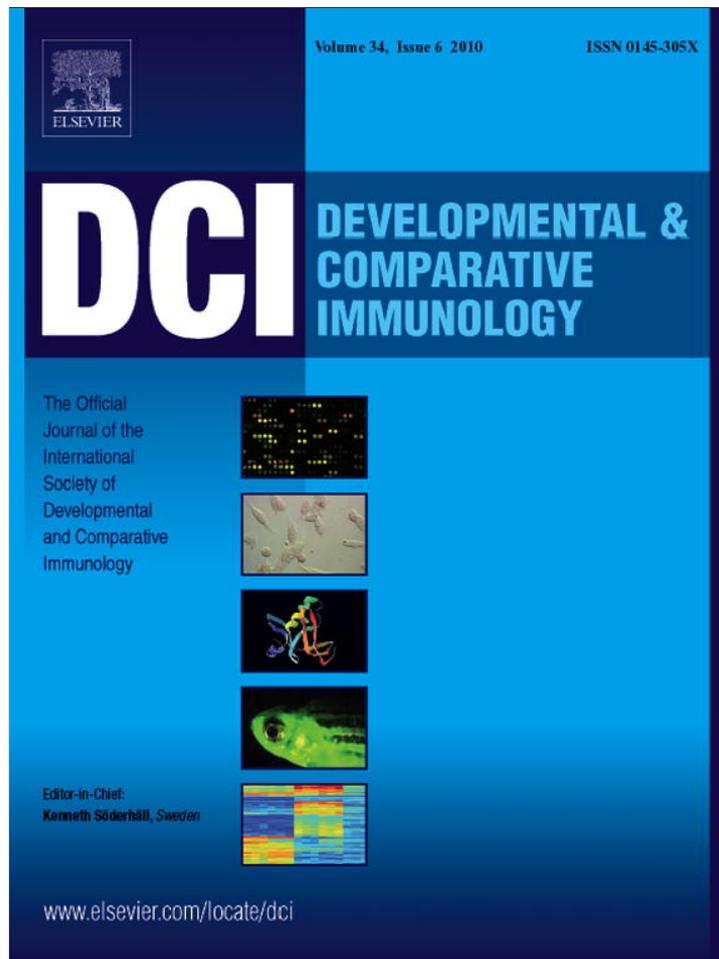


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Functional analysis of four processing products from multiple precursors encoded by a lebocin-related gene from *Manduca sexta*

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ABSTRACT

Antimicrobial peptides (AMPs) are a crucial component of the natural immune system in insects. Five types of AMPs have been identified in the tobacco hornworm *Manduca sexta*, including attacin, cecropin, moricin, gloverin, and lebocin. Here we report the isolation of lebocin-related cDNA clones and antibacterial activity of their processed protein products. The 17 cDNA sequences are composed of a constant 5' end and a variable 3' region containing 3–16 copies of an 81-nucleotide repeat. The sequence of the corresponding gene isolated from a *M. sexta* genomic library and Southern blotting results indicated that the gene lacks introns and exists as a single copy in the genome. The genomic sequence contained 13 complete and one partial copy of the 81-nucleotide repeat. Northern blot analysis revealed multiple transcripts with major size differences. The mRNA level of *M. sexta* lebocin increased substantially in fat body after larvae had been injected with bacteria. The RXXR motifs in the protein sequences led us to postulate that the precursors are processed by an intracellular convertase to form four bioactive peptides. To test this hypothesis, we chemically synthesized the peptides and examined their antibacterial activity. Peptide 1 killed Gram-positive and Gram-negative bacteria. Peptide 2, similar in sequence to a *Galleria mellonella* AMP, did not affect the bacterial growth. Peptide 3 was inactive but peptide 3 with an extra Arg at the carboxyl terminus was active against *Escherichia coli* at a high minimum inhibitory concentration. Peptide 4, encoded by the 81-bp repeat, was inactive in the antibacterial tests. The hypothesis that posttranslational processing of the precursor proteins produces multiple bioactive peptides for defense purposes was validated by identification of peptides 1, 2, and 3 from larval hemolymph via liquid chromatography and tandem mass spectrometry. Comparison with the orthologs from other lepidopteran insects indicates that the same mechanism may be used to generate several functional products from a single precursor.

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1. Introduction

Antimicrobial peptides (AMPs) participate in innate immunity of various organisms ranging from prokaryotes to plants, invertebrates, and vertebrates [1,2]. Most of them are less than 5 kDa, hydrophobic, membrane-active, and carry positive net charge at physiological pH. These peptides are either absent or present at low constitutive levels in naïve insects. Upon microbial infection, association of host recognition molecules and microorganisms triggers extracellular serine proteases to activate a spätzle precursor via limited proteolysis [3]. Spätzle then binds to

the Toll receptor to initiate an intracellular pathway that relays the signal into nucleus, where transcription factors of the Rel family induce the AMP gene expression [4]. Identification of orthologous genes in the *Anopheles gambiae*, *Aedes aegypti*, *Apis mellifera*, *Tribolium castaneum*, and *Bombyx mori* genomes suggests that similar signaling pathways exist in other insects to induce the production of defense proteins by fat body and hemocytes [5–9]. Fat body, a tissue analogous to liver, synthesizes AMPs and secretes these heat-stable compounds into the plasma to kill the invading microbes.

Insect AMPs can be categorized into the following groups: α -helical peptides (e.g. cecropin and moricin), disulfide-stabilized peptides (e.g. defensin and drosomycin), proline-rich peptides (e.g. lebocin and drosocin), glycine-rich peptides (e.g. gloverin and dipterin), and others [2]. In the tobacco hornworm *Manduca sexta*, at least five types of AMPs have been identified, including attacin, cecropin, moricin, gloverin, and lebocin [10–13]. As a step

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towards understanding the role of AMPs in humoral immune responses of *M. sexta*, we have characterized a lebecin-like protein, its processing products, and corresponding gene in this work.

Lebecins are proline-rich AMPs first purified from the silkworm, *B. mori* [14]. These 32-residue peptides are glycosylated at Thr¹⁵ and this modification is important for the antimicrobial activity against Gram-negative bacteria. cDNA and gene cloning indicates that the active peptide is located near the carboxyl terminus, after a signal peptide and a 102-residue pro-segment [15,16]. Lebecins increase the permeability of liposomes at a low ionic strength and have weak antibacterial activities under physiological conditions. They seem to function as synergists by reducing the minimum inhibitory concentration of cecropin D [17]. Lebecin cDNAs have been isolated from *Trichoplusia ni* [18] and *Pseudoplusia includens* [19]. In lebecin homologs of *Samia cynthia* [20], the pro-segment aligns well with the *B. mori* sequences, but the part corresponding to the mature lebecin differs significantly. Two peptides, purified from hemolymph of *Helicoverpa armigera* and *Galleria mellonella*, are similar to a region in the pro-segment of *B. mori* lebecins [21,22]. The 42-residue anionic peptide-1 from the greater wax moth *G. mellonella* is active against *Micrococcus luteus*, *Listeria monocytogenes* and filamentous fungi, but neither of these peptides inhibits the growth of Gram-negative bacteria (e.g. *Escherichia coli*). It is unclear how these peptides are derived from their protein precursors (pro-lebecins). Here we report the cDNA and genomic cloning of lebecin-related proteins from *M. sexta*, which suggests a conserved mechanism in Lepidoptera to generate structural/functional diversity in products derived from pro-lebecins. This mechanism is validated by identification in hemolymph of three processing products by mass spectrometry. For simplicity, we use “lebecin” in parts of the paper to describe the entire gene, cDNA, and protein that are related but not the same as the mature lebecin peptide.

2. Materials and methods

2.1. Insect rearing and bacterial challenge

M. sexta (eggs purchased from Carolina Biological Supply) were reared on an artificial diet [23]. Day 2, fifth instar larvae were injected with formalin-killed *E. coli* (2×10^8 cells/larva). Alternatively, day 2, fifth instar larvae or day 1, male adults were injected with 100 μ g *M. luteus* (Sigma) or with sterile water as a control. At various time points after injection, hemocytes and fat body were collected for RNA preparation. Muscle tissues or gut-removed carcasses were used for genomic DNA isolation.

2.2. Screening for full-length lebecin cDNA clones

A 280 bp *M. sexta* lebecin cDNA fragment [11] was labeled with [α -³²P]-dCTP using Multiprime DNA Labeling System (GE Healthcare Life Science). A *M. sexta* induced fat body cDNA library [24] was screened according to Sambrook and Russell [25]. Positive plaques were purified to homogeneity via secondary and tertiary screening. Plasmids, *in vivo* excised from the positive bacteriophages, were sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Sequences were assembled using MacVector Sequence Analysis Software (Oxford Molecular Ltd.).

2.3. Multiple sequence alignment and phylogenetic analysis

Based on BLAST search of GenBank (<http://www.ncbi.nlm.nih.gov/>) and ButterflyBase (<http://butterflybase.ice.mpg.de/>) using *M.*

sexta lebecin as query, homologous protein sequences were retrieved and compared using ClustalX 1.83 (<ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/>) [26]. A Blosum 30 matrix [27], with a gap penalty of 10 and an extension gap penalty of 0.1 were selected for multiple sequence alignment and unrooted phylogenetic tree was constructed based on neighbor-joining algorithm. Treeview (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>) [28] was used to display phylogram.

2.4. Genomic library screening, subcloning, and DNA sequencing

The insert from a full-length lebecin cDNA clone (NC2), obtained after digestion with EcoRI and XhoI, was labeled with [α -³²P]-dCTP and used as a probe to screen a *M. sexta* genomic library in λ GEM11, kindly provided by Dr. Yucheng Zhu at the Southern Insect Management Research Unit (USDA ARS). Following plaque purification and amplification, phage DNA was isolated using Wizard Lambda Preps DNA Purification System (Promega). To determine its restriction map, the DNA was digested with one, two or three of the enzymes (XhoI, ApaI, EcoRI, HindIII, KpnI, SacI, Sall and XbaI) and separated by 0.8% agarose gel electrophoresis. After transferring onto a GenScreen Plus membrane (NEN Life Science Products), the DNA fragments were hybridized with the full-length lebecin cDNA, labeled by DIG-High Prime DNA Labeling Detection Kit (Roche Applied Science). Fragments of the lebecin gene were subcloned, sequenced, and assembled as described above.

2.5. Genomic Southern blot analysis

M. sexta genomic DNA was extracted from muscles of a single fifth instar larva using a DNeasy Blood and Tissue Kit (Qiagen). The genomic DNA was also isolated from two larval carcasses according to Bradfield and Wyatt [29]. About 10 μ g of DNA was digested with XhoI and ScaI at 37 °C overnight and separated by 1% agarose gel electrophoresis. After capillary transfer onto a nitrocellulose membrane, hybridization was carried out using ³²P-labeled XhoI-ScaI fragment of the lebecin cDNA.

2.6. Northern blot and RT-PCR analysis

Fat body RNA samples were prepared from the individual insects using Micro-to-Midi Total RNA Purification System (Invitrogen) or the method described by Chinzei et al. [30]. Denatured total RNA samples were separated on a 1% agarose gel containing 2.2 M formaldehyde. After electrophoresis, RNA was transferred to a membrane and hybridized with ³²P-labeled XhoI-ScaI fragment of the cDNA. Similarly, the time course of lebecin expression in larvae and adults were examined. For RT-PCR analysis, hemocyte and fat body total RNA samples were prepared from naïve and injected larvae. In each reaction, RNA (2–4 μ g), oligo(dT) (0.5 μ g) and dNTPs (1 μ l, 10 mM each) were mixed with DEPC-treated H₂O in a final volume of 12 μ l and denatured at 65 °C for 5 min. M-MLV reverse transcriptase (1 μ l, 200 U, Invitrogen), 5 \times buffer (4 μ l), 0.1 M dithiothreitol (2 μ l), and RNase OUT (1 μ l, 40 U) were added to the RNA for cDNA synthesis at 37 °C for 50 min. The *M. sexta* ribosomal protein S3 mRNA was used as an internal control to normalize the cDNA samples in a PCR using primers j501 (5'-GCCGTTCTTCCCTGTT-3') and j504 (5'-CGCGAGTTGACTTCGGT-3'). The lebecin cDNA fragment was amplified using forward (5'-CTGATTTTGGCGTTGCGCTG-3') and reverse (5'-GCGGTATCTTCTATCTGGA-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 lebecin mRNA in the normalized samples were determined by 1.5% agarose gel electrophoresis.

2.7. Peptide synthesis

Eight lebecin-related peptides (~10 mg each) (LP1, QRFSQPTFKLPQGRLLTSRKF; LP1A, QRFSQPTFKLPQGRLLTSRKF; LP2, ESGNEPLWLYQGDNIKAPSTAEHPFLPSIIDVVKFNPDRRYAR; LP2A, ESGNEPLWLYQGDNIKAPSTAEHPFLPSIIDVVKFNPDRRYA; LP3, SLGTPDHYHGGRHSISIRGSQSTGPTHGYNRRNAR; LP3A, SLGTPDHYHGGRHSISIRGSQSTGPTHGYNRRRNA; LP4, SVETLASQEHLSSLPMDSQETLLRGTR; LP4A, SVETLASQEHLSSLPMDSQETLLRGT) were prepared by stepwise solid-phase synthesis using Fmoc-amino acid derivatives (Bio-Synthesis, Inc.). Following deprotection and cleavage, the peptides were purified by reversed-phase HPLC to >95% purity and analyzed by mass spectrometry.

2.8. Antibacterial activity assay

The synthetic peptides were separately tested against pathogenic strains of *Salmonella typhimurium*, *E. coli* O157:H7, *Klebsiella pneumoniae*, *S. typhimurium* DT104, *L. monocytogenes*, *Staphylococcus aureus*, and two strains of methicillin-resistant *S. aureus*, kindly provided by Dr. Guolong Zhang in the Department of Animal Science at Oklahoma State University. The minimum inhibitory concentrations (MICs) were determined in a broth micro-dilution assay [31]. 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 4 °C and washing with 10 mM Tris-HCl (pH 7.4), the cells were suspended in 5% Trypticase Soy Broth (5 × 10⁵ 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 37 °C 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 the *M. sexta* lebecin-related peptides against the bacterial strains.

2.9. Identification of lebecin-related peptides in plasma by mass spectrometry

Synthetic peptides and biological samples were characterized using a hybrid LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled to a New Objectives PV-550 nanoelectrospray ion source and an Eksigent NanoLC-2D chromatography system. MS and MS/MS spectra of LP1A through LP4A were first collected by infusion using 1-μm infusion tips (New Objective). These peptides were further analyzed by trapping on a 2.5 cm ProteoPrepII pre-column (New Objective), followed by analytical separation on a 75 μm ID fused silica column packed in house with 10 cm of Magic C18 AQ, terminated with an integral fused silica emitter pulled in house. The peptides were eluted using a 3–70% acetonitrile (AcCN)/0.1% formic acid gradient performed over 33 min at a flow rate of 300 nl/min. During each one-second full-range FT-MS scan (nominal resolution of 60,000 FWHM, 300–2000 *m/z*), the three most intense ions were analyzed via MS/MS in the linear ion trap. MS/MS spectra were collected using a trigger threshold of 1000 counts and monoisotopic precursor selection. Parent ions were rejected for MS/MS if their charge states were unassignable, if they were previously identified as contaminants on blank gradient runs, or if they had already been twice selected for MS/MS (data dependent acquisition using a dynamic exclusion for 150% of the typical chromatographic peak width). Column performance was monitored using the peptide standards, and via blank injections between samples to assay for contamination. A 100 μl aliquot of pooled hemolymph from bacteria-injected *M. sexta* larvae was heated at 95 °C for 5 min and centrifuged at 4 °C for 10 min at 12,000 × g. Polypeptides in the supernatant (40 μl)

was cleaned up on a PepClean™ C-18 Spin Column (Pierce) according to the manufacturer's instructions. Peptide samples eluted with 5–40% AcCN and 40–70% AcCN were lyophilized, reconstituted in 0.1% formic acid, and combined for LC-MS/MS analysis as described above. The resultant LC-MS/MS files were inspected manually, using the synthetic peptide's retention time, *m/z*, charge states, isotopic distributions, and characteristic MS/MS decay spectra as identifying criteria. For use as a standard to show that the natural LPA1 has a pyroGlu at the amino terminus, the first Gln in the synthetic peptide was partly converted to pyroGlu by incubating LPA1 (1 μl, 1 mg/ml) with 50 μl, 100 mM NH₄HCO₃ (pH 8.2) overnight at 37 °C [32]. The peptide mixture was enriched on a ZipTipC18 (Millipore) and analyzed by LC and tandem mass spectrometry as described above.

3. Results

During the cDNA cloning of *M. sexta* hemolin [33], we isolated a false positive clone (pP4-7) which encodes a region 48% identical to *B. mori* lebecin in the amino-terminal pro-segment followed by 12 copies of a 27-residue repeated amino acid sequence. In a separate project to identify bacteria-induced genes by suppression subtractive hybridization and molecular cloning, Zhu et al. [11] identified 5 cDNA fragments (BI262584, BI262626, BI262686, BI262688, and BI262708) encoding sequences similar to pP4-7 and the proregion of silkworm lebecins. Intrigued by the unique structure of pP4-7 and similarity of the PCR-derived clones to lebecin, we screened an induced fat body cDNA library using the 5' unrepeated region as a probe and isolated fifteen positive clones.

Sequencing from both ends of the positive clones indicated that all of the cDNAs contained a 5' region identical to that found in pP4-7, which includes a 5' untranslated region (5'-UTR) and a 363-nucleotide sequence encoding a 20-residue signal peptide and a 101-residue segment similar to the proregion of *B. mori* lebecin (Fig. 1A). Following this region, an 81-nucleotide sequence was repeated 3–16 times in different clones. Four clones (NC2, NC3, NC10 and NC11) contain 13 repeats and the other 12 (NC7, G6, G1, NC6, NC9, NC13, NC1, NC5, G2, NC8, pP4-7 and C5) have 3, 3, 5, 7, 8, 8, 9, 10, 11, 12, 12 and 16 repeats, respectively. We have identified in DQ115324 an open reading frame of the pro-lebecin with the constant region and 6.3 repeats, out-of-frame fused with *M. sexta* HP23 cDNA as an artifact of library construction [34]. The repeats in these seventeen clones are nearly identical at the nucleotide level and they encode peptide sequences that are highly conserved. Following these complete repeats is a constant region consisting of a 33 bp partial repeat, a stop codon, and a 3'-UTR.

Amino acid sequence comparison shows that the 101-residue pro-segment in *M. sexta* lebecin is similar to its counterpart in *T. ni* (45%), *P. includens* (49%), *Papilio dardanus* (68%), *Spodoptera frugiperda* (66% for 1, 64% for 2, 70% for 3), *B. mori* (64%), *Heliopsis virescens* (72%), *S. cynthia* (78%), *Antheraea mylitta* (67% for 1 and 75% for 2), *Antheraea pernyi* (70% for 1 and 73% for 2), *Lonomia obliqua* (81%) (Fig. 2). In the remaining carboxyl-terminal region, the *T. ni* and *P. includens* sequences are 70% identical to each other, the identity between *A. mylitta*-1 and *A. pernyi*-1 is 76%, and the *S. cynthia*, *A. mylitta*-2 and *A. pernyi*-2 sequences are ~80% identical. However, this high similarity in the carboxyl terminus is not conserved in lepidopterans from different families. It appears that rapid evolution has given rise to major variations in length and sequence of the *M. sexta* repeat, *B. mori* mature lebecin, and their counterparts in the above three groups. In fact, this region in *S. cynthia*, *A. mylitta*-2 and *A. pernyi*-2 are Ser/Thr, Gly, His-rich (instead of Pro-rich), whereas the 27-residue repeat in *M. sexta* lebecin contains eight Ser or Thr, five Leu, and only one Pro.

Three mechanisms that might account for variable numbers of the 81-nucleotide repeats in *M. sexta* sequences are: (1) multiple

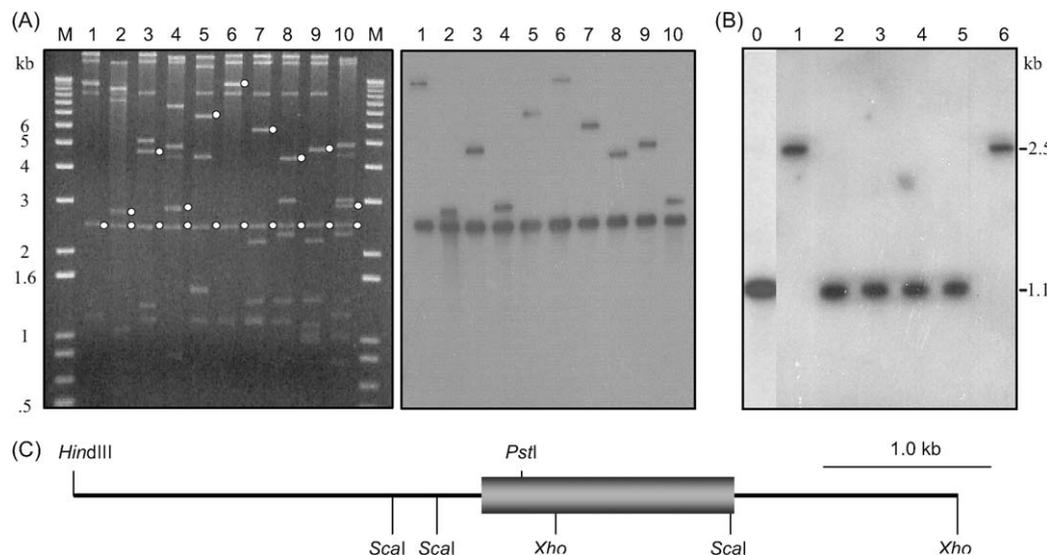


Fig. 3. Subcloning strategy (A), genomic Southern blot (B), and structure (C) of the *M. sexta* lebecin gene. (A) The bacteriophage DNA isolated from the positive λ clone was digested by XhoI (lane 1), XhoI and ApaI (lane 2), XhoI and EcoRI (lane 3), XhoI and HindIII (lane 4), XhoI and KpnI (lane 5), XhoI and SacI (lane 6), XhoI and Sall (lane 7), XhoI and XbaI (lane 8), XhoI, EcoRI and Sall (lane 9), or XhoI, HindIII and XbaI (lane 10). The reaction mixtures and DNA size markers were subjected to gel electrophoresis, capillary transfer, and hybridization using the full-length lebecin cDNA as a probe. Left panel, stained gel with hybridizing fragments marked with white dots; right panel, chemiluminescence detection of the hybridization signals. (B) Following XhoI–Scal double digestion, genomic DNA (10 μ g) from multiple (lane 0) or single (lanes 1–6: A1 to A6) insects was separated by gel electrophoresis, transferred onto a membrane, and hybridized with 32 P-labeled XhoI–Scal fragment of lebecin cDNA corresponding to the repetitive region. Positions and sizes of the DNA markers are indicated. (C) Restriction map of the subcloned HindIII–XhoI and XhoI–XhoI fragments with the single exon of *M. sexta* lebecin gene shown as a box.

major difference between the gene and cDNA sequences. For instance, clone NC6 contains 7.4 (instead of 13.4) repeats (Fig. 1A).

Computer analysis of the 5' flanking sequence allows us to identify potential regulatory elements in the lebecin gene. Two imperfect NF- κ B motifs (9/10 match) [35] are present at nucleotides 634 (minus strand) and 764 (plus strand). One GATA box is located on the plus strand at position 1308 and two are on the minus strand at positions 1523 and 2305. There is an interferon-stimulated response element at nucleotide 115, matching 12 of the 13 positions in the consensus motif. The CCAAT sequence at position 1406 closely resembles the consensus (TCAGT) which typically resides within 10 nucleotides before or after the transcription initiation site in arthropod genes [36]. At 38 bp upstream of CCAAT, there is a TATAAT sequence, reminiscent of the TATA or Goldberg–Hogness box.

In Southern blot analysis of the genomic DNA isolated from multiple insects (Fig. 3B, lane 0), digestion with XhoI–Scal released a 1.1 kb fragment hybridizing with a cDNA probe that only recognized the repeats. Since no other hybridization signal was found, this result suggests a single lebecin gene in the genome, which contains an XhoI–Scal fragment identical in size to the genomic clone (Fig. 3C) and to the cDNAs (NC2, NC3, NC10, and NC11) with 13.4 repeats. We repeated the experiment using DNA samples isolated from six different insects randomly chosen from the colony. Four of them (A2–A5) showed the 1.1 kb band that corresponded to 13.4 repeats, but the other two (A1 and A6) exhibited a single band at 2.5 kb (Fig. 3B, lanes 1–6). Further analyses indicated that the 2.5 kb band resulted from XhoI cleavage (Fig. 3C), rather than XhoI–Scal double digestion. Sequencing of the PCR products of genomic DNA from A1 and A6 revealed 13.4 repeats and a point mutation that abolished the Scal site (data not shown).

Northern blot analysis of fat body RNA from insects A1, A4, A5, and A6 showed three bands at 1.9, 2.5 and 4.2 kb positions (Fig. 4A). The 1.9 kb band represented the major transcript, consistent in size with the cDNAs containing 13.4 repeats. The other two bands, due to their large sizes, may result from transcription at an alternative initiation or termination site rather

than containing more than 20 repeats. There was no significant variation in the transcript sizes among these four insects. Neither was there a ladder of mRNA with different numbers of repeats, although we cannot rule out that smaller transcripts were present at a low level. We carried out RT-PCR to test whether lebecin transcription occurred in fat body and hemocytes in response to the bacterial injection. The lebecin mRNA in hemocytes or fat body of naïve larvae was below the detection limit, and a PCR product at the expected size was amplified from fat body but not hemocytes of the insects after injection of *E. coli* (Fig. 4B). This is consistent with the results from Northern analysis of fat body RNA from *M. luteus*-injected larvae and adults, indicating that lebecin gene expression is highly induced upon immune challenges (Fig. 4C). The mRNA level and its increase in larvae seem to be higher than those in adults.

Although its transcription pattern suggests a role in defense, we do not know the exact function of *M. sexta* lebecin, especially those repeats which correspond to the silkworm mature lebecins in position but not sequence. Do these repeats function in a whole or separately as a processed 27-residue peptide? If processing does occur, where is the scissile bond located? Does the conserved pro-segment carry any function? To address these questions, we synthesized the peptide (RSVETLASQEHLSSLPMDSQETLLRGT). The synthetic peptide did not kill any bacteria (data not shown). Recombinant expression of the pro-lebecin was unsuccessful using *E. coli* or insect cells. These negative results stimulated us to closely examine the precursor sequences and propose that processing of the repeats yields 27-residue peptides ending with RGTR. This prediction is based on an RXXR motif recognized by intracellular processing enzymes [37,38], often followed by a hydrophilic residue (e.g. Ser). We have identified three RXXR motifs in the pro-segment of *M. sexta* lebecin: R¹⁹KFR²²*E, R⁶³YAR⁶⁶*S and R⁹⁸NAR¹⁰¹*S (Fig. 1). These Arg residues at the –4 and –1 positions are 100% conserved in the lebecin-related proteins from other insects (Fig. 2A). A convertase in the secretory pathway may cleave the lebecin precursors to form five peptides: LP1, Q¹-R²² (22 mer); LP2, E²³-R⁶⁶ (44 mer); LP3, S⁶⁷-R¹⁰¹ (35 mer); LP4, S¹⁰²-R¹²⁸ (27 mer); LP5, SVETLASQEVL (11-residue partial

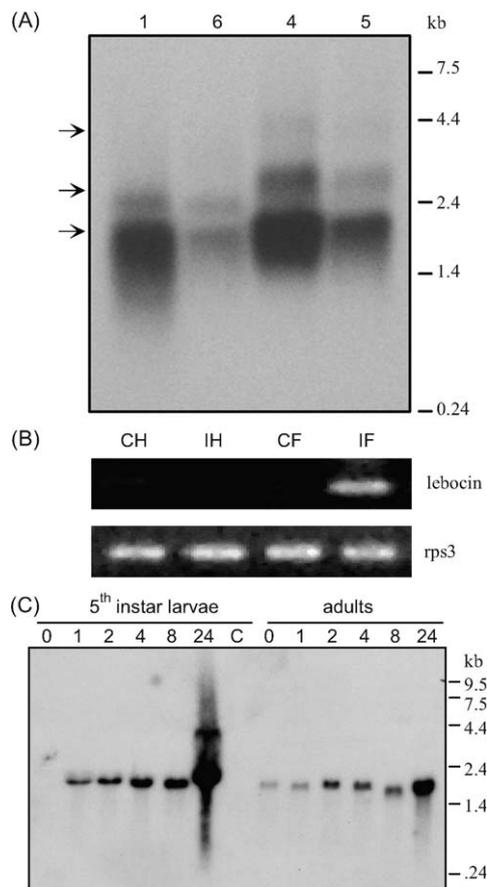


Fig. 4. Detection of lebecin mRNA in fat body of *M. sexta* injected with bacteria. (A) Transcript sizes. Fat body total RNA samples (6 µg) from the four *E. coli*-injected larvae (A1, A4, A5, A6 in Fig. 3B) were individually separated by agarose gel electrophoresis, transferred onto a membrane, and hybridized with the ³²P-labeled repetitive region in the *M. sexta* lebecin cDNA. Positions of the 1.9, 2.5, and 4.2 kb bands are marked with arrows. (B) Reverse transcription-PCR. CH and CF: hemocytes and fat body from naïve larvae (control); IH and IF: hemocytes and fat body collected from larvae at 24 h after *E. coli* injection. The cDNA samples were normalized with *M. sexta* ribosomal protein S3 (rpS3) transcripts. (C) Time course. Fat body total RNA (10 µg), isolated from *M. sexta* larvae or adults at different time after injection with *M. luteus*, were subjected to Northern blot analysis using the full-length cDNA (pP4-7) as the probe. Lane labels indicate hours after injection. The positions and sizes of the RNA markers are indicated.

repeat). LP4 (SVETLASQEHLSSLPMDSQETLLRGTR) would be produced in multiple copies since each polypeptide contains 3–16 of the 27-residue repeats.

To test their biological functions, we chemically synthesized and purified four of the peptides. Their experimental masses were

2691.95 (LP1), 5056.39 (LP2), 3814.12 (LP3) and 2988.89 (LP4), nearly identical to the theoretical values (2691.19, 5054.64, 3814.09 and 2985.33 Da). LP1 is highly cationic with a calculated isoelectric point of 12.9. LP2 (pI = 4.8) is similar in sequence to the anionic AMP isolated from *G. mellonella* [22] (Fig. 2B). LP3 has a calculated pI of 11.8, whereas LP4, the repetitive sequence of 27 residues (SVET...RGTR), is anionic (pI = 4.6). We tested these peptides for antibacterial activity against Gram-negative and Gram-positive bacterial strains using a broth micro-dilution assay [31]. LP1 was active against both Gram-positive and Gram-negative bacteria. Its MIC was 25 µg/ml against all the bacteria except for *S. aureus*, whose growth was completely blocked by LP1 at 100 µg/ml (Table 1). LP2 did not exhibit antimicrobial activity against the bacterial strains tested, differing from its homolog in *G. mellonella* [22]. LP3 had a low activity against *E. coli* O157:H7 with a high MIC (200 µg/ml). LP4 showed no activity against the bacteria. After realizing that the carboxyl-terminal Arg in these four peptides is likely removed by a carboxyl peptidase *in vivo*, we synthesized another set of peptides LP1A, LP2A, LP3A, and LP4A, one residue shorter than the corresponding LPs, and tested antimicrobial activities against the same panel of bacteria strains. The only difference we observed was between LP3A and LP3: missing the terminal Arg rendered LP3A inactive against the *E. coli* strain (Table 1). The extra positively charged residue in LP3 may have facilitated the interaction between the peptide and negatively charged bacterial cell surface.

To identify lebecin-related peptides in larval plasma, we raised a polyclonal antiserum against a mixture of the four synthetic peptides, which only detected one immunoreactive band with mobility similar to that of synthetic LP2 (data not shown). Concrete evidence for the existence of LP2 and other peptides came from analysis liquid chromatography (LC) and tandem mass spectrometry. We first assayed synthetic LP1A through LP4A to obtain chromatographic retention times, peptide *m/z*'s, and MS/MS fragmentation fingerprints (Table 2, left columns). For instance, LP2A eluted at 22 min and the peak was composed primarily of +5 and +6 ions with monoisotopic *m/z* of 980.489 and 817.242, respectively. These ions corresponded to a neutral molecular mass of 4897.45 Da, a value close to the calculated LP2A mass of 4897.42 Da. Upon MS/MS fragmentation, each ion of synthetic LP2A ion yielded a characteristic fingerprint identical to that of a peptide in the induced plasma (Table 2, right columns). This peptide, with the same retention time of 22 min, represented a major peptide constituent of the hemolymph (data not shown). While LP3A was found in the same way (Table 2), we did not detect LP4A in the induced plasma. The identification of LP1A in the induced plasma was complicated by a posttranslational modification. Synthetic LP1A eluted at 19 min and yielded +4 and +5 ions that correspond to a neutral mass of 2534.43 Da

Table 1
MICs of *M. sexta* lebecin-related peptides.^a

	ATCC	MIC of peptides (µg/ml)							
		LP1	LP1A	LP2	LP2A	LP3	LP3A	LP4	LP4A
Gram-negative									
<i>E. coli</i> O157:H7	25,922	25	25	–	–	200	–	–	–
<i>S. typhimurium</i>	14,028	25	25	–	–	–	–	–	–
<i>K. pneumoniae</i>	13,883	25	25	–	–	–	–	–	–
<i>S. typhimurium</i> DT104	700,408	25	25	–	–	–	–	–	–
Gram-positive									
<i>L. monocytogenes</i>	19,115	25	25	–	–	–	–	–	–
<i>S. aureus</i>	25,923	100	100	–	–	–	–	–	–
<i>S. aureus</i> (MRSA) ^b	43,300	100	100	–	–	–	–	–	–
<i>S. aureus</i> (MRSA) ^b	BAA-39	100	100	–	–	–	–	–	–

^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 micro-dilution 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*.

Table 2
LC-MS/MS analysis of lebecin-related peptides from chemical synthesis or *M. sexta* hemolymph.^a

Peptide name (calculated mass)	Synthetic peptide			Hemolymph		
	Intact peptide ion (<i>m/z</i>)	Elution time (min)	Predominant MS/MS fragments	Intact peptide ion (<i>m/z</i>)	Elution time (min)	Predominant MS/MS fragments
LP1A (2534.41)	507.887*	19	508 → 473 508 → 498 508 → 625 508 → 630 508 → 631	504.484*	19	505 → 473 505 → 498 505 → 625 505 → 630 505 → 631
	634.608**	19	635 → 594 (635 → 626) 635 → 652 635 → 709 635 → 773 635 → 846	630.353**	19	(630 → 589) 630 → 652 630 → 709 630 → 773 630 → 846
LP2A (4897.42)	817.242***	22	817 → 776 817 → 798 817 → 877 817 → 969	817.241***	22	817 → 776 817 → 798 817 → 877 817 → 969
	980.489*	22	980 → 969 980 → 1044 980 → 1096	980.486*	22	980 → 969 980 → 1044 980 → 1096
LP3A (3656.73)	610.459***	15–18	611 → 693 611 → 689 611 → 661	610.462***	14–17	611 → 693 611 → 689 611 → 661
	732.353*	15–18	733 → 661 733 → 689 733 → 773 733 → 826 733 → 866	732.350*	14–17	733 → 661 733 → 689 733 → 773 733 → 826 733 → 866

^a Peptides were analyzed by LC-MS/MS as described in Section 2. Characteristic precursor and fragment ions are listed. $m/z = (M + n)/n$, wherein m/z is ratio of ion mass to ion charge, M is neutral molecular mass, z is charge (*: +5; **: +4; ***: +6).

(Table 2). When the biological sample was analyzed, the 19-min major peak was dominated by ions corresponding to a neutral mass of 2517.42 Da. The mass decrease of 17.01 Da suggested that the amino-terminal Gln of LP1A was cyclized *in vivo* to form pyroGlu. Consistent with this interpretation, the predominant MS/MS fragments generated by the biological ions were nearly identical with those observed for synthetic LP1A (Table 2). We converted the first Gln of synthetic LP1A to pyroGln and analyzed the peptide mixture by NanoLC-MS/MS, which yielded ions corresponding to 2534.43 Da (original) and 2517.42 Da (converted) peptides (data not shown). Fragmented +4 and +5 ions (630.35 and 504.47 Da) from the modified LP1A were identical in mass fingerprints to those from the 19-min fraction of the biological sample.

4. Discussion

Insects and other multicellular organisms rely on a network of host defense mechanisms to survive and prosper in microbe-rich environments [4]. One such mechanism involves the synthesis and secretion into hemolymph of AMPs that are effective against a broad spectrum of pathogens including bacteria, fungi, and protozoa [1,39]. Gene duplication and sequence divergence has given rise to a sizable repertoire of AMPs in various insect groups. For instance, analysis of the silkworm genome has revealed over thirty AMP genes in the cecropin, moricin, gloverin, attacin, lebecin, and enbocin families [40]. X-tox genes in lepidopteran species encode proteins with multiple defensin-like domains that are not processed or active as AMPs [41–44].

M. sexta lebecin was first reported as a differentially expressed gene in response to bacterial injection in a subtractive suppression hybridization experiment [11]. In this study, we confirmed its inducible transcription and discovered an interesting feature of the cDNAs: 3–16 copies of an 81 bp repeat between the 5' and 3'

constant regions (Fig. 1). Gene sequencing and Southern blotting (Figs. 3 and 4) ruled out three potential mechanisms (i.e. multiple genes, allelic variations, and alternative splicing) for generating these variations. The negative results led us to suspect that the variation in copy numbers was an artifact caused by the tandem repeats when these cDNAs were first synthesized. No change was observed during DNA subcloning or propagation.

Regardless of the number of repeats in the transcript, five different peptides can be derived from the protein precursors via proteolytic processing after RXXR (Fig. 2). LP1 and LP3 were both active against bacteria, although the latter had a high MIC. These two peptides, as well as probably also *G. mellonella* anionic peptide 1, result via proteolytic processing from the amino-terminal region previously thought to be a pro-segment in lebecin-related proteins. In the initial biochemical study, *B. mori* lebecin-1/2 and -3 were names for 32-residue polypeptides active against Gram-negative bacteria [14], which are now known to be processed from the carboxyl-terminal end of a larger precursor. However, the carboxyl-terminal region in their counterparts in the *M. sexta*, *T. ni*, *P. includens*, *S. cynthia*, *A. mylitta*, *A. pernyi*, *S. frugiperda*, *P. dardanus*, *H. virescens*, and *L. obliqua* homologs differ significantly from the *B. mori* lebecins in amino acid sequence (Fig. 2). We propose to expand the original definition of lebecin to include the entire precursor proteins. Then, the processed products can be sequentially named from amino to carboxyl terminus as lebecin peptide 1 (LP1) through LPn. For instance, the *M. sexta* peptides are named LP1 through LP5: LP1, LP2 and LP3 come from the “pro-segment”; LP4 (27 mer) and LP5 (11 mer) correspond to the complete and partial repeats (Fig. 1A). *B. mori* LP4 is the same as lebecin by the original definition and LP5 represents the 25-residue fragment at the carboxyl terminus. While *B. mori* LP5 results from processing at R¹⁵¹YRR¹⁵⁴*H, there is no LP5 counterpart in *T. ni*, *P. includens*, *S. cynthia*, *A. mylitta*, *A. pernyi*, *S. frugiperda*, *H. virescens*, or *L. obliqua* lebecins.

B. mori LP4 variants were thought to be encoded by members of a gene family in the genome [15,16]. Since the genome analysis only revealed a single gene [40], the minor differences in the cDNA and gene sequences may reflect allelic variations in the silkworms used for protein purification and library construction. No evidence was available for the existence of such multi-gene family in *T. ni* [18], *H. virescens*, *L. obliqua*, or *P. dardanus*. On the other hand, *A. pernyi* and *A. mylitta* have two genes: lebocin-2's are most similar to the *S. cynthia* lebocin [20] and lebocin-1's to the *B. mori* variants (Fig. 2C). Lebocin gene duplication must have occurred in the evolution of Saturniidae and, in another species from the same family (*S. cynthia*), a separate lebocin gene may exist to encode a precursor protein more similar to *A. pernyi*-1, *A. mylitta*-1 and *B. mori* lebocins. In one lineage of the superfamily Noctuoidea, gene duplication has generated two to three lebocins in *S. frugiperda*, which form a clade with their homologs of *P. includens* and *T. ni* (Fig. 2C). In the pyrosequencing analysis of immunity-related cDNAs from *M. sexta* [44], we identified four contigs (#5813, #6639, #6760 and #6851) coding for polypeptides similar to a part of the pro-segment (LP1–LP2 region). Sequence comparison suggested two additional lebocin-like genes in the *M. sexta* genome (data not shown). One or both of them may be more similar to silkworm lebocins than the one reported herein. Taken together, the sequence-based phylogenetic relationships are consistent with those derived from morphological characteristics.

B. mori, *T. ni*, *P. includens* and *S. cynthia* lebocin genes were mainly expressed in fat body of immune challenged insects [15,18–20]. Their mRNA levels were much lower in hemocytes even after bacterial injection. Consistent with the presence of immune regulatory elements in its gene (Fig. 1B), *M. sexta* lebocin expression was up-regulated during antimicrobial responses in larvae and adults (Fig. 4C). Similarly, cecropin B of *M. sexta* was mainly produced in fat body [10]. These results are consistent with the observation that lebocin and cecropin expression was up-regulated by injecting *B. mori* spätzle into *M. sexta* larvae [45].

Although direct evidence is limited, it seems to be a common mechanism in lepidopteran insects to generate several active peptides from a lebocin polypeptide precursor. This is supported by the purification of LP2 from *G. mellonella* and *H. armigera* hemolymph [21,22] and by the detection of antibacterial activities of *M. sexta* LP1, LP3, and *G. mellonella* LP2 (i.e. anionic peptide 1) (Fig. 2B). The purification and functional characterization of *B. mori* LP4 variants (i.e. lebocin-1/2 and 3) further supported this hypothesis [14]. In this study, our antiserum prepared toward a mixture of the four synthetic lebocin peptides recognized synthetic LP2 efficiently and labeled a polypeptide in the induced plasma, which is similar to synthetic LP2 in apparent M_r (data not shown). Direct evidence for the existence of LP2A (as well as LP1A and LP3A) at a high level was obtained by LC–MS/MS analysis of the larval hemolymph (Table 2): (1) two plasma polypeptides were identical to synthetic LP2A and LP3A in all molecular properties; (2) a peptide with MS/MS fragments identical to those of synthetic LP1A had its amino-terminal Gln converted to a pyroGlu *in vivo*. Surprisingly, we did not detect any LP4A, which should be approximately 13 times as abundant as the other peptides. There are three possible reasons for this negative result: (1) the repeated region is resistant to processing, (2) LP4A attaches to hemocytes or proteins that precipitate during sample preparation; (3) LP4A is rapidly degraded in the plasma. The existence of the perfect repeats in multiple copies, which is not found in the other lepidopteran species, suggests that LP4A plays a unique role in the *M. sexta* defense system. On the other hand, the high sequence similarity of the LP1, LP2 and LP3 regions in other species suggest that these predicted processing products are functionally conserved. A very recent peptidomics study of AMPs in hemolymph of

G. mellonella [46] has revealed the same mechanism discovered in this investigation.

Processing of large precursors is a strategy commonly used by neuroendocrine systems of vertebrates and invertebrates to generate biologically active peptides. Rules have been proposed to predict the processing sites in insect neuropeptide precursors [37,38]. This study provides initial evidence that the insect immune system may use the same tactic to produce functional products by processing protein precursors. This hypothesis would support the notion that parts of the nervous, hormonal, and immune systems have a common evolutionary origin.

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