbial cell wall components (Furukawa et al., 1999).

Lebocins

Proline-rich lebocin-like AMPs were first isolated from the silkworm, *Bombyx mori* (Hara and Yamakawa, 1995). Lebocin-like proteins were also identifed in the cabbage looper, *Trichoplusia ni* (Liu et al., 2000) and the eri-silkworm, *Samia cynthia* (Bao et al., 2005). Lebocins act on bacterial membranes and *O*-glycosylation is required for their antibacterial activity (Hara and Yamakawa, 1995).

Lebocins show very weak antibacterial activity under physiological conditions. They primarily serve to reduce the minimum inhibitory concentrations (MIC) of other AMPs (Ponnuvel and Yamakawa, 2002). *Bombyx mori* lebocin 3 acts in synergism with cecrpin D and reduces the MIC of cecropin D (Hara and Yamakawa, 1995).

CHAPTER III

MATERIALS AND METHODS

Insect rearing and bacterial challenge

M. sexta eggs (Carolina Biological Supply Co., Burlington, NC) were hatched to larvae and reared on an artificial diet (Dunn & Drake, 1983). Day 2, fifth instar larvae were injected with formalin-killed *Escherichia coli* XL-1-blue (2×10^8 cells/larvae). After 24h hemolymph and fat body samples were collected for RNA preparation. Muscle tissues were used for isolating Genomic DNA.

Probe labeling

A 280 bp *M. sexta* lebocin (Zhu et al, 2003) fragment cloned in pGEM-T vector (Promega) was obtained from Dr. Kanost at Kansas State University. The cDNA fragment was retrieved from the plasmid by digeston with *SacII* and *SpeI*. The digested DNA was separated on 1% agarose gel. The cDNA insert was excised from the gel and purified using Qiagen Gel Purification Kit. The DNA fragment was labeled with α^{32}-P-dCTP using Multiprime DNA labelling system (Amersham Biosciences). About 25ng of DNA, 5µl of random hexamers and water were mixed in a final volume of 33µl. The mixture was denatured at 100^{o}C for 10 min and quickly chilled on ice.

Labeling buffer (10µl), Klenow enzyme (2U) and α^{32}-P-dCTP (5µl) were added to the DNA sample. After incubating for 30 min at 37^{o}C, the labeled DNA was separated from unincorporated α^{32}-P-dCTP on a Sephadex G-25 column (Pharmacia Biotech). Specific radioactivity of the labeled DNA was determined on a liquid scintillation counter.

Screening of M. sexta fat body cDNA library

M. sexta induced fat body cDNA library (Jiang et al., 2003a) was screened for full length lebocin cDNA clones. About 1x10^5 plaques were grown on each 150mm NZY agar plates using *E. coli* XL1-Blue(MRF) as the host cells. A 135mm nitrocellulose membrane was laid on the surface of the agar plate for 2min. The membrane was carefully lifted and subsequently denatured in 1.5M NaCl , 0.5M NaOH solution for 2min and neutralized in 1M Tris-Hcl, 1.5M NaCl, pH 8.0 for 5 min. Then the membrane was soaked in 2 X SSC (0.3 NaCl and 0.03 M sodium citrate) for less than 30 sec. The DNA was then cross-linked to the membranes using UV irradiation (1.2 x 10^5 µJ/cm^2).

Prehybridization and Hybridization

Nitrocellulose membranes were soaked in 30ml of 6XSSC for 15min in hybridization oven at 45^{o}C. The membranes were then pre-hybridized in the hybridization solution (0.1% SDS, 6XSSC, 5 X Denhardt's and 0.1mg/ml denatured fish sperm DNA) for 2 h at 58^{o}C. The ^{32}P-labeled DNA probe was denatured at 100^{o}C for 10min, quickly chilled on ice, was added to the hybridization solution (5 x 10^6 cpm/ml). The DNA blot was hybridized overnight at 58^{o}C. Post-hybridization washes were performed in 0.1 X SSC and 0.1%SDS solution at 58^{o}C (10min x 2, 20min x 2, 30 min x

2). Nitrocellulose membranes were exposed to X-ray film with two intensifying screens at -80^{o}C for 48-72 h. Positive plaques were purified to homogeneity by secondary and tertiary screening.

In vivo excision:

In a 15ml Falcon tube 200 µl of *E. coli* XL1-Blue cells (OD_{600} = 1.0), 1 x 10^5 pfu/ml of phage stock and 1 µl of ExAssist^{TM} (Stratagene) helper phage were mixed and incubated at 37^{o}C for 15min. About 3ml of LB medium was added to the mixture and incubated for 3-5h at 37^{o}C with shaking. The tubes were then heated at 70^{0}C for 20min and centrifuged at 4000 × *g* for 15min. The supernatant was transferred to a new falcon tube and stored at 4^{o}C. One µl of 10^{-4} dilution supernatant was mixed with 200 µl of *E. coli* SOLR^{TM} (Stratagene) cells and incubated at 37^{o}C for 15min. Around 100 µl was spread on LB-ampicillin plate and incubated overnight at 37^{o}C. One single colony carrying plasmid was inoculated into 3ml of LB/ampicillin solution and incubated at 37^{o}C with shaking at 220 rpm. Plasmid DNA was isolated using Wizard Plus DNA Purification System (Promega).

Sequencing and bioinformatic analysis:

The positive plasmids were sequenced using BigDye v2.0 Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA) at OSU Protein/Nucleic acid Resource facility using ABI373 Automated DNA Sequencer. Sequence editing, assembly, and the analysis of transcription factor binding site in the

promoter region were performed using MacVector Sequence Analysis Software (Version 6.5, Oxford Molecular Ltd, Oxford, UK).

M. sexta genomic library screening:

A full-length lebocin cDNA clone (NC2) was digested with *EcoRI* and *XhoI.* The insert was separated from the vector by 1% agarose gel electrophorosis, recovered using Qiagen Gel Purification Kit and labeled with α^{32}-P-dCTP as described above. The probe was used to screen the *M. sexta* genomic library in λGEM11 (kindly provided by Dr. Yucheng Zhu at the Southern Insect Management Research Unit, USDA-IRS).

The plaque lifting, hybridization, and washing was performed as described above for cDNA screening, except using, *E. coli* LE372 as host cells.

Isolation of λ DNA and Subcloning:

Positive bacteriophages were amplified and λ DNA was extracted using Qiagen Lambda DNA Extraction Kit (midi). Phage DNA was subjected to single and double digestion using restriction enzymes *Xho*I*, Apa*I*, Eco*RI*, Hind*III*, Kpn*I*, Sac*I*, Sal*I, and *Xba*I. The digested λ DNA samples were separated on 0.8% agarose gel and restriction map was determined. The DNA was then transferred onto a GenScreen Plus nitrocellulose membrane (NEN Life Science Products) and hybridized with full-length lebocin cDNA, labelled with DIG-High Prime DNA Labeling Detection Kit (Roche Applied science). After washing the membrane was exposed to X-ray film at room temperature for 20min. Lebocin gene fragments identified by autoradiography were sub cloned into pBluescript-KS (Stratagene). Recombinant plasmids were then transformed

into *E. coli* DH5α competent cells and cultured on agar plates containing 50 μg/ml ampicillin, 0.2 mM IPTG and 40 μg/ml X-gal for selection. Positive clones were picked and plasmid DNA was isolated with a Wizard Plus Miniprep Plasmid DNA Kit (Promega). The DNA was then digested with the same restriction enzymes and analyzed by 1% agarose gel electrophoresis to verify the insert.

Southern blot analysis:

M. sexta genomic DNA was extracted from the muscle tissue of a single fifth instar larva using Qiagen DNeasy Blood and Tissue Kit. About 10 μg of DNA was incubated with *Xho*I and *Sca*I restriction enzymes at 37^{o}C overnight. The digested DNA sample was separated by electrophoresis on a 1% agarose gel and transferred onto a GeneScreen Plus nitrocellulose membrane (NEN Life Science Products). The DNA blot was hybridized as described by Sambrook and Russell (2001), with ^{32}P labeled lebocin cDNA, initially digested with *Xho*I and *Sca*I.

Northern blot analysis:

Fat body total RNA samples were prepared from the induced, day 3, fifth instar larvae using Invitrogen Micro-to-Midi Total RNA Purification System. Six μg of total RNA was mixed with 2 μl 10 x MOPS running buffer (0.4M MOPS, 0.1M sodium acetate, 0.01M EDTA), 1.5 μl of 37% formaldehyde, 4.5 μl formamide and 1μl of ethidium bromide (2mg/ml) to a final volume of 20 μl with DEPC treated water. The mixture was incubated for 5min at 65^{o}C and quickly chilled on ice. RNA samples mixed with 4 μl of loading buffer (1mM EDTA, pH 8.0, 0.25% bromophenol blue, 0.25%

xylene cyanol, and 50% glycerol) were separated on 1% agarose gel containing 2.2 M formaldehyde with 1 x MOPS running buffer. After electrophoresis, the gel was rinsed with DEPC treated water and transferred onto a GeneScreen Plus nitrocellulose membrane (NEN Life Science Products). The RNA blot was hybridized as described by Sambrook and Russell (2001), with ^{32}P labeled lebocin cDNA, initially digested with *Xho*I and *Sca*I.

RT-PCR analysis:

Hemocytes and fat body total RNA samples were prepared from the naïve and induced, day 3, fifth instar larvae using Invitrogen Micro-to-Midi Total RNA Purification System. The RNA sample (2-4 µg), oligo(dT) (0.5 µg) and dNTPs (1 µl, 10 mM each) were mixed with diethylpyrocarbonate-treated H_2O in a final volume of 12 µl, denatured at 65°C for 5 min, and quickly chilled on ice for 3 min. M-MLV reverse transcriptase (1 µl, 200 U/µl, Invitrogen), 5×buffer (4 µl), 0.1 M dithiothreitol (2 µl), and RNase OUT (1 µl, 40 U/µl, Invitrogen) were added to the denatured RNA sample (12 µl) for cDNA synthesis at 37°C for 50 min. The *M. sexta* ribosomal protein S3 (rpS3) mRNA was used as an internal control to normalize the cDNA samples in a preliminary PCR using primers 501 (5′-GCCGTTCTTGCCCTGTT-3′) and 504 (5′-CGCGAGTTGACTTCGGT-3′). Lebocin cDNA fragment was amplified using forward (5′-CTGATTTTGGGCGTTGC GCTG-3′) and reverse (5′-GCGCGTATCTTCTATCTGGA-3′) primers under conditions empirically chosen to avoid saturation: 30 cycles of 94°C, 30 s; 50°C, 30 s; 72°C, 30 s. The relative levels of lebocin transcripts in the normalized samples were determined by 1.5% agarose gel electrophoresis.

Peptide synthesis and bacterial strains

Four lebocin-related peptides (~10 mg each) (#1, QRFSQPTFKLPQGRLTLSRK FR; #2, ESGNEPLWLYQGDNIPKAPSTAEHPFLPSIIDDVKFNPDRRYAR; #3 SLGT PDHYHGGRHSISRGSQSTGPTHPGYNRRNAR; #4 SVETLASQEHLSSLPMDSQET LLRGTR) were prepared by automated protocols of stepwise solid-phase synthesis using Fmoc-amino acid derivatives (Bio-Synthesis Inc). Pathogenic strains of *Salmonella typhimurium* ATCC 14028*, Escherichia coli* O157:H7 ATCC 25922, *Listeria monocytogenes* ATCC 19115*, Staphylococcus aureus* ATCC 25923, were kindly provided by Dr. Guolong Zhang in Department of Animal Science at Oklahoma State University for antibacterial assay.

Antibacterial activity assay

The synthetic peptides were separately tested against two different strains each of the Gram-negative and Gram-positive bacteria including *E. coli, S. typhimurium, L. monocytogenes,* and *S. aureus*. The minimum inhibitory concentrations (MICs) were determined in a broth micro dilution assay (NCLS, 2003). Briefly, overnight bacterial cultures were subcultured into 4 ml of Trypticase Soy Broth for 3-5 h until the bacteria reached mid-log phase. After centrifugation at 1000 × *g* at 4ºC and washing with 10 mM Tris Hcl, the cells were suspended in 5% Trypticase Soy Broth (5×10^5 cfu/ml). Aliquots of the diluted cultures (90 µl) were mixed with 10 µl of the synthetic peptide at 1000, 500, 250, 125, 62.5 µg/ml. All bacteria were cultured at 37ºC for overnight in a 96-well cell culture plate, and the lowest concentration of peptide that caused no visible growth

was recorded. The experiment was performed at least three times for each strain to obtain MICs of *M. sexta* lebocin-related peptides against the bacterial strains.

CHAPTER IV

RESULTS AND DISCUSSION

Lebocin cDNA cloning and sequencing

M. sexta lebocin was initially identified as a differentially expressed gene in response to bacterial injection in a subtractive suppression hybridization experiment (Zhu et al., 2003). Its expressed sequence tag of length 280bp, cloned into pGEM-T vector was obtained from Dr. Kanost at Kansas State University. The fragment was used as a probe to screen the *M. sexta* fat body cDNA library in λZAP (Stratagene) vectors, to obtain full-length lebocin cDNA clones.

Thirteen positive clones were isolated and subcloned by *in vivo* excision (Short et al., 1988) of pBluescript phagemids. Sequencing from both ends using primers T3 (5′-GCAATTAACCCTCACTAAAGG-3′), T7 (5′-AATACGACTCACTATA G-3′) J957 (5′-CATCCGGGCTATAATCGCCGT-3′), and J958 (5′-GACGCTTACTTGACTGACT ATG-3′) revealed that, all the cDNAs contain a constant region at the 5’-terminal and vary in the length at the 3’ end (Fig. 1). Sequence comparison showed that after a 121-residue N-terminal segment, which has a significant similarity to *B. mori* lebocin 4 cDNA (Hara and Yamakawa, 1995), a 27 amino acid fragment was repeated multiple times followed by a 11-residue partial repeat. This repetition of 27 amino acids varied in each positive clone from 3 to 16 times (Table 1) and is rich in Ser, Thr and Leu (13/27).

No similar sequence was found when we did the BLAST search using the 27 amino acid repetitive segment as query. Within the repeats two synonymous substitutions were identified, AGG - AGA encodes Ser and ACC - ACT encodes Arg. Interestingly, *B. mori* lebocin precursor also contains a 27-residue-long extra sequence at the carboxyl terminus which was assumed to be processed during maturation of lebocin (Chowdhury et al., 1995). A 24 amino acid additional sequence was also observed in *D. melanogaster* drosocin precursor (Bulet et al., 1993). *Trichoplusi ni* lebocin cDNA also contains a 39 bp short repetitive sequence at the C-terminal untranslated region immediately after the coding region (Liu et al., 2000).

M. sexta lebocin contains a predicted signal peptide of 20 amino acids. The N-terminal 22-amino acid segment following the signal peptide is similar to *B. mori* mature lebocin peptide. Residues 43-85 of the *M. sexta* lebocin precursor is nearly identical (Fig. 2) to *B. mori* lebocin and anionic antimicrobial peptides isolated from *Galleria mellonella* (Cytrynska et al., 2007) and *Helicoverpa armigera* (Mackintosh et al., 1998). Identification of more than one mature peptide with antibacterial activity in the same protein itself, suggests a role of *M. sexta* lebocin-like protein in eliminating the microbes from the hemolymph. Residues 86-121 contains a highly conserved sequence THPGYNRR which shows significant similarity (Fig. 3) to lebocin-like proteins isolated from *T.ni* (Liu et al., 2001) and *S. ricini* (Bao et al., 2005) but not to *B. mori* lebocin. A recognition sequence for poly-adenylation, AATAAA, was also found 89 bp after the stop codon (TGA). The complete nucleotide sequence and the deduced amino acid sequence are shown in Figure 1. Like *B. mori, T.ni* and *S. ricini* lebocins, *M. sexta*

lebocin-like protein lacks the PRP triplet common in other proline-rich peptides (Liu et al., 2001). Like *S. ricini* lebocin (Bao et al., 2005), no *O*-glycosylation site was observed in *M. sexta* lebocin-like protein whereas a possible site was present in *B. mori* (Hara and Yamakawa, 1995) and *T. ni* (Liu et al., 2001) lebocins. Multiple sequence alignments of lebocin like proteins in lepidopteran insects is shown in Figure 3.

Lebocin gene structure

How could a gene generate a variable number of repeats at the 3' end in the cDNA clone? Is it the result of splicing mechanism? In order to examine the mechanism that results in the variable numbers of repeats in the cDNA clones and also to study the gene expression mechanism of *M. sexta* lebocin-like gene, we screened *M. sexta* genomic library using lebocin cDNA as a probe and obtained one positive clone, namely L45. Southern blot using full length cDNA probe identified two fragments (Fig. 4) of sizes nearly 2.5 kb (*Xho*I and *Xho*I) and 2.9 kb (*Xho*I and *Hind*III) which were subcloned and completely sequenced (Figs. 5 and 6). Comparison of cDNA and genomic sequences indicated that *M. sexta* lebocin is an intron-less gene. *B. mori* lebocin (Furukawa et al., 1997), defensin (Dimarcq et al., 1994) and diptericin (Reichart et al., 1992) from *D. melanogaster* are also intron-less. Conversely, other antibacterial peptide genes contain 1-2 introns. These include attacins (Sun et al., 1991), cecropins A, B and D (Xanthopoulos et al., 1988; Gudmundsson et al., 1991) from the giant silk-moth, *Hyalophora cecropia*, Sarcotoxin I and II (Kanai and Natori, 1989; Kanai and Natori, 1990) from the flesh fly, *Sarcophaga peregrina*, cecropin A1, A2 and B (Kylsten et al., 1990) from *D. melanogaster*, cecropin B (Taniai et al., 1995) from *B. mori*.

M. sexta lebocin-like gene has a coding sequence encoding 483 amino acids containing a probable signal peptide of 20 amino acids, followed by 121 amino acids prosegment and an additional 27 amino acids repeated 13 times at its carboxyl-terminus. A poly-adenylation signal, AATAAA, was present 89 bp after translation stop codon (TGA). Although, we isolated the genomic clone and analyzed its exon-intron organization, we failed to establish the mechanism that results in the variable numbers of repeats in the cDNA clones.

Much work has been done in *D. melanogaster* and *H. cecropia* to elucidate the DNA sequences that regulate the anti-bacterial protein induction (Hultmark, 1993; Hultmark, 1994). Most of the antimicrobial peptides whose gene sequences have been determined, contains NF-κB like motifs in their upstream region. Besides the NF-κB motif, several other response elements involved in mammalian actue-phase inflammation were also found in the upstream regions of *D. melanogaster* antibacterial genes (Reichhart et al., 1992; Georgel et al., 1993). In addition to NF-κB, upstream region of *B. mori* lebocin gene also contains IL-6 responsive element which is present in acute phase immunity-related genes in the mammalian immune system (Furukawa et al., 1997). To identify DNA sequences that might be involved in the regulation of lebocin-like gene of *M. sexta*, upstream sequence of 2.5 kb was searched for sequence motifs by computer analysis (Table 2). Typical TATA and CAAT (Kadalayil et al., 1997) were observed. Three GATA motifs were found, one on plus strand and two on minus strand. Two NF-κB motifs with one mismatch were found, one on plus strand and one on minus strand. There are two interferon stimulated response elements with one mismatch for each on the plus strand (Fig 5).

Southern blot analysis

To explain the relationship between cDNA and genomic clones, we hypothesize that a family of lebocin genes may exist in *M. sexta* and be responsible for the variations in the cDNA clones. *B. mori* lebocin form a multiple gene family (Chowdhury et al., 1995; Furukawa et al., 1997; Cheng et al., 2007) whereas in *T. ni* no evidence for such a gene family was identified (Liu et al., 2000). Southern blot analyses was performed to determine the gene copy no. of lebocin in *M. sexta* genome, using repetitive region as the probe. Probe hybridized with a single fragment nearly 1.1 kb (Fig. 7) suggesting the repetitive region of ~1.1kb is a part of a single copy gene. As we cannot exclude the possibility of having differences in the lebocin gene in *M. sexta* individuals, we have extracted genomic DNA from six different insects A1, A2, A3, A4, A5, A6 and performed southern blot analysis with repetitive region as a probe as described previously. Probe hybridized with single fragment ~1.1kb in insects A2, A3, A4 and A5 and ~2.5kb in insects A1 and A6 (Fig. 8) indicating a single copy gene. In insects A1 and A6 due to a possible mutation at the internal *Sca*I site, the hybridizing genomic fragment migrated to 2.5kb as an *Xho*I-*Xho*I fragment (Fig. 6). The results are consistent with the sequencing results of genomic clone (L45) obtained by screening the genomic library.

Northern blot analysis:

Does variation in the cDNA clones occur at the transcriptional level or is it due to a reverse transcriptase artefact? In order to explore this, Northern blot analysis was performed using repetitive region as a probe. Fat body total RNA was extracted from

insects A1, A6, A4 and A5. We specifically selected these insects because in the southern blot analysis for insects A1 and A6 probe reacted with ~1.1kb fragment and for A4 and A 5 it reacted with ~2.5kb fragment (Fig.8). To our surprise, in the Northern blot the probe reacted with multiple bands (Fig. 9) suggesting the variations of repeats occurring at the transcriptional level rather than during cDNA synthesis. Nevertheless, we do not understand the mechanism for the generation of multiple transcripts.

RT-PCR analysis

B. mori, *T. ni* and *Samia cynthia ricini* lebocin genes were mainly expressed in fat bodies, whereas in hemocytes the expression level is very low (Chowdhury et al., 1995; Liu et al., 2000; Bao et al., 2005). Cecropin B of *M. sexta* is also mainly expressed in the fat body (Dickson et al., 1988). In contrast many antibacterial proteins like attacins are expressed similarly in fat bodies and hemocytes (Yamano et al., 1994; Kishimoto et al., 2002). No lebocin mRNA was observed in the hemocytes and fat body from the naïve larvae. After an immune challenge, the transcripts became abundant in fat body, consistent with the observation that lebocin expression was up-regulated by injecting *B. mori* spätzle-1 into *M. sexta* larvae (Wang et al., 2007). Semi-quantitative RT-PCR was performed to examine the inducibility of *M. sexta* lebocin gene expression in hemocytes and fat bodies. As a control *M. sexta* ribosomal protein S3 (rps3) transcripts were normalized. Relative band intensities indicated that lebocin-like protein mRNA levels in fat body of bacteria-injected larvae were significantly higher than those of the naïve insects (Fig. 10).

Chemical synthesis of Lebocin-related peptides

Regulatory peptides are synthesized as large precursors which will be later processed into active peptides. After the cleavage of the signal peptide, further proteolytic processing occurs predominantly at basic amino acid residues. In order to predict the putative proteolytic processing sites in insect neuropeptide precursors rules have been proposed (Devi, 1991; Veentra, 2000). Cleavage of peptide precursors can occur at mono basic sites if a basic amino acid is present in the -4 position, usually an Arg i.e RXXR and not followed by aliphatic amino acids (Met, Leu, Ile, Val) at the +1 position. Excluding the signal peptide three plausible cleavage sites are observed in *M. sexta* lebocin-like protein. Basing on the cleavage prediction four peptides were chemically synthesized which have a purity >95%. Peptide #1 is 22amino acids in length and has an experimental mass of 2691.95 Da nearly identical to its theoretical value (2691.19 Da). This peptide is highly cationic with a calculated isoelectric point of 12.9. Peptide #2 is 44 amino acids in length and has an experimental mass of 5056.39 Da, nearly identical to the theoretical value (5054.64 Da). This peptide is anionic with a calculated isoelectric point of 4.8. Peptide #2 is similar to (Fig. 2) anionic antimicrobial peptides isolated from the hemolymph of *Galleria mellonella* (Cytrynska et al., 2007) and *Helicoverpa armigera* (Mackintosh et al., 1998). Peptide #3 is 35 amino acids in length and has an experimental mass of 3814.12 Da, nearly identical to its theoretical value (3814.09 Da). This peptide is cationic with a calculated isoelectric point of 11.8. Peptide #4, the repetitive sequence of 27 amino acids has an experimental mass of 2988.89, nearly identical to its theoretical value (2985.33 Da). This peptide is anionic with a calculated isoelectric point of 4.6.

Antibacterial activity and spectrum

M. sexta lebocin-related peptides were tested for their antibacterial activity against two Gram-negative and two Gram-positive bacterial strains using broth micro dilution assay (NCLS, 2003). Peptide #1 is active against both Gram-positive and Gram-negative bacteria. Its MIC is determined as 25 μg/ml except for *S. aureus* which required 100 μg/ml. Peptide #3 showed activity only against *E. coli* O157:H7 among the strains we tested. Its MIC against *E. coli* is determined as 200 μg/ml. Peptide #2 and # 4 showed no activity against the strains we tested (Table 3).

CHAPTER V

CONCLUSIONS

M. sexta fat body cDNA library was screened for full length lebocin cDNA clones. Thirteen cDNA clones were isolated. All the clones contained a constant 5'-terminus and varied in length at the 3' end. In each clone a 81 bp fragment was repeated multiple times followed by a 33 bp partial fragment. This repetition varied in each clone from 3 to 16 times. In order to identify the mechanism for the generation of variable numbers of repeats at the 3' end of the cDNA clones, *M. sexta* genomic library was screened for lebocin gene. The lebocin gene is intron-less and single copy. Northern blot analysis revealed variations in transcript sizes. However, the mechanism for the generation of repeats was not understood. *Manduca* lebocin is highly induced in the fat body. Based on its peptide sequence, *M. sexta* lebocin may go through post transcriptional processing to generate five peptides. Four of the lebocin derived peptides were chemically synthesized and tested for their antibacterial activity. Peptide #1 is active against Gram-positive and Gram-negative bacteria. Peptide # 3 is active against *E. coli* at a high MIC.

Table 1: *M. sexta* lebocin-like protein cDNA clones with variable number of repeats.

No. of repeats	Clone name
3	NC7
5	G1
7	NC6
8	NC9
9	NC1
10	NC5
11	G2
12	NC8
13	NC2, NC3, NC10, NC11
16	C5

Table 2: Sequence analysis of the 5' flanking region of the *M. sexta* lebocin gene

Motif name and consesensus*	Sequence found**	Strand	Matched
NF-κB:GGGRAYYYYY	GGGGACTTTg	-	9/10
	GGGAAaCTTT	+	9/10
GATA:WGATAA	TGATAA	+	6/6
	AGATAA	-	6/6
	AGATAA	-	6/6
ISRE:GGAAANNGAAANN	GGAAATTcAAAC	+	11/12
	aGAAAAAGAAGT	+	11/12

* *R*: A, G; *Y*: C, T; *W*: A, T; *K*: G, T; *M*: A, C. *W*: A, T; *N*: A, C, G, T.
** the nucleotides in lower case do not match with the consensus.
ISRE, interferon-stimulated response elements.

Table 3: MICs of *Manduca* lebocin-related peptides

Bacteria	ATCC	MIC* of Peptides (µg/ml)			
		#1	#2	#3	#4
Gram negative					
E. coli O157:H7	25922	25	-	200	-
S. typhimurium	14028	25	-	-	-
Gram positive					
L. monocytogenes	19115	25	-	-	-
S. aureus	25923	100	-	-	-

*MIC is defined as the lowest peptide concentration that gives no visible growth after overnight incubation in 100% Muller-Hinton broth. Conditions for the broth microdilution assay are specified by Clinical Laboratory Standard Institute. The experiments were repeated at least three times for each bacterial strain.

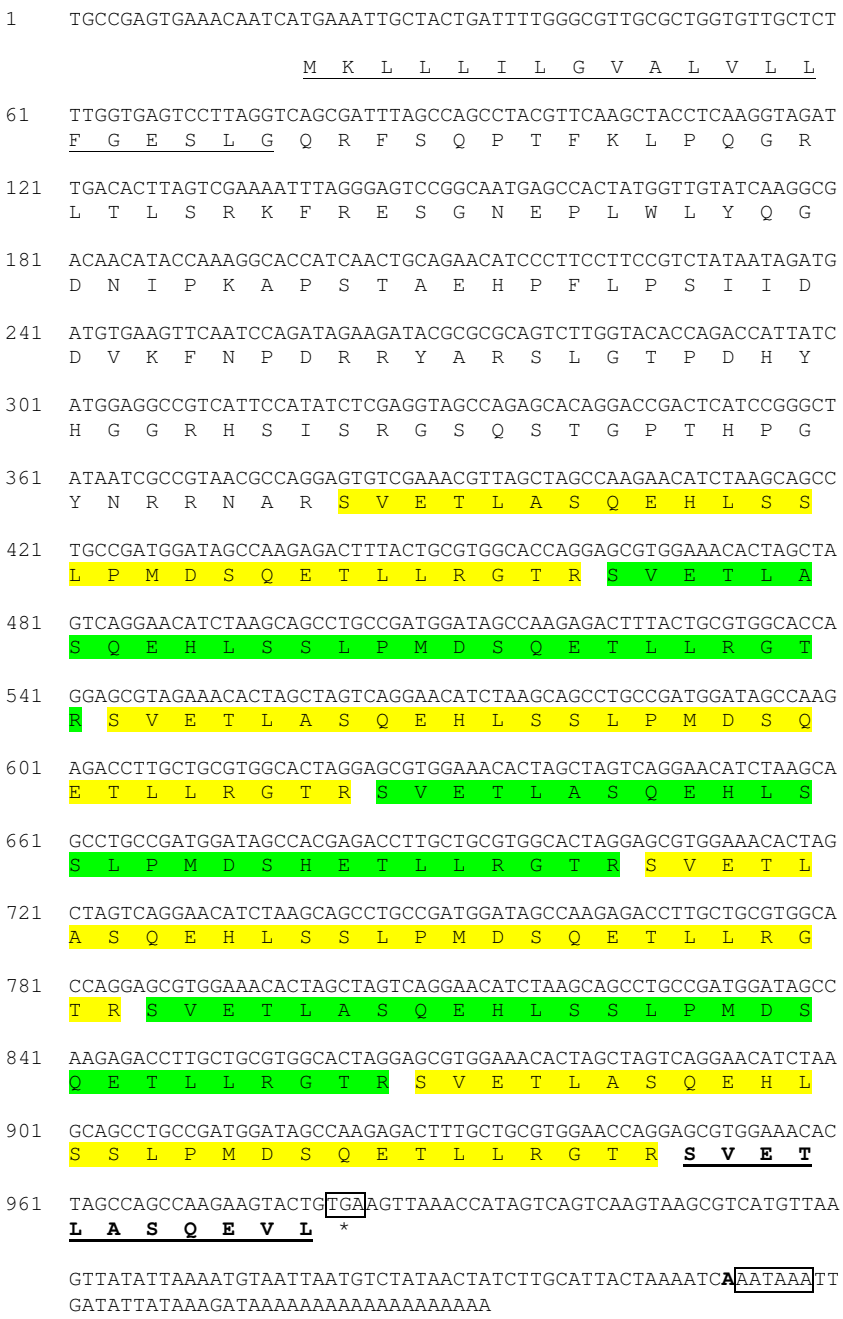

Figure 1: **Nucleotide and deduced aminoacid sequence of *M. sexta* Lebocin-like protein cDNA clone (NC6)** Segment of 27 amino acids which is repeated 7 times is highlighted. Partial repeat of 11 amino acids is bold and underlined. The predicted signal peptide is underlined. Translation stop codon (TGA) and polyadenylation signal (AATAAA) are boxed.

```
Manduca lebocin           42 ESGNEPLWLYQGDNIPKAPSTAEHPFLPSIIDDVKFNPDRRYAR 85
Galleria anionic peptide   1  EAD------K----ER--T--D--I---------LD-N----  42
Bombyx  lebocin 1         43 QA-Q----------V-R-----D--I---K----QLD-N---V  85
Bombyx  lebocin 3         43 QA-Q----------V-R-----D--I---K----QLD-N---V  85
Bombyx  lebocin 4         43 QA-Q-----------R-----D--I---K-----LD-N---V  85
Helicoverpa P1 peptide     1 GADD-----------ER--TA-D--S---V-              31
```

Figure 2: Comparison of *Manduca* lebocin-like protein residues 42-85 with *Galleria* anionic peptide (Cytrynska et al., 2007) *Bombyx* lebocin (Chowdhury et al., 1995; Furukawa et al., 1997) and *Helicoverpa* P1 peptide (Mackintosh et al., 1998) Hyphens denote aminoacids identical to respective residues in the compared peptide.

```
Manduca           -MKLLLILGVALVLLFGESLGQR----FSQPTFK-LPQGRLTLSRKFRESGNEPLWLYQG
Bombyx-1/2        -MYKFLVFSSVLVLFFAQASCQR----FIQPTFRPPPTQRP-IIRTARQAGQEPLWLYQG
Bombyx-3          -MYKFLVFSSVLVLFFAQASCQR----FIQPTFRPPPTQRP-ITRTVRQAGQEPLWLYQG
Bombyx-4          -MYKFLVFSSVLVLFFAQASCQR----FIQPTYRPPPTRRP-IIRTARQAGQEPLWLYQG
Pseudoplusia      MSKYILILCVLSAFLIAEATCQR----IILPTYRPPPAPRRPVIMRARREAEEPLIFHGE
Trichoplusia      MSKYILVLCVLSAFLIAEATCQR----IILPTYRPPPAPRRPVIMRARRELQLQPV-HE
Samia             MSKVIFTLTVVAVLFVAETTCWRRDLPVIYPTYRPRPTVGPVTMRAKRSADDEPLWLFKD
                      :: :     ::  ::   *    .  **::  *          *   :

Manduca           DNIPKAPSTAEHPFLPSIIDDVKFNP-DRRYARSLGTPDHYHGGRHSISRGSQSTGPTHP
Bombyx-1/2        DNVPRAPSTADHPILPSKIDDVQLDP-NRRYVRSVTNPENNE-ASIEHSHHTVDTGLDQP
Bombyx-3          DNVPRAPSTADHPILPSKIDDVQLDP-NRRYVRSVTNPENNE-ASIEHSHHTVDIGLDQP
Bombyx-4          DNIPRAPSTADHPILPSKIDDVKLDP-NRRYVRSVTNPENNE-ASIESSHHTVDIGLDRP
Pseudoplusia      ETYS----------EPGYVEVSEIEH-GERVERSLGTPSRSRGGGGSRPSGSRDTGPTHP
Trichoplusia      ETY-----------PGFEEVSEVDHHGERVERSLGTPSRSRGGGSSRPSSSLDTGPTHP
Samia             NNEPRAPSTGDHPVLPSIIDDIKLNP-NTRYARSLSTPNKYHGGSHTISKSSQSTGPTHP
                  :              *   :  : :    *  **:  *            :   *  :*

Manduca           GYNRRNARSVETLASQEHLSSLPMDSQETLLRGTR-------------------------
Bombyx-1/2        IESHRNTRDLRFLYPRGKLPVPTPPPFNPKPIYIDMGNRYRRHASDDQEELRQYNEHFLI
Bombyx-3          IESHRNTRDLRFLYPRGKLPVPTLPPFNPKPIYIDMGNRYRRHASEDQEELRQYNEHFLI
Bombyx-4          IESHRNTRDLRFWNPREKLPLPTLPPFNPKPIYIDMGNRYRRHASDDQEELRHHNEHFLI
Pseudoplusia      GYNRRNARSLPDFKLPGMKYPIPATTPPFVPKRSRFPIYA--------------------
Trichoplusia      GYNRRNARSLPSLRLPGRNFPIPPTTPPFVPKPRRFPIYV--------------------
Samia             GYNRRHVRSFDSRSSKHHGGSPSTSSGSKNTGQTHPG-YNRRNA----------------
                     :*: *

Manduca           -------
Bombyx-1/2        PRDIFQE
Bombyx-3          PRDIFQE
Bombyx-4          PRDILQD
Pseudoplusia      -------
Trichoplusia      -------
Samia             -------
```

Figure 3: CLUSTAL W (1.81) multiple sequence comparison of lebocin like proteins in lepidopteran insects. *Manduca* lebocin-like protein is compared with lebocins 1, 3 , 4 from *Bombyx* (Chowdhury et al., 1995; Furukawa et al., 1997) Lebocin-like proteins from *Pseudoplusia* (AY533675), *Trichoplusia* (Liu et al., 2000) and *Samia* (Bao et al., 2005) Identical positions were indicated with *. Residues identical and are exclusively found in *Manduca, Pseudoplusia, Trichoplusia* and *Samia* are underlined.

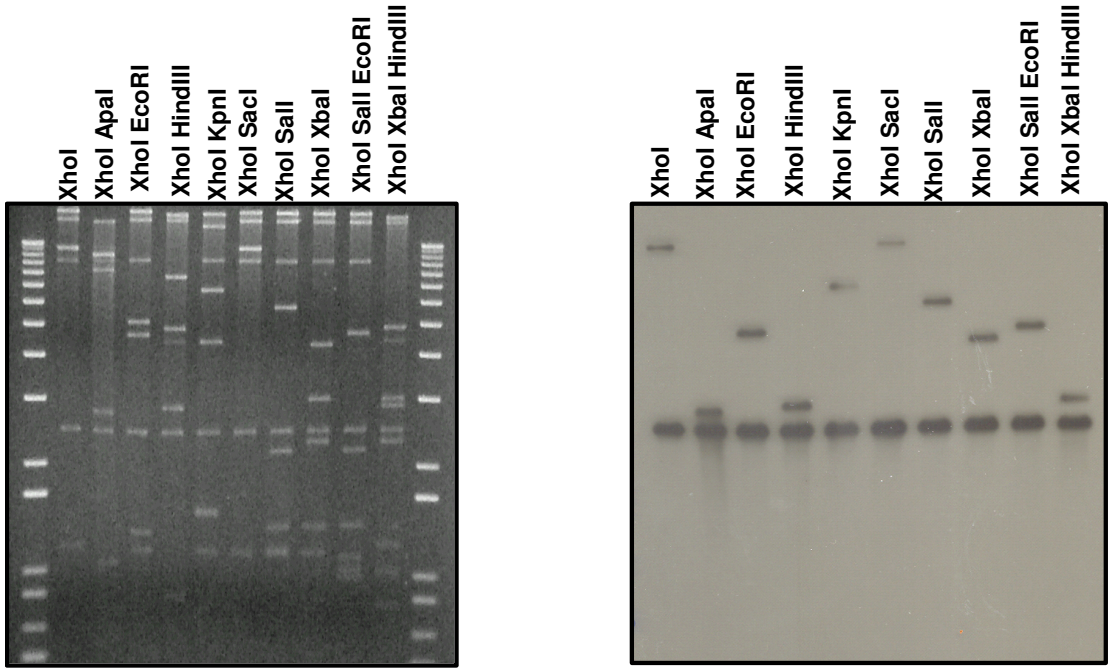

Figure 4: Southern blot analysis of *M. sexta* lebocin genomic clone. (A) Gel picture. (B) X-ray film. Samples of DNA (genomic clone, L45) 10µg each were digested with *Xho*I, *Apa*I, *Eco*RI, *Hind*III, *Kpn*I, *Sac*I, *Sal*I, and *Xba*I. After separation by agarose gel electrophorosis and transfer to a nylon membrane, the DNA fragments were hybridized with DIG-labelled lebocin cDNA.

Figure 5: Complete nucleotide sequence of *M. sexta* lebocin gene: Single exon sequence is underlined. Amino acid sequences are listed below translated exon using the one letter code under the middle nucleotide of each codon. GATA motifs are boxed. NF-κB and ISRE sites were highlighted. TATA and Poly adenylation signal are bold and underlined.

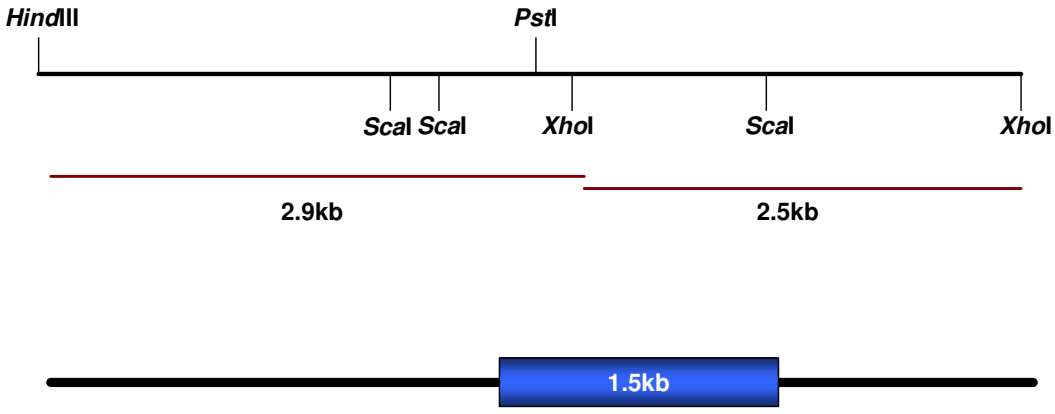

Figure 6: Structure of *M. sexta* lebocin gene and subcloning strategy. Upper panel: restriction map of the lebocin genomic insert in a positive λ bacteriophage. Horizontal bars mark the lebocin genomic fragments for subcloning. Lower panel exon-intron organization of the lebocin gene.

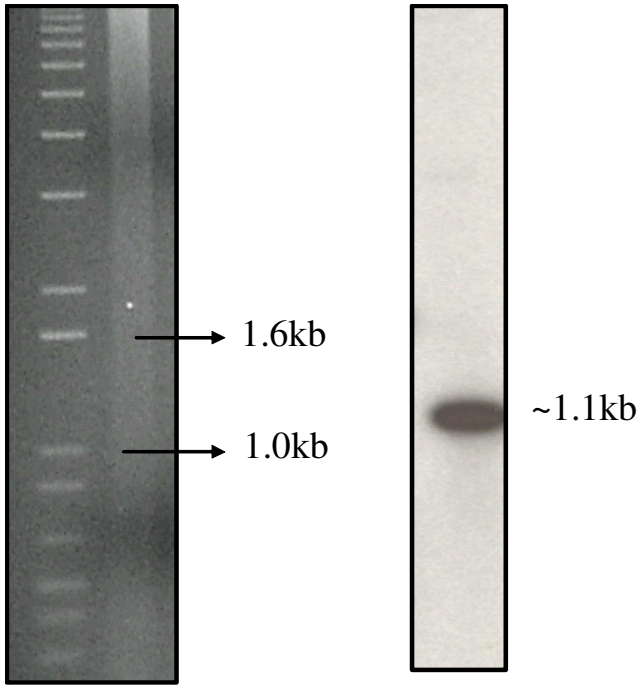

Figure 7: Southern blot analysis of *M. sexta* genomic DNA. (A) Gel picture. (B) X-ray film. *M. sexta* DNA (10µg) was digested with *Xho*I and *Sca*I. After separation by agarose gel electrophorosis and transfer to a nylon membrane, the DNA fragments were hybridized with ^{32}P-labelled lebocin cDNA initially digested with *Xho*I and *Sca*I (repetitive region). The size of the fragment with which the probe hybridized is marked on the right.

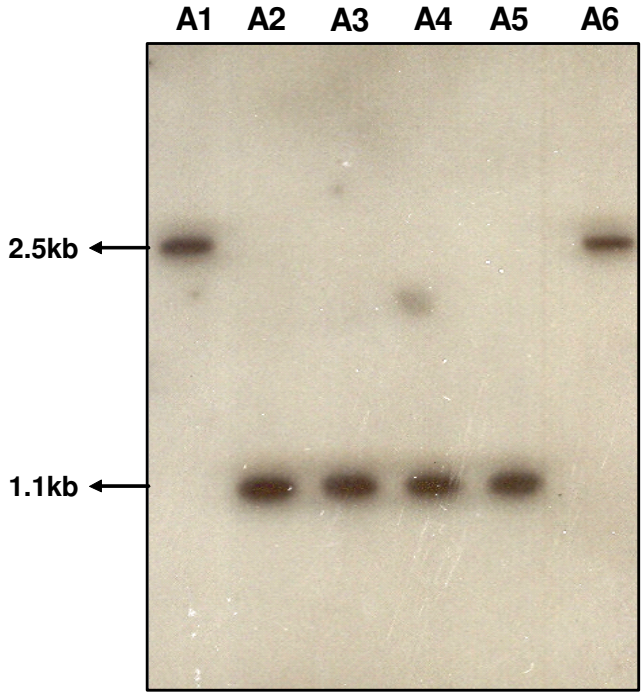

Figure 8: Genomic southern blot of six different insects of *M. sexta*. Samples of *M. sexta* DNA (10µg) was digested with *Xho*I and *Sca*I. After separation by agarose gel electrophorosis and transfer to a nylon membrane, the DNA fragments were hybridized with ^{32}P-labelled lebocin cDNA initially digested with *Xho*I and *Sca*I (repetitive region). The size of the fragment with which the probe hybridized is marked on the left.

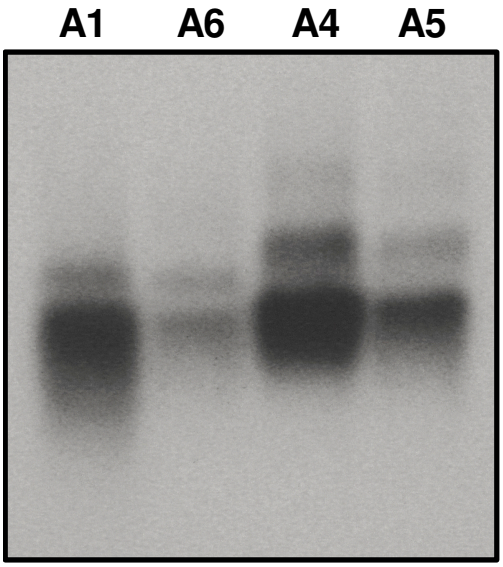

Figure 9: Northern blot of four different insects of *M. sexta*. Fat body total RNA (10μg) was separated by agarose gel electrophorosis containing formaldehyde and transfered to a nylon membrane, the RNA fragments were hybridized with ^{32}P-labelled Lebocin cDNA initially digested with *Xho*I and *Sca*I (repetitive region).

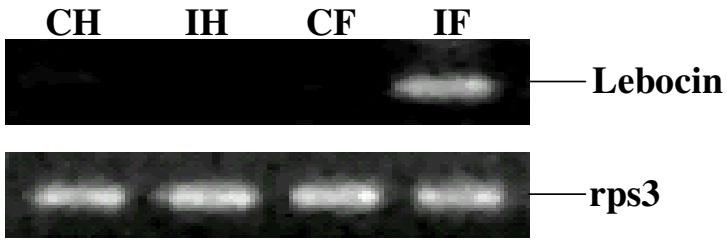

Figure 10: Inducibitility of *M. sexta* lebocin m-RNA in fat body and hemocytes upon bacterial infection. CH and CF: hemocytes and fat body from the naïve larvae; IH and IF: hemocytes and fat body collected from the larvae at 24h after injection of *E. coli. M. sexta* ribosomal protein S3 (rpS3) transcripts were normalized for the analysis.

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APPENDIX

Antibacterial activity, and expression profile of *Manduca sexta* moricin

Introduction:

In response to wounding or infection, insects produce a battery of antimicrobial peptides and other defense molecules to kill the invading pathogens. Exclusively found in lepidopteran insects so far, moricin-like AMPs are active against both Gram-positive and Gram-negative bacteria (Hara and Yamakawa, 1995). They affect structural integrity of bacterial plasma membrane. Moricin synthesis is induced by microbial cell wall components (Furukawa et al., 1999). To determine the role of AMPs in humoral responses of the tobacco hornworm, we chemically synthesized *M. sexta* moricin, a 42-residue peptide (GKIPVKAIKQAGKVIGKGLRAINIAGTTHDVVSFFRPKKKKH, 4,539 Da) and tested its biological activity. The inducibility, tissue-specificity, and developmental profile of the moricin gene expression were also examined.

The compound exhibited potent antimicrobial activities against a broad spectrum of Gram-positive and negative bacteria with a minimum inhibitory concentration of 1.4 μM. The mRNA levels of *M. sexta* moricin increased substantially in fat body and hemocytes after the larvae were challenged with bacterial cells.

Antibacterial activity and spectrum

We examined the antimicrobial activity of *M. sexta* moricin against four Gram-negative and four Gram-positive bacterial strains using broth micro dilution assay (NCLS, 2003) and found that the synthetic peptide killed all these bacteria at low micromolar range (Table 1). Except for *S. aureus* BAA-39, which required 12.5 μg/ml of the peptide for complete growth inhibition, the moricin was effective at 6.25 μg/ml or 1.4 μM against all the strains tested, including multidrug-resistant *S. typhimurium* DT104 and methicillin-resistant *S. aureus* ATCC 43300.

Tissue specificity, immune inducibility and developmental regulation of moricin expression

M. sexta moricin transcripts were present at low levels in nerve tissue, salivary gland, Malpighian tubule, trachea, midgut, fat body, integument or muscle from day 3, fifth instar insects (Fig. 1). A moderate level of moricin mRNA was observed in the hemocytes from the naïve larvae. After an immune challenge, the transcripts became more abundant in hemocytes and fat body, consistent with the observation that moricin expression was up-regulated by injecting B. mori spätzle-1 into the M. sexta larvae (Wang et al., 2007). The transcription of moricin gene may also be controlled by developmental signals: we detected moderate levels of its mRNA in fat body from the 4th instar larvae (day 0), 5th instar larvae (days 3 and 6), wandering larvae (day 6), and adults in the absence of infection.

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Table 1 MICs of *Manduca sexta* moricin [a]

bacterial species	ATCC No.	moricin (μg/ml)
Gram-negative		
E. coli O157:H7	25922	6.25
S. typhimurium	14028	6.25
K. pneumoniae	13883	6.25
S. typhimurium DT104	700408	6.25
Gram-positive		
L. monocytogenes	19115	6.25
S. aureus	25923	6.25
S. aureus (MRSA) [b]	43300	6.25
S. aureus (MRSA) [b]	BAA-39	12.5

[a] MIC is defined as the lowest peptide concentration that gives no visible growth after overnight incubation in 100% Muller-Hinton broth. Conditions for the broth microdilution assay are specified by Clinical Laboratory Standard Institute. The experiments were repeated at least three times for each bacterial strain.

[b] Methicillin-resistant *S. aureus.*

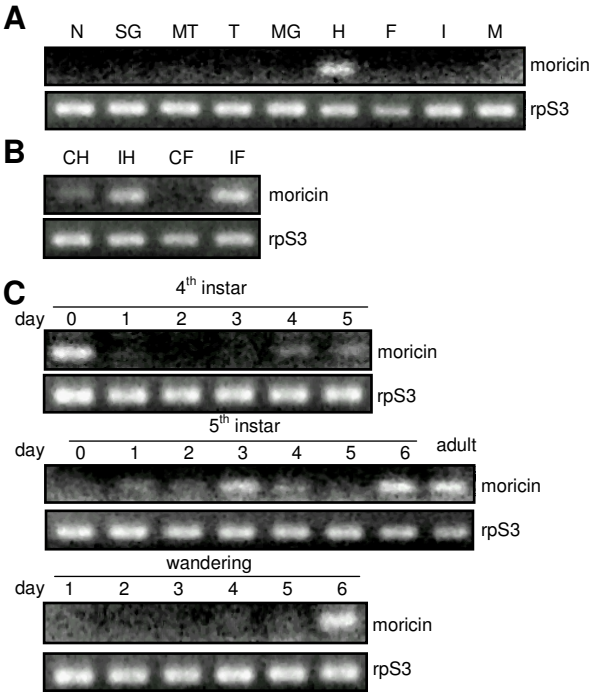

Fig. 1. Profiling of M. sexta moricin transcript levels by reverse transcription-polymerase chain reaction. (A) expression in different tissues from day 3, 5th instar naïve larvae. N, nerve tissue; SG, salivary gland; MT, Malpighian tubule; T, trachea; MG, midgut; H, hemocytes, F, fat body; I, integument; M, muscle. (B) induced transcription in hemocytes (H) and fat body (F) from naïve (C) and bacteria-injected (I) larvae. (C) mRNA level changes in fat body from insects at different developmental stages.

VITA

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Scope and Method of Study: Antimicrobial peptides (AMPs) are a critical component of the natural defense system in various organisms. Over 200 AMPs have been identified in arthropods. Most of them are less than 10 kDa, hydrophobic, membrane active, and have net positive charges. At least five different types of AMPs are identified in tobacco hornworm, *M. sexta* which include attacins, cecropins, moricin, gloverin and lebocin. Here we report screening and isolation of *Manduca* lebocin cDNA and Genomic clones and also the antibacterial activity of *Manduca* lebocin derived peptides.

Findings and Conclusions: Thirteen cDNA clones were isolated. All the clones contained a constant 5'-terminus and varied in length at the 3' end. In each clone a 81 bp fragment was repeated multiple times followed by a 33 bp partial fragment. This repetition varied in each clone from 3 to 16 times. In order to identify the mechanism for the generation of variable numbers of repeats at the 3' end of the cDNA clones, M. sexta genomic library was screened for lebocin gene. The lebocin gene is intron-less and single copy. Northern blot analysis revealed variations in transcript sizes. However, the mechanism for the generation of repeats was not understood. Manduca lebocin is highly induced in the fat body. Based on its peptide sequence, M. sexta lebocin may go through post transcriptional processing to generate five peptides. Four of the lebocin derived peptides were chemically synthesized and tested for their antibacterial activity. Peptide #1 is active against Gram-positive and Gram-negative bacteria. Peptide # 3 is active against E. coli at a high MIC.

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