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Insect immunity: molecular cloning, expression, and characterization of cDNAs and genomic DNA encoding three isoforms of insect defensin in *Aedes aegypti*

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Abstract

Aedes aegypti **were immune activated by injection with bacteria, and the expression of insect defensins was measured over time. Northern analyses indicated that defensin transcriptional activity continued for at least 21 days after bacterial injection, and up to 10 days after saline inoculation. Mature defensin levels in the haemolymph reached approximately 45 μM at 24 h post inoculation. cDNAs encoding the preprodefensins of three previously described mature *Ae. aegypti* defensins were amplified by PCR, cloned and sequenced. Genomic clones were amplified using primers designed against the cDNA sequence. Sequence comparison indicates that there is significant inter- and intra-isoform variability in the signal peptide and prodefensin sequences of defensin genes. Preprodefensin sequences of isoforms A and B are very similar, consisting of a signal peptide region of twenty amino acids, a prodefensin region of thirty-eight amino acids and a forty amino acid mature peptide domain. The sequence encoding isoform C is significantly different, comprising a signal peptide region of twenty-three amino acids, a prodefensin region of thirty-six amino acids, and the mature protein domain of forty amino acids. Analysis of the genomic clones of each isoform revealed one intron spatially conserved in the prodefensin region of all sequences. The intron in isoforms A and B is 64 nt long, and except for a 4 nt substitution in one clone, these intron sequences are identical. The intron in isoform C is 76 nt long and does not share significant identity with the intron sequences of isoforms A or B. The defensin gene mapped to chromosome 3, between two known loci, blt and LF168.**

Keywords: ***Aedes aegypti*****, defensin, insect immunity.**

Introduction

The success of insects in exploiting diverse ecological niches is due in part to their ability to defend themselves against harmful pathogens and parasites (Lowenberger, 1996a). Insects mount a very effective humoral and cellular response to prokaryotic and eukaryotic organisms (Dunn, 1986; Lackie, 1988), involving phagocytosis of bacteria or a melanotic encapsulation response to metazooan parasites (Christensen & Forton, 1986; Li & Christensen, 1990).

Insects also respond to bacterial invasion or the disruption of cuticular integrity with the synthesis of an array of potent antibacterial peptides (Brey *et al.*, 1993; Hoffmann *et al.*, 1996; Hetru *et al.*, 1998). In recent years these compounds have been studied extensively and classified into distinct families. For reviews on the cecropins, attacins, diptericins, defensins, proline-rich compounds and glycine-rich compounds that show bactericidal or bacteriostatic activity see Boman *et al.* (1991), Cociancich *et al.* (1994), Hetru *et al.* (1994, 1998) and Hoffmann *et al.* (1996). The predominant antibacterial proteins produced by *Aedes aegypti* in response to bacterial inoculation or sterile injury are defensins (Lowenberger *et al.*, 1995; Chalk *et al.*, 1995; Cho *et al.*, 1996). In the Diptera, defensin genes are expressed mainly in the fat body and in certain haemocytes (Dimarcq *et al.*, 1990; Hoffmann & Hetru, 1992). Recently, transcriptional activity for insect defensins has been reported in the midguts of blood-fed *An. gambiae* by Richman *et al.* (1997) in *Stomoxys calci-*

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trans by Lehane *et al.* (1997) and in *Ae. aegypti* by Lowenberger *et al.* (1999).

Insect defensins are small cationic molecules thirty-four to forty-three amino acids in length, except for the fifty-one residue royalisin isolated from royal jelly (Fujiwara *et al.*, 1990) and the forty-six residue Smd1 isolated from *S. calcitrans* (Lehane *et al.*, 1997). Defensins contain six cysteine residues arranged in three intramolecular bridges (Hetru *et al.*, 1998) and are active primarily against Gram-positive bacteria (Hoffmann & Hetru, 1992; Hetru *et al.*, 1998). Insect defensins are present in ancient insect orders such as the Odonata (Bulet *et al.*, 1992) and modern insects such as Culicidae (Lowenberger *et al.*, 1995; Chalk *et al.*, 1995; Cho *et al.*, 1996; Richman *et al.*, 1996). Functional analogues have been identified from molluscs (Charlet *et al.*, 1996; Hubert *et al.*, 1996), scorpions (Cociancich *et al.*, 1993; Ehret-Sabatier *et al.*, 1996), mammals (Ganz & Lehrer, 1995; Evans & Harmon, 1995) and plants (Broekaert *et al.*, 1995; Penninckx *et al.*, 1996), although the three-dimensional structure and disulphide bond linkages are different for defensins from mammals and plants (Hanazawa *et al.*, 1990) and they share only limited sequence identity.

It has been suggested that these inducible components of the insect immune system may also play a role in limiting the development of parasites that cause diseases such as malaria and lymphatic filariasis. These parasite–vector relationships represent tightly co-evolved systems in which the genetics of both host and parasite define the potential for a parasite to develop and be transmitted. These host–parasite associations are complex and essential for disease transmission; consequently, the mechanisms controlling these associations serve as potential targets for direct intervention to reduce compatibility between vectors and parasites. Certain species or strains of mosquitoes are naturally resistant to specific parasites, and numerous physiological factors are undoubtedly involved in determining susceptibility and/or resistance (Richman & Kafatos, 1995), but the molecular and genetic basis for this resistance, and the mechanisms by which mosquitoes recognize parasites as non-self, remain unclear (Christensen & Severson, 1993).

Insect antibacterial peptides are generally considered inactive against eukaryotic parasites (Cociancich *et al.*, 1994), but the injection of synthetic immune peptides into mosquitoes has been reported by Gwadz *et al.* (1989) and Chalk *et al.* (1995) to reduce the establishment and development of *Plasmodium* sp. and *Brugia pahangi* respectively. Lowenberger *et al.* (1996b) reported that immune activation of *Ae. aegypti* by inoculation with bacteria or sham inoculation with sterile saline significantly reduced the ability of ingested *Brugia malayi* to establish a successful infection, and the same authors reported that immune activation of *Ae. aegypti* or *An. gambiae* by inoculation with bacteria reduced the prevalence and mean intensity of infection with oocysts of *Plasmodium gallinaceum* and *P. berghei* respectively (Lowenberger *et al.*, 1999). However, because the injection of insects induces the expression of many compounds that may play dual roles in wound healing response physiology and parasite killing, the compounds specifically responsible for parasite killing demonstrated in these studies have not been determined. However, these studies suggest that susceptible mosquitoes, that normally permit the development of ingested parasites, can kill or reduce the prevalence and mean intensity of infection if their immune responses are activated prior to ingesting parasites. Although there is no direct evidence that immune peptides are killing the parasites, it is crucial to understand the processes that occur in immune activation and to isolate and characterize the promotors and regulators of immune activation events. The presence of high levels of defensin in immune-activated mosquitoes (Lowenberger *et al.*, 1995) may be indicative of an overall immune activation and may be used as a measurement or predictor of immune status. The aim of reducing disease transmission by developing transgenic mosquitoes that are refractory to ingested parasites requires that we first identify immune compounds that can kill parasites as well as the mechanisms required to express these compounds to coincide temporally and spatially with susceptible parasite stages. Therefore a major research emphasis is now being placed on identifying endogenous inducible immune peptides from vector insects with the aim of understanding their induction and activation, and the role they may play in parasite killing.

We report herein two cDNA allelic clones for each of the three isoforms of mature inducible insect defensins described previously in *Ae. aegypti* (Lowenberger *et al.*, 1995) and descriptions of genomic clones for each of the isoforms. We also present a temporal profile for defensin transcriptional activity in the fat bodies of bacteria-challenged mosquitoes and mature defensin protein levels in the haemolymph.

Results

Northern analyses

Northern analyses of fat body RNA removed from naive adult mosquitoes and from mosquitoes at various times post inoculation with bacteria, showed that no discernible defensin transcriptional activity was found

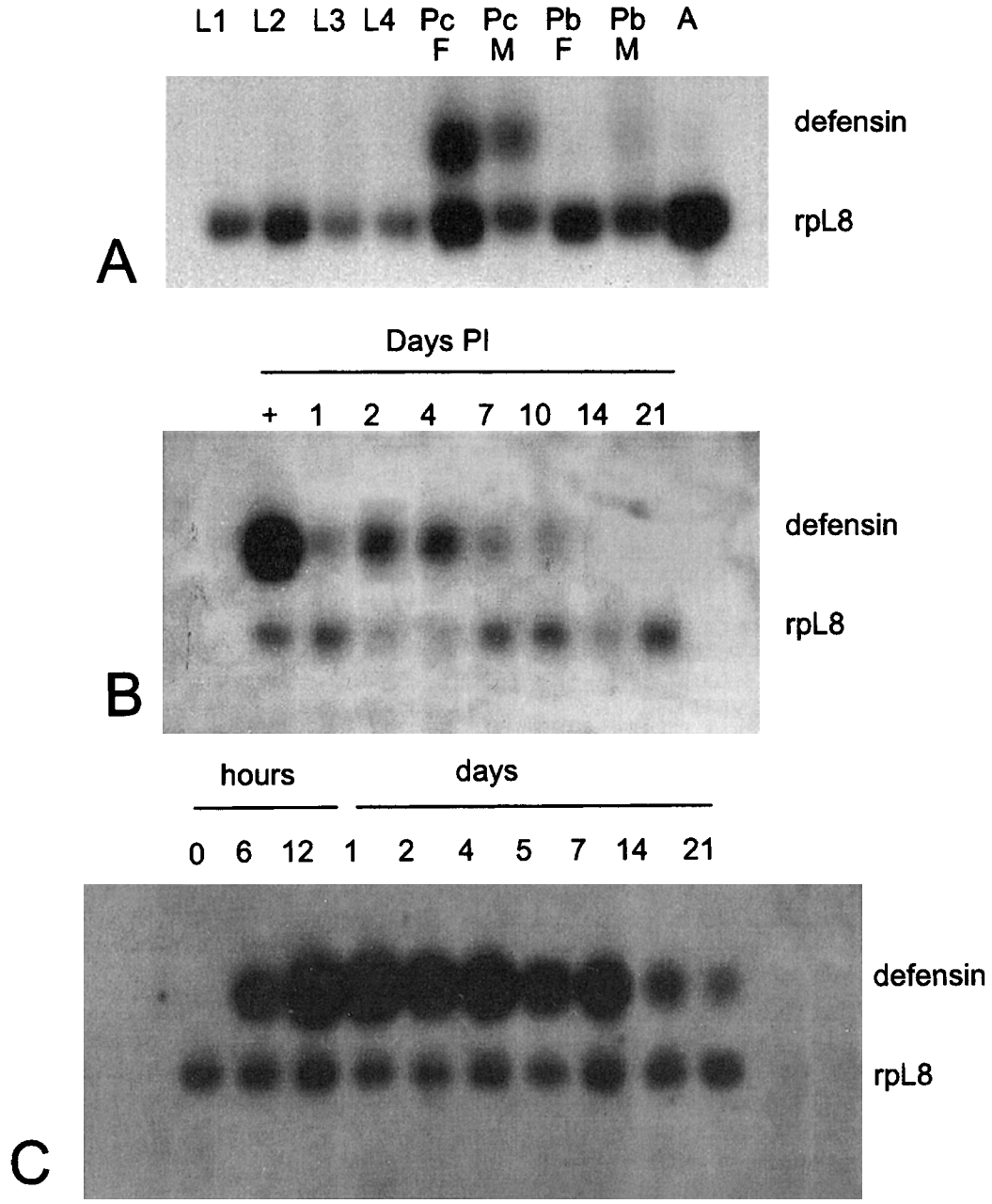


Figure 1. Northern blot radiographs of fat body RNA from adult and whole body RNA from immature stages of naive *Aedes aegypti* and mosquitoes immune activated with injection of bacteria. (A) Naive mosquitoes: L1–L4 indicates larval stages 1–4, PcM = callow male pupae; PcF = callow female pupae; PbM = black male pupae; PbF = black female pupae; (A) = adults. (B) Saline-inoculated mosquitoes from which RNA was collected 1–21 days post inoculation. + = Positive control (5 μg RNA from bacteria-inoculated adult *Ae. aegypti*). (C) Bacteria-inoculated adult mosquitoes. Each lane contains 5 μg of fat body (adults) or whole body (immature stages) RNA as explained in Experimental procedures. A probe generated from a ribosomal protein-encoding cDNA (rPL8) was used as a loading control.

in naive mosquitoes, nor during the first 15 min post-inoculation. By 30 min post inoculation, transcriptional activity was detectable (data not shown) and remained present through 21 days (Fig. 1). Defensin transcriptional activity was seen up to 10 days after inoculation with sterile saline (Fig. 1). Northern analysis of immature stages of mosquitoes determined that defensins are expressed constitutively in the white or callow pupae during metamorphosis, but no expression of defensin was seen in larvae (Fig. 1).

Features of the defensin sequences

A total of ninety-four clones encoding partial regions of the putative mosquito cDNA or genomic sequences

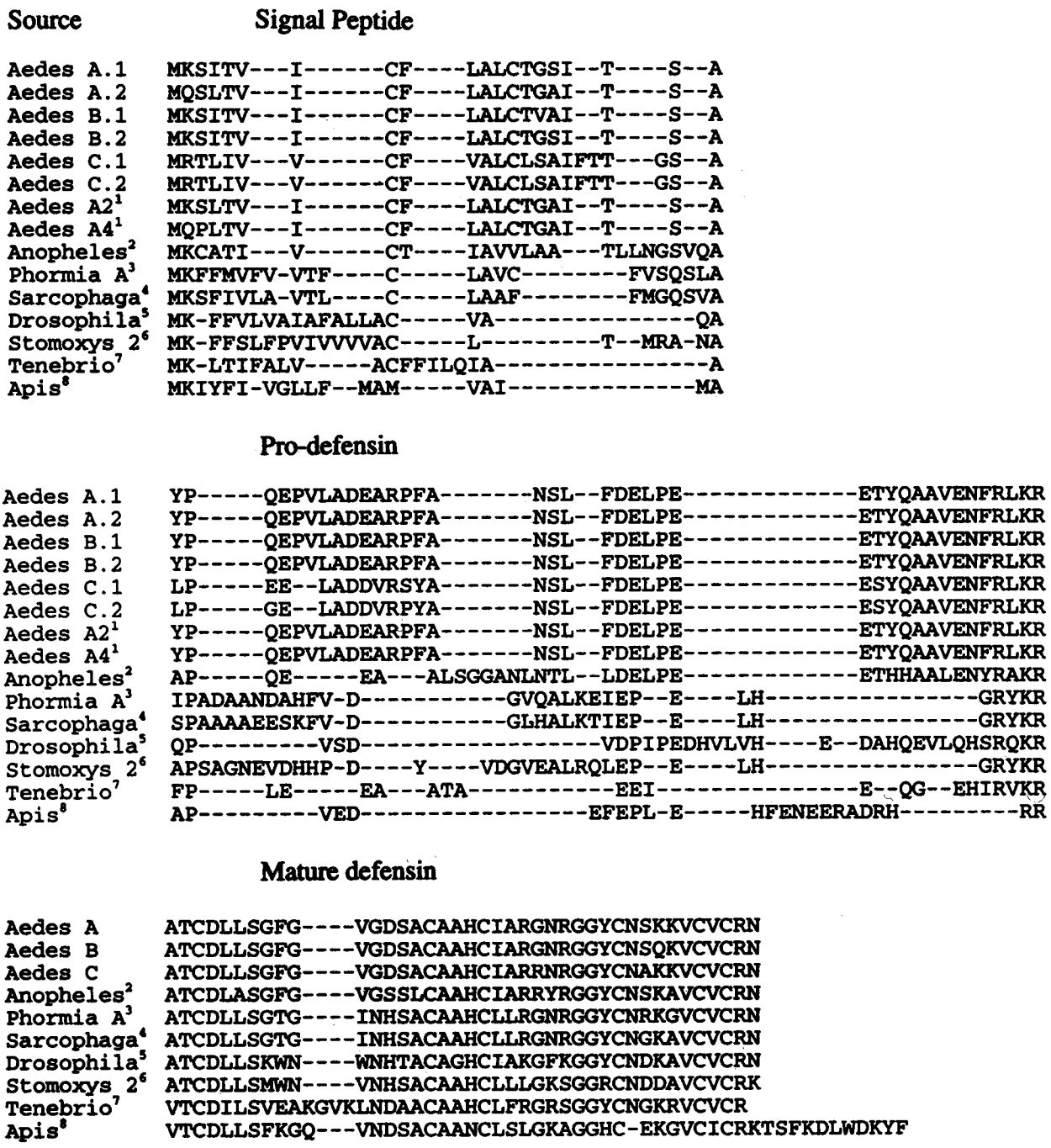
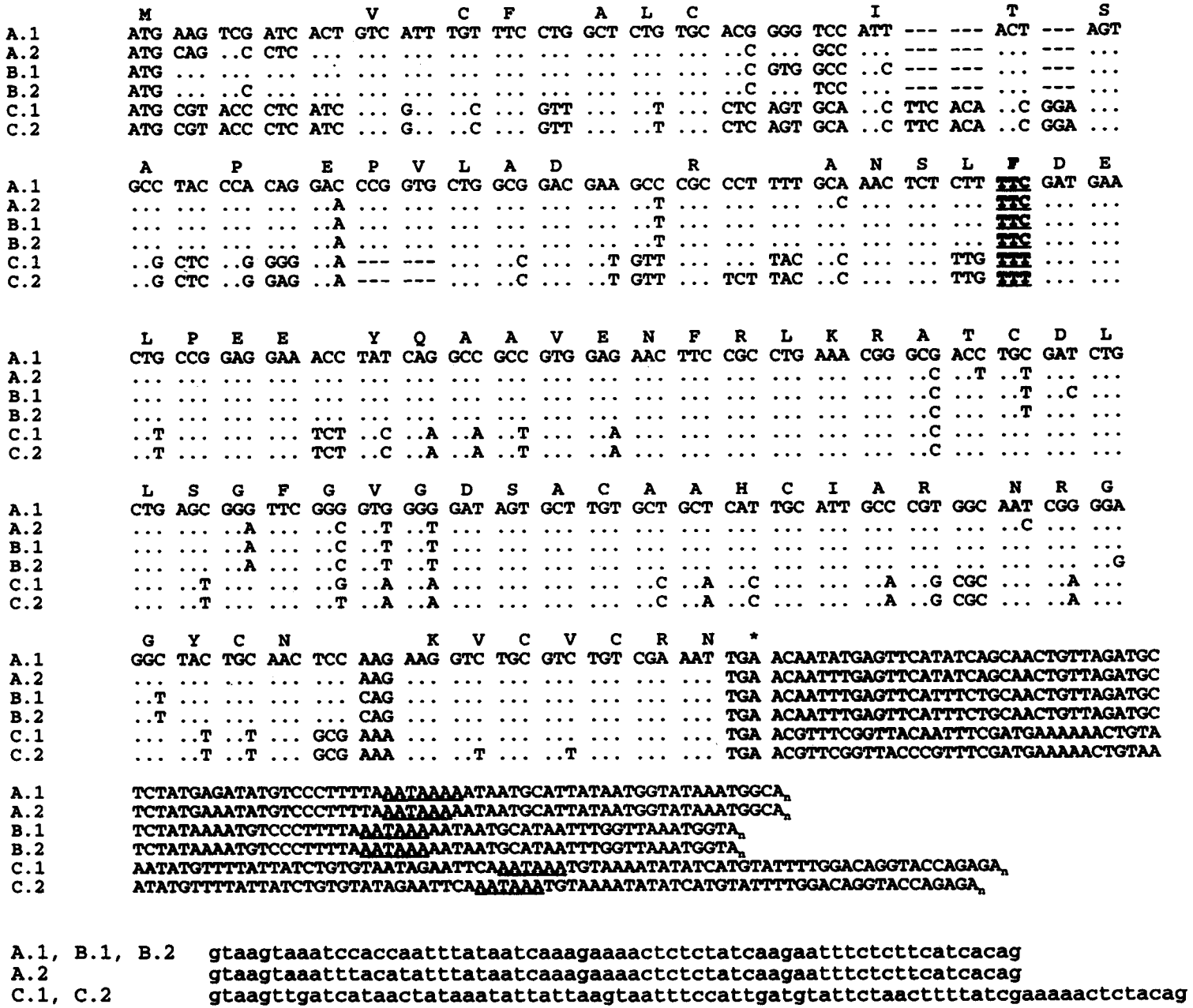


Figure 2. Comparative alignment of deduced amino acids of preprodefensins from *Aedes aegypti* and selected examples from the orders Diptera, Coleoptera and Hymenoptera. Alignment was done using Clustal analysis with PAM250 residue weight table (DNA Star, Madison, Wis.). Dashes were introduced to maintain maximum alignment. [1]*Aedes aegypti* (Cho *et al.*, 1996); [2]*Anopheles gambiae* (Richman *et al.*, 1996); [3]*Phormia terranovae* (Dimarcq *et al.*, 1990); [4]*Sarcophaga peregrina* (Matsuyama *et al.*, 1988); [5]*Drosophila melanogaster* (Dimarcq *et al.*, 1994); [6]*Stomoxys calcitrans* (Lehane *et al.*, 1997); [7]*Tenebrio molitor* (Moon *et al.*, 1994); [8]*Apis mellifera* (Casteels-Josson *et al.*, 1994).

Figure 3. (A) Alignment of the cDNA sequences encoding three isoforms of *Aedes aegypti* defensin. Two sequences that differ in the signal peptide or prodefensin region are presented here for each isoform of mature defensin. Dots represent identical nucleotides in each sequence; dashes are introduced to maintain alignment. Deduced amino acids are presented in single-letter codes over each codon only when the same amino acid occurs in all isoforms. Each sequence ends with a stop codon (TGA) indicated with an asterisk. The putative polyadenylation consensus sequence is underlined. The phenylalanine residue in which the introns were found in genomic sequences is indicated in bold. (B) Intron structure found in genomic clones of *Ae. aegypti* defensin genes. The introns in isoforms A and B contain sixty-four base pairs and differ only by four nucleotides in the sequence of isoform A2. The intron in the gene for defensin isoform C is 76 nt long.



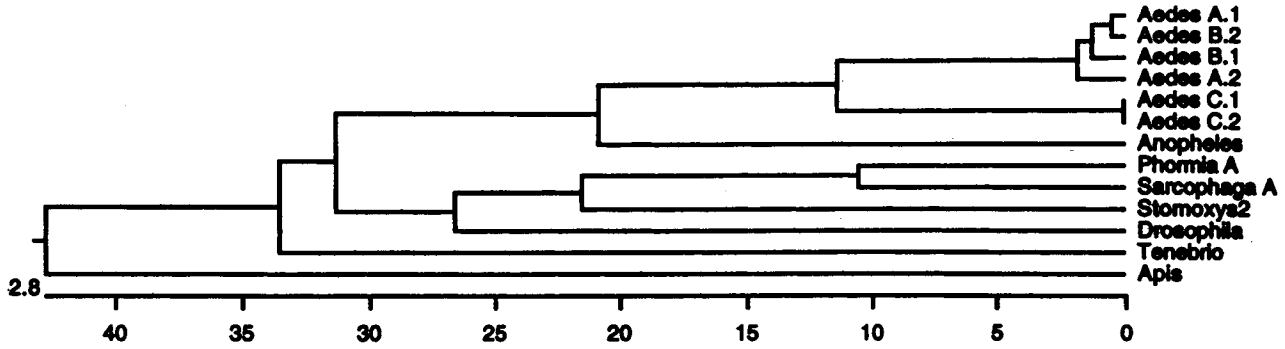


Figure 4. Phylogenetic tree analysis of preprodefensin sequences of defensins isolated from different insect orders. Analysis was done using Clustal analysis with PAM 250 residue weight table (DNA STAR, Madison, Wis.). Values on the *X* axis indicate the number of substitutions. [1]*Anopheles gambiae* (Richman *et al.*, 1996); [2]*Phormia terranovae* (Dimarcq, 1990); [3]*Sarcophaga peregrina* (Matsuyama *et al.*, 1988); [4]*Drosophila melanogaster* (Dimarcq *et al.*, 1994); [5]*Tenebrio molitor* (Moon *et al.*, 1994); [6]*Apis mellifera* (Casteels-Josson *et al.*, 1994); [7]*Stomoxys calcitrans* (Lehane *et al.*, 1997).

were examined and sequenced. There is a deduced open reading frame of 294 nucleotides (nt) for isoforms A and B, and 297 nt for isoform C (Fig. 2). All sequences begin with an ATG codon and encode a total protein of ninety-eight (isoforms A and B) or ninety-nine (isoform C) residues with a calculated molecular mass of 10.6, 10.6 and 10.8 kDa (isoforms A, B and C respectively). The protein contains a putative signal sequence comprising the first twenty residues in isoforms A and B, and twenty-three residues in isoform C (Fig. 3). These signal peptides are highly hydrophobic and, in all sequences shown here, probably end in an alanine residue similar to insect defensins isolated from *Phormia terranovae* by Dimarcq *et al.* (1990), *Sarcophaga peregrina* by Matsuyama & Natori, 1988), *Drosophila melanogaster* by Dimarcq *et al.* (1994), *Anopheles gambiae* by Richman *et al.* (1996), *Apis mellifera* by Casteels-Josson *et al.* (1994) and *Tenebrio molitor* by Moon *et al.* (1994), as well as other small antimicrobial peptides isolated from insects; diptericin isolated from *P. terranovae* by Reichhart *et al.* (1989) and cecropins isolated from *Hyalophora cecropia* by Lidholm *et al.* (1987) and *Sarcophaga peregrina* by Matsumoto *et al.* (1986). Cho *et al.* (1996), however, suggest that the signal peptide of a clone of *Ae. aegypti* cDNA for defensin A terminates with a threonine. If we assume that the alanine is the cleavage site, then the coding region of the pro-defensin sequence is thirty-eight residues for isoforms A and B and thirty-six residues for isoform C. In all sequences the pro-defensin ends in a potential K-R cleavage site that is present in all insect defensins isolated from Diptera with the exception of Smd1 in *S. calcitrans* (Fig. 2). This cleavage site is followed by the sequence coding for the forty amino acid mature insect defensins reported previously (Lowenberger *et al.*, 1995). The coding region contains a stop codon, a 3′ untranslated region of fifty-five to sixty-five nucleotides, depending on the isoform, the putative polyadenylation consensus signal (AATAAA), and the poly-A tail. As was described by Cho *et al.* (1996), we have demonstrated that there are different coding sequences for the same isoform of mature defensin (Fig. 3) that differ mainly in the signal peptide region and that exhibit different codon usage or variation in the 'wobble' position of the codons throughout the sequences. The sequences of the signal and pro-defensin coding regions are very similar for each of the forms of defensin isoforms A and B. However, signal peptides that code for isoform C are significantly different from those of isoforms A and B (Fig. 3).

In all sequences there is an intron contained within the codon for phenylalanine in the pro-defensin region (T- intron-TT isoforms A and B, and T-intron-TC in isoform C [see Fig. 3]). The introns in isoforms A and B are all 64 nt long, and forms A.1, B.1 and B.2 are identical. The intron from isoform A.2 differs at positions 11–12 and 15–16. The intron in sequences coding for isoform C is 76 nt long and is significantly different from the other introns (Fig. 3b). The phylogenetic analysis of defensin sequences from several insects (Fig. 4) demonstrates the similarities and conservation of these proteins among insect orders. As expected, sequences from the Culicidae share the greatest identity of the insects presented.

Mature protein in haemolymph

As demonstrated in Fig. 5, there were no detectable levels of defensin found in naive mosquitoes 3 days after emergence. However, 1 day after inoculation with bacteria, defensin levels reached approximately 45 μM in the insect haemolymph. Such levels remained

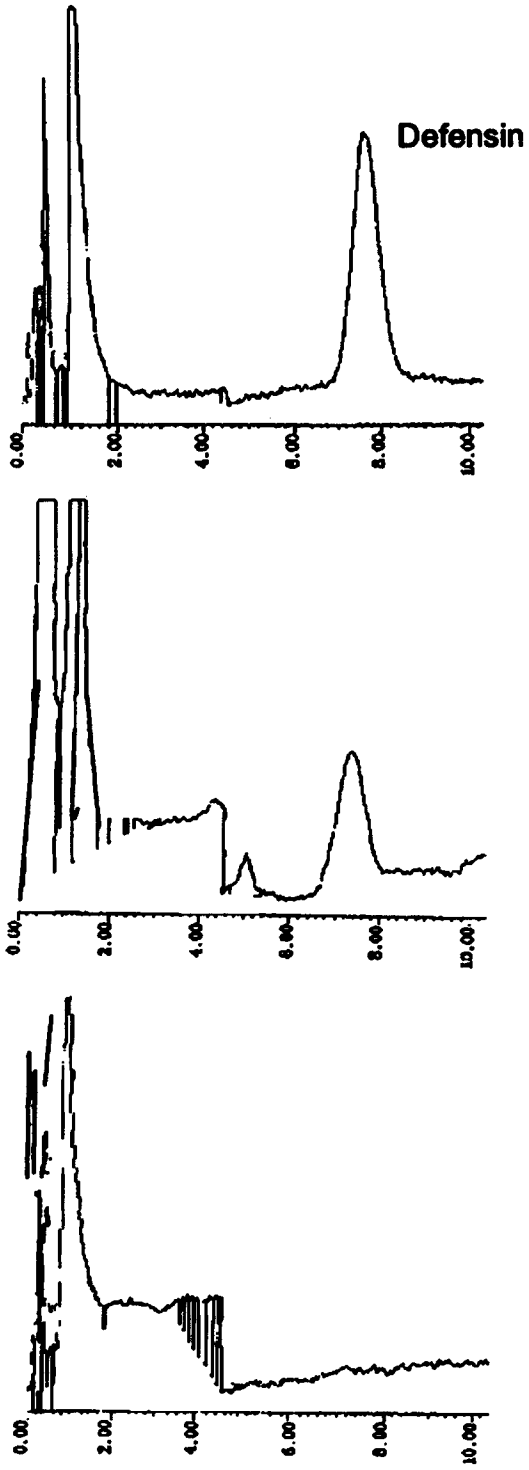


Figure 5. HPLC profiles of mature defensin protein found in the haemolymph of *Aedes aegypti*. Haemolymph was collected from five mosquitoes per time period. The samples were prepared as described in Experimental procedures, and were resuspended in 25 μl of acidified water. 5 μl samples were analysed. Upper panel: purified defensin; middle panel: haemolymph from a bacteria-inoculated mosquito 24 h after inoculation with bacteria; lower panel: haemolymph from a naive mosquito. The asterisk indicates the peak corresponding to purified defensin.

steady in the mosquito for at least 3 days after immune activation, and haemolymph defensin levels represent approximately 6% of total haemolymph proteins.

Mapping of the defensin gene in Aedes aegypti

The defensin probe mapped to a region on Chromosome 3 between loci blt and LF168 (Severson *et al.*, 1993). Distances in Kosambi cM were: blt-(20.2 cM)-defensin-(7.5 cM)-LF26-(5.3 cM)-LF168. The segregation pattern of defensin RFLP was indicative of a single locus or tightly linked loci between which no recombination was detectable.

Discussion

Transcription of the genes coding for insect defensins in *Ae. aegypti* is extremely rapid, and results in the production of high levels of mature protein in the haemolymph (Lowenberger *et al.*, 1995). The fact that we can find a strong signal present 21 days after bacteria challenge suggests that (1) once transcription for defensin is initiated it remains on, (2) that bacteria inoculated into the haemocoel are never cleared completely, thus maintaining a constant stimulus for transcription, or (3) the message is stable for long periods. The mosquitoes injected with a sterile needle also demonstrated transcription for defensin. This may be due to the introduction of small amounts of bacteria into the insects. Alternatively, defensin, and other immune peptides, may be expressed in response to injury, and may be integral in the wound healing response. Immune peptides such as cecropin have been found in insects in which the cuticle was abraded, but not broken (Brey *et al.*, 1993), suggesting that immune peptides may be expressed in all tissues to prevent the establishment of pathogens. The reduction in transcriptional activity after 10–14 days post inoculation in saline-injected controls suggests that the amount of bacteria introduced significantly influences both the level and duration of transcriptional activity. Although this and previous studies (Lowenberger *et al.*, 1996; Cho *et al.*, 1996) found significant transcriptional activity and levels of mature protein in the haemolymph of immune activated *Ae. aegypti*, Richman *et al.* (1996) found significantly less transcriptional activity and mature defensin protein in *An. gambiae* larvae inoculated with bacteria. Whereas transcriptional activity for defensin returned to normal in *An. gambiae* larvae 30 h after inoculation with bacteria (Richman *et al.*, 1996), we have demonstrated that in adult *Ae. aegypti* the message remains detectable 21 days after immune activation. When we inject *Ae. aegypti* larvae with bacteria we also find a significantly reduced transcriptional profile for defensin

as compared with adults (data not shown), which suggests a differential transcription pattern between immature and adult stages. Alternatively, these differences in transcriptional activity between *Ae. aegypti* and *An. gambiae* may represent species-specific differences. In some insects the gene for defensin is expressed constitutively during larval development (Matsuyama & Natori, 1988; Dimarcq *et al.*, 1994), and in immature stages of *Ae. aegypti* there is endogenous expression of defensin in the white or callow pupae prior to sclerotization (Fig. 1). We can only speculate that this gene may be expressed during moults from larvae to larvae or from larvae to pupae to prevent the entry and establishment of pathogens from the bacteria-laden larval habitat through tears that might occur in the very soft cuticle prior to sclerotization, or during the histolysis of specific tissues during metamorphosis from larva to pupa to adult mosquito. Alternatively, defensins may have a dual role in defence and development in *Ae. aegypti* as suggested for sapecin in *S. peregrina* by Matsuyama & Natori (1988). The concentration of mature defensin in the haemolymph of immune activated mosquitoes, approximately 45 μM, is significantly higher than that of *Drosophila* in which only approximately 1–2 μM concentrations are found (P. Bulet, unpublished data), is higher than the 1–10 μM concentrations recorded for most insect immune peptides, but is significantly lower than the 100 μM levels of drosomycin found in *D. melanogaster* (Hetru *et al.*, 1998).

We have demonstrated that there are different amino acid sequences in the signal peptide region of the cDNAs that encode the same mature protein. The signal peptide is characteristically highly hydrophobic to ensure transport through membranes. The substitutions K_2/Q_2, I_4/L_4, G_{15}/V_{15} and S_{16}/A_{16} probably do not significantly affect the overall hydrophobicity of the signal peptide and its function *in vivo*. Although there is some modification in the codon usage in the pro-defensin region of sequences coding for isoforms A and B, there is no amino acid substitution in our sequences nor in those four sequences coding for isoform A reported by Cho *et al.* (1996), suggesting an extremely conserved region. The signal peptide/pro-defensin region of sequences coding for defensin isoform C is quite different. In this sequence there is an additional amino acid, and the signal peptide and pro-defensin sequences are of different lengths compared with those of isoforms A and B. Based on the estimation of the cleavage site between the signal-prodefensin, the sequence coding for isoform C has three more amino acids in the signal peptide and two fewer in the pro-defensin region as compared to sequences coding for isoforms A and B. We may speculate that these differences indicate a tissue specificity for the different isoforms as Lowenberger *et al.* (1999) reported an up-regulation of message for defensin C, but not such an increase in isoforms A or B in the midguts of *Ae. aegypti* after ingestion of a *P. gallinaceum* infectious bloodmeal.

The structure of the intron isolated from the different isoforms also is significantly different. The intron for each isoform described here was found in the same location, internal to the codon for phenylalanine in the prodefensin region. Except for four substitutions in the intron in sequence A.2 (Fig. 3), all 64 nt of the introns in sequences coding for isoforms A and B were identical. The intron in pro-defensin C is 76 nt long and the sequence does not share significant identity with the other introns. Although introns have been found in the signal peptide region of an insect defensin isolated from *T. molitor* (Lee *et al.*, 1996), Dimarcq *et al.* (1994) reported that *D. melanogaster* contains a single copy of an intronless gene coding for defensin. Our data suggest that in *Ae. aegypti* there are at least two tandemly linked genes for defensin that map to one locus on chromosome 3, between loci blt and LF168.

Analysis of the alignment of the cDNAs of defensins from various insects for which we have complete protein sequences demonstrates the similarity between closely related species (Fig. 4). Sequences from the Culicidae are more closely related to each other than to other groups. This relationship is altered only minimally when signal peptides alone, signal peptides plus pro-defensin sequences, pro-defensin plus mature defensin, or mature defensin sequences alone are compared (data not shown). In these analyses only the relative positions of *Apis*, *Tenebrio* and *Drosophila* are altered. These comparisons suggest that changes made in the signal peptide, pro-defensin, and mature protein regions of insect defensin genes isolated from different insect orders probably represent alterations of a common ancestor molecule modified after the separation of the different insect orders.

Genetic linkage maps generated for *Ae. aegypti* can determine linkage and gene order both within and among species (Severson *et al.*, 1995). The use of map-based technology has resulted in the identification of quantitative trait loci (QTL) that influence vector competence (Severson *et al.*, 1994) and enable us to identify regions of the chromosome responsible for specific traits. Although Beerntsen *et al.* (1995) demonstrated a QTL that influences filarial worm intensity is linked to QTL responsible for susceptibility to other mosquito-borne pathogens, the locus to which defensin maps has not been demonstrated to have any influence on the ingestion, establishment or develop-

ment of eukaryotic parasites transmitted by *Ae. aegypti.*

In summary, we have demonstrated that there are at least two, and possibly more, genes for *Ae. aegypti* defensins. These are located on the third chromosome between two known loci. There are different sequences in the signal peptides and pro-defensin regions of clones that code for the same mature defensin protein; however, the signal peptide of defensin isoform C is significantly different from those of A and B. Northern analyses suggest that once mosquitoes are immune activated, they may remain so for significant portions of their adult life and mature levels of defensin in the haemolymph reach very high levels within 24 h of immune activation. These data provide us with more information on the activation of mosquito immune systems and the subsequent release of defensin into the haemolymph. Studies are ongoing to investigate *in vivo* the direct effects of these immune peptides on the establishment and development of ingested eukaryotic parasites.

Experimental procedures

Mosquito maintenance

Aedes aegypti (Liverpool strain) were obtained originally from the University of London in 1977 and were reared as described previously (Beerntsen & Christensen, 1990).

Immune activation

Escherichia coli K12 strain and *Micrococcus luteus* were grown in Luria-Bertani's rich nutrient medium (LB medium) overnight at 37°C. Following incubation, 0.5 ml samples of each culture were combined in a 1.5 ml microfuge tube and centrifuged. The supernatants were removed, leaving a moist pellet. Mosquitoes were anaesthetized with CO_2 and kept cold-inactivated on wet ice. Individual mosquitoes were held vertically in place by fine suction on a vacuum saddle as described previously (Beerntsen & Christensen, 1990). A sterile stainless steel probe (0.15 mm) was dipped into the moist bacterial pellet and inserted into the body cavity through the neck membrane (Lowenberger *et al.*, 1995). A control group of mosquitoes was not inoculated and all mosquitoes were used within 48 h of eclosion.

Haemolymph collection and mature protein analysis

Haemolymph from bacteria-inoculated mosquitoes and controls was collected 1–120 h post-inoculation by tearing the last abdominal segments and perfusing the haemocoel contents as described by Lowenberger *et al.* (1996). Three drops of haemolymph were collected per mosquito and haemolymph from five mosquitoes was combined per tube. Haemolymph samples were filtered through 30,000 molecular mass filters (Centricon 30, Amicon, Beverly, Mass., USA) for 10 min at 12,000 rpm. The resultant haemolymph was vacuum dried, and the pellet resuspended in 25 μl distilled water containing 0.05% TFA. Protein samples were analysed in a Gilson HPLC (Gilson, USA) using an Aquapore OD 300, 7 μm, 220 × 2.1 mm column (Brownlee™, PE Applied Biosystems, USA). Haemolymph samples (5 μl) were injected into the HPLC and the areas under the peak corresponding to defensin were compared with the peaks from known concentrations of purified *Ae. aegypti* defensin. Defensin levels in haemolymph were compared to overall protein content of mosquito haemolymph by calculating haemolymph protein levels using the Biorad Protein Assay Kit (Bio-Rad Laboratories, Hercules, Calif., USA) and BSA as a standard.

Northern analyses

In order to assess defensin transcriptional activity we assayed a subset of each group of immune activated mosquitoes 0.5 h to 21 d post inoculation. Five adult mosquitoes from each treatment group were cold anaesthetized and total RNA was collected from dissected fat bodies. For studies on immature stages, RNA was extracted from approximately 100 first and second instars, fifty whole bodies of third or fourth instars, ten pupae or five adults. All RNA extraction was done using the single-step acid guanidinium thiocyanate–phenol–chloroform extraction isolation method (Chomczynski & Sacchi, 1987). RNA (5.0 μg/lane as determined by spectrophotometry OD_{260}) from each group of mosquitoes was separated on formaldehyde-agarose gels (Sambrook *et al.*, 1989). Following transfer, membranes were air-dried and baked *in vacuo* at 80°C for 3–5 h. ^{32}P probes were generated using specific primers to amplify 50 ng of the 120 nt sequence of the defensin A clone in a PCR reaction described previously (Severson & Kassner, 1995). Membranes were hybridized with the defensin probe and either simultaneously or sequentially with a ribosomal probe, rpL8 (Durbin *et al.*, 1988), as a loading control. Free dNTPs were separated by selective precipitation of the labelling reaction using column chromatography (Severson & Kassner, 1995). Hybridizations and washes were conducted at 60°C in glass bottles in a rotating oven (National Labnet). Membranes were washed initially in 2× SSC with 0.1% SDS at room temperature for 15 min and then at 65°C for 15 min, and then twice in 0.2× SSC with 0.1% SDS at 60°C for 15 min each. The membranes then were exposed to Kodak XAR film (Eastman-Kodak) at −70°C with an intensifying screen.

Molecular cloning

Degenerate primers were designed initially to the amino acid sequence of defensin isoforms A and C described by Lowenberger *et al.* (1995). RNA from the fat bodies of mosquitoes 8 h post-inoculation was extracted and reverse transcription-PCR (RT-PCR) (Sambrook *et al.*, 1989) was used to amplify the mature defensin sequence using degenerate primers at the 5′ end, and an oligo-dT primer. Subsequently, cDNA amplification was done using a Marathon cDNA Amplification Kit (Clontech, Palo Alto, Calif.), using primers internal to the mature defensin sequence, and linkers supplied with the kit to amplify regions upstream of the cDNA region coding for the mature peptides. PCR reactions used in this operation were: 94°C for 2 min (one cycle), 94°C for 1 min, 58°C for 1 min, 60°C for 4 min (×30 cycles). The products of these reactions were size-fractionated on a 1% agarose gel. The bands were excised from the gel and cloned into pGEM-T vector (Promega, Madison, Wis.) using the manufacturer's protocols. Blue-white screening of XL1-blue cells (Stratagene, USA) was used to identify potential transfor-

mants, and these colonies were grown overnight in 5 ml LB medium with 5 μl Ampicillin (100 μg/μl) and purified using the Wizard Plus Minipreps DNA Purification system (Promega, Madison, Wis.). Sequencing of these clones was done on an ABI 310 automatic sequencer (Perkin Elmer, USA) following the manufacturer's instructions. All sequencing reactions of confirmed full-length sequences were done at least twice from each direction using internal primers and SP6 and T7 flanking primers. Genomic clones were amplified using the same PCR protocol with primers designed from the cDNA sequences and 50 ng of genomic DNA (Severson *et al.*, 1993) from the Liverpool strain of *Ae. aegypti*. Nucleotide and deduced amino acid sequences were compared and aligned using the MEGALIGN program of DNASTAR (DNASTAR, Madison, Wis.).

Genetic linkage mapping of defensin

A PCR-generated probe coding for the mature protein region of isoform A, as described for Northern analyses, was used to probe a screening blot containing EcoR1 digested bulk genomic DNA from several strains of *Ae. aegypti* (see Severson *et al.*, 1993). The sequence used to generate this probe shares significant identity with isoforms B and C, suggesting that this probe would hybridize to DNA from all three isoforms of defensin. Two strains of *Aedes aegypti*, Red and Formosus, were found to be polymorphic for this probe. Subsequently, this probe was used for hybridization with a segregating F2 population Southern blot of a Formosus X Red cross backcrossed to Red, consisting of ninety-four lanes of DNA from individual mosquitoes. DNA extraction, digestion, size fractionation, transfer, hybridization and membrane washes have been described previously (Severson *et al.*, 1993; Severson & Kassner, 1995). The autoradiograph was scored and compared to other known markers for this genetic cross. Chi-square goodness-of-fit values were calculated for segregation and independent assortment ratios. Multipoint linkage analyses were performed using MapMaker computer program (Lander *et al.*, 1987). A minimum LOD threshold of 3 was used to identify linkage between markers.

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References

Beerntsen, B.T. and Christensen, B.M. (1990) *Dirofilaria immitis*: effect on hemolymph polypeptide synthesis in *Aedes aegypti* during melanotic encapsulation reactions against microfilariae. *Exp Parasitol* **71**: 406–414.

Beerntsen, B.T., Severson, D.W., Klinkhammer, J.A., Kassner, V.A. and Christensen, B.M. (1995) *Aedes aegypti*: a quantitative trait locus (QTL) influencing filarial worm intensity is linked to QTL for susceptibility to other mosquito-borne pathogens. *Exp Parasitol* **81**: 355–362.

Boman, H.G., Faye, I., Gudmundsson, G.H., Lee, J.Y. and Lidholm, D.A. (1991) Cell free immunity in *Cecropia*: a model system for antibacterial proteins. *Eur J Biochem* **201**: 23–31.

Brey, P.T., Lee, W.J., Yamakawa, M., Koizumi, Y., Perrot, S., Francois, M. and Ashida, M. (1993) Role of the integument in insect immunity: epicuticular abrasion and induction of cecropin synthesis in cuticular epithelial cells. *Proc Natl Acad Sci USA* **90**: 6275–6279.

Broekaert, W.F., Terras, F.R.G., Cammue, B.P.A. and Osborn, R.W. (1995) Plant defensins: novel antimicrobial peptides as components of the host defense system. *Plant Physiol* **108**: 1353–1358.

Bulet. P., Cociancich, S., Reuland, M., Sauber, S., Bishoff, R., Hegy, G., Van Dorsselaer, A., Hetru, C. and Hoffmann, J.A. (1992) A novel insect defensin mediates the inducible antibacterial activity in larvae of the dragonfly, *Aeschna cyanea* (Paleoptera Odonata). *Eur J Biochem* **209**: 977–984.

Casteels-Josson, K., Zhang, W., Capaci, T., Casteels, P. and Tempst, P. (1994) Acute transcriptional response of the honeybee peptide-antibiotics gene repertoire and required post-translational conversion of the precursor structures. *J Biol Chem* **269**: 28569–28575.

Chalk, R., Albuquerque, C.M.R., Ham, P.J. and Townson, H. (1995) Full sequence and characterization of two insect defensins: immune peptides from the mosquito *Aedes aegypti*. *Proc R Soc Lond* B **261**: 217–221.

Chalk, R., Townson, H. and Ham, P.J. (1995) *Brugia pahangi*: the effects of cecropins on microfilariae in vitro and in *Aedes aegypti*. *Exp Parasitol* **80**: 401–406.

Charlet, M., Chernysh, S., Philippe, H., Hetru, C., Hoffmann, J.A. and Bulet, P. (1996) Innate immunity: isolation of several cysteine-rich antimicrobial peptides from the blood of a mollusc, *Mytilus edulis*. *J Biol Chem* **271**: 21808–21813.

Cho, W.L., Fu, Y.C., Chen, C.C. and Ho, C.M. (1996) Cloning and characterization of cDNAs encoding the antibacterial peptide, Defensin A, from the mosquito, *Aedes aegypti*. *Insect Biochem Mol Biol* **26**: 395–402.

Chomczynski, P. and Sacchi, N. (1987) Single step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analyt Biochem* **162**: 156–159.

Christensen, B.M. and Severson, D.W. (1993) Biochemical and molecular basis of mosquito susceptibility to *Plasmodium* and filarioid nematodes. *Parasites and Pathogens of Insects*, Vol. 1. *Parasites* (Beckage, N.E., Thompson, S.E. and Federici B.A., eds), pp. 245–266. Academic Press, San Diego.

Christensen, B.M. and Forton, K.F. (1986) Hemocyte-mediated melanization of microfilariae in *Aedes aegypti*. *J Parasitol* **72**: 220–225.

Cociancich, S., Bulet, P., Hetru, C. and Hoffmann, J.A. (1994) The inducible antibacterial peptides of insects. *Parasitol Today* **10**:132–139.

Cociancich, S., Goyffon, M., Bontems, F., Bulet, P., Bouet, F., Mendez, A. and Hoffmann, J.A. (1993) Purification and characterization of a scorpion defensin, a 4 kDa antibacterial peptide presenting structural similarities with insect defensins and scorpion toxins. *Biochem Biophys Res Commun* **194**: 17–22.

Dimarcq, J.L., Hoffmann, D., Meister, M., Bulet, P., Lanot, R., Reichhart, J.M. and Hoffmann, J.A. (1994) Characterization and transcriptional profiles of a *Drosophila* gene encoding an insect defensin. *Eur J Biochem* **221**: 201–209.

Dimarcq, J.L., Zachary, D., Hoffmann J.A., Hoffmann, D. and Reichhart, J.M. (1990) Insect immunity: expression of the two major inducible antibacterial peptides, defensin and diptericin, in *Phormia terranovae*. *EMBO J* **9**: 2507–2515.

Dunn, P.E. (1986) Biochemical aspects of insect immunology. *Annu Rev Entomol* **31**: 321–329.

Durbin, J.E., Swerdel, M.R. and Fallon, A.M. (1988) Identification of cDNAs corresponding to mosquito ribosomal protein genes. *Biochim Biophys Acta* **950**: 182–192.

Ehret-Sabatier, L., Loew, D., Goyffon, M., Fehlbaum, P., Hoffmann, J.A., Dorsselaer, A. van and Bulet, P. (1996) Characterization of novel cysteine-rich antimicrobial peptides from scorpion blood. *J Biol Chem* **271**: 29537–29544.

Evans, E.W. and Harmon, B.G. (1995) A review of antimicrobial peptides: defensins and related cationic peptides. *Vet Clin Pathol* **24**: 109–116.

Fujiwara, S., Imai, J., Fujiwara, M., Yaeshima, T., Kawashima, T. and Kobayashi, K. (1990) A potent antibacterial protein in royal jelly: purification and determination of the primary structure of royalisin. *J Biol Chem* **265**: 11333–11337.

Ganz, T. and Lehrer, R.I. (1995) Defensins. *Pharmac Ther* **66**: 191–205.

Gwadz, R.W., Kaslow, D., Lee, J.Y., Maloy, W.L., Zasloff, M. and Miller, L.H. (1989) Effects of magainins and cecropins on the sporogonic development of malaria parasites in mosquitoes. *Infect Immun* **57**: 2628–2633.

Hanazawa, H., Shimada, I., Kuzuhara, T., Komano, H., Kohda, D., Inagki, F., Natori, S. and Arata, Y. (1990) ^{1}H nuclear magnetic resonance study of the solution conformation of an antibacterial protein, Sapecin. *FEBS Lett* **269**: 413–420.

Hetru, C., Bulet, P., Cociancich, S., Dimarcq, J.L., Hoffmann, D. and Hoffmann, J.A. (1994) Antibacterial peptides/polypeptides in the insect host defense. *Phylogenetic Perspectives in Immunity: the Insect Host Defense* (Hoffmann, J.A., Janeway, C.A. and Natori, S., eds), pp. 43–65. S.R.G. Landes Company, Austin, Texas.

Hetru, C., Hoffmann, D. and Bulet, P. (1998) Antimicrobial peptides from insects. *Molecular Mechanisms of Immune Responses in Insects* (Brey, P.T. and Hultmark, D., eds), pp. 40–66. Chapman and Hall, London.

Hoffmann, J.A. and Hetru, C. (1992) Insect defensins: inducible antibacterial peptides. *Immunol Today* **13**: 411–415.

Hoffmann, J.A., Reichhart, J.M. and Hetru, C. (1996) Innate immunity in higher insects. *Curr Opin Immunol* **8**: 8–13.

Hubert, F., Noel, T. and Roch, P. (1996) A member of the arthropod defensin family from edible Mediterranean mussels (*Mytilus galloprovincialis*). *Eur J Biochem* **240**: 302–306.

Lackie, A.M. (1988) Immune mechanisms in insects. *Parasitol Today* **4**: 98–105.

Lander, E.S., Green, P., Abrahamson, J., Barlow, A., Daly, M., Lincoln, S. and Newberg, L. (1987) MapMaker: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* **1**: 174–181.

Lee, Y.L., Chung, T.J., Park, C.W., Hahn, Y., Chung, J.H., Lee, B.L., Han, D.M., Jumg, Y.H., Kim, S. and Lee, Y. (1996) Structure and expression of the tenecin 3 gene in *Tenebrio molitor*. *Biochem Biophys Res Commun* **218**: 6–11.

Lehane, M.J., Wu, D. and Lehane, S.M. (1997) Midgut-specific immune molecules are produced by the blood-sucking insect *Stomoxys calcitrans*. *Proc Natl Acad Sci USA* **94**: 11502–11507.

Li, J. and Christensen, B.M. (1990) Immune competence of *Aedes trivittatus* hemocytes as assessed by lectin binding. *J Parasitol* **76**: 276–278.

Lidholm, D.A., Gudmundsson, G.H., Xanthopoulos, K.G. and Boman, H.G. (1987) Insect immunity: cDNA clones coding for the precursor forms of cecropins A and D, antibacterial proteins from *Hyalophora cecropia*. *FEBS Lett* **226**: 8–12.

Lowenberger, C.A., Bulet, P., Charlet, M., Hetru, C., Hodgeman, B., Christensen, B.M. and Hoffmann, J.A. (1995) Insect immunity: isolation of three novel inducible antibacterial defensins from the vector mosquito, *Aedes aegypti*. *Insect Biochem Mol Biol* **25**: 867–873.

Lowenberger, C.A. (1996) Insect immunology: mosquito immune responses. *Lab Animal* **25**: 30–33.

Lowenberger, C., Ferdig, M.T., Bulet, P., Khalili, S., Hoffmann, J.A. and Christensen, B.M. (1996) *Aedes aegypti*: induced antibacterial proteins reduce the establishment and development of *Brugia malayi*. *Exp Parasitol* **83**: 191–201.

Lowenberger, C.A., Kamal, S., Chiles, J., Paskewitz, S., Bulet, P., Hoffmann, J.A. and Christensen, B.M. (1999) Mosquito–*Plasmodium* interactions in response to immune activation of the vector. *Exp Parasitol* (in press).

Matsumoto, N., Okada, M., Takahashi, H., Qu, X.M., Nakajima, Y., Nakanishi, Y., Komano, H. and Natori, S. (1986) Molecular cloning of a cDNA and assignment of the C-terminal of sarcotoxin IA, a potent antibacterial protein of *Sarcophaga peregrina*. *Biochem J* **239**: 717–722.

Matsuyama, K. and Natori, S. (1988) Molecular cloning of cDNA for sapecin and unique expression of the sapecin gene during the development of *Sarcophaga peregrina*. *J Biol Chem* **263**: 17117–17121.

Moon, H.J., Lee, S.Y., Kurata, S., Natori, S. and Lee, B.L. (1994) Purification and molecular cloning of cDNA for an inducible antibacterial protein from larvae of the Coleopteran, *Tenebrio molitor*. *J Biochem* **116**: 53–58.

Penninckx, I.A.M.A., Eggermont, K., Terras, F.R.G., Thomma, B.P.H.J., De Samblanx, G.W. Buchala, A., Metraux, J.P., Manners, J.M. and Broekaert, W.F. (1996) Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. *Plant Cell* **8**: 2309–2323.

Reichhart, J.M., Essrich, M., Dimarcq, J.L., Hoffmann, D., Hoffmann, J.A. and Lagueux, M. (1989) Insect immunity: isolation of cDNA clones corresponding to diptericin, an inducible antibacterial peptide from *Phormia terranovae* (Diptera) transcriptional profiles during immunization. *Eur J Biochem* **182**: 423–427.

Richman, A.M., Bulet, P., Hetru, C., Barillas-Mury, C., Hoffmann, J.A. and Kafatos, F.C. (1996) Inducible immune factors of the vector mosquito *Anopheles gambiae*: biochemical purification of a defensin antibacterial peptide and molecular cloning of preprodefensin cDNA. *Insect Molec Biol* **5**: 203–210.

Richman, A.M., Dimopoulos, G., Seeley, D. and Kafatos, F.C. (1997) *Plasmodium* activates the innate immune response of *Anopheles gambiae* mosquitoes. *EMBO J* **16**: 6114–6119.

Richman, A.M. and Kafatos, F.C. (1995) Immunity to eukaryotic parasites in vector insects. *Curr Opin Immunol* **8**: 14–19.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York.

Severson, D.W. and Kassner, V.A. (1995) Analysis of mosquito genome structure using graphical genotyping. *Insect Mol Biol* **4**: 279–286.

Severson, D.W., Mori, A., Zhang, Y. and Christensen, B.M. (1993)

Linkage map for *Aedes aegypti* using restriction fragment length polymorphisms. *J Hered* **84**: 241–247.

Severson, D.W., Mori, A., Zhang, Y. and Christensen, B.M. (1994) Chromosomal mapping of two loci affecting filarial worm susceptibility in *Aedes aegypti*. *Insect Mol Biol* **3**: 67–73.

Severson, D.W., Mori, A., Kassner, V.A. and Christensen, B.M. (1995) Comparative linkage maps for the mosquitoes, *Aedes albopictus* and *Ae. aegypti*, based on common RFLP loci. *Insect Mol Biol* **4**: 41–45.