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Insect immunity: expression of the two major inducible antibacterial peptides, defensin and diptericin, in *Phormia terranovae*

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Injections of low doses of bacteria into larvae of *Phormia terranovae* induce the appearance of potent bactericidal peptides in the blood, among which predominate the anti-Gram positive insect defensins and the anti-Gram negative diptericins. Insect defensins show significant homologies to mammalian (including human) microbicidal peptides present in polymorphonuclear leukocytes and macrophages. We report the molecular cloning of cDNAs and primer extension studies which indicate that insect defensin is produced as a prepro-peptide yielding mature defensin A (40 residues) after cleavage of a putative signal peptide (23 residues) and a prosequence (34 residues). Previous studies have established that diptericin (82 residues) is matured from a pre-peptide by cleavage of a putative signal peptide (19 residues) and C-terminal amidation. Using oligonucleotide probes complementary to the sequences of the mRNAs for defensin and diptericin, we show by *in situ* hybridization that both antibacterial peptides are concomitantly synthesized by the same cells: thrombocytoids, a specialized blood cell type, and adipocytes. Transcriptional studies based on hybridization of RNAs to cDNAs of defensin and diptericin indicate that the transcription of both genes is induced regardless of the nature of the stimulus (injection of Gram positive or Gram negative bacteria, lipopolysaccharides). Even a sterile injury applied to axenically raised larvae is efficient in inducing the transcription of both genes suggesting that the local disruption of the integument aspecifically initiates a signalling mechanism which the thrombocytoids and the adipocytes are able to interpret. The transcription of immune genes is relatively short lived and a second challenge yields a response similar to that of the first stimulus, indicating that the experimental insects do not keep a 'memory' of their first injection.

Key words: antibacterial peptides/defensin/diptericin/insect immunity

Introduction

The antibacterial defence reactions in higher insect orders, namely Lepidoptera, Diptera and Hymenoptera, are characterized by the synthesis of a battery of potent bactericidal peptides. Several families of peptides have been isolated and their primary structures characterized in recent years: cecropins (4 kd) (Steiner *et al.*, 1981), attacins (>20 kd) (Hultmark *et al.*, 1983), diptericins (9 kd) (Dimarcq *et al.*, 1988), insect defensins (4 kd) (Lambert *et al.*, 1989) and apidaecins (2 kd) (Casteels *et al.*, 1989) [and several homologues referred to as sarcotoxins and sapecins, see Okada and Natori (1985); Ando and Natori (1988); Matsuyama and Natori (1988a)]. These bactericidal molecules are mostly cationic peptides with a broad spectrum of activity against Gram positive and/or Gram negative bacteria.

Among these inducible antibacterial peptides, insect defensins represent a remarkable group. They are small cationic molecules (40 residues) with six cysteines engaged in three intramolecular bridges (Lambert *et al.*, 1989). So far they have been identified in only two dipteran insects (immune *Phormia terranovae*, Lambert *et al.*, 1989; culture medium of a *Sarcophaga* cell line, Matsuyama and Natori, 1988a). They are, however, probably widespread in the animal kingdom as they have structural and functional analogues in mammals. Lehrer and associates have shown that polymorphonuclear leukocytes contain an array of antibacterial peptides that contribute to their host defence (Klebanoff, 1988; Ganz *et al.*, 1986). Six rabbit, one guinea pig and three human defensin peptides have been purified and their amino-acid sequence determined (Selsted *et al.*, 1985a,b; Selsted and Harwig, 1987). Although among these mammalian defensins, the interspecies amino-acid sequence homology is low (27 to 45%), they all contain eight interspersed conserved amino-acids, including six cysteines involved in intrachain disulfide bonds, as is the case for insect defensins.

Defensins are primarily active against Gram positive bacteria, but various mammalian defensins have been reported to show significant *in vitro* activity against fungi (Ganz *et al.*, 1985; Lehrer *et al.*, 1985) and enveloped viruses (Lehrer *et al.*, 1985; Daher *et al.*, 1986). In the Dipteran *P.terranovae*, the anti-Gram positive defensins, together with the anti-Gram negative diptericins, account for the bulk of the inducible antibacterial response as evidenced by growth inhibition assays (data from Keppi *et al.*, 1986; Dimarcq *et al.*, 1988; Lambert *et al.*, 1989).

In the present paper, we have addressed the following questions: (i) which cell types express the defensin and the diptericin genes during the immune response; previous studies from *Hyalophora* (Faye and Wyatt, 1980; Trenczek, 1988) and *Manduca* (Dickinson *et al.*, 1988) strongly suggested that the fat body was involved in the synthesis of immune peptides whereas studies by Natori and associates found expression of some immune peptides in fat body and undefined blood cells of *Sarcophaga* (Matsumoto *et al.*, 1986; Ando and Natori, 1988; Matsuyama and Natori, 1988b). In particular, we were interested to know whether both genes are expressed in the same cell types. (ii) Is the immune response influenced by the stimulus? In other words, are the two types of genes, coding for peptides with markedly

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different antibacterial spectra, induced concomitantly regardless of the stimulus (e.g. injection of Gram positive or Gram negative bacteria, lipopolysaccharides or injury) or can a preferential induction be detected? (iii) Once stimulated do insects react differently to a second challenge, i.e. do they keep a 'memory' of the first induction? We had previously cloned cDNAs encoding a diptericin of *P.terranovae* (Reichhart *et al.*, 1989) and have now cloned a cDNA encoding defensin A, in order to address these questions.

Results

Molecular cloning of defensin cDNA

Wandering, crop-half-empty, 3rd instar larvae of *P.terranovae* were injected with live *Enterobacter cloacae* β12 to enrich the mRNA for defensins. After 6 h, the fat bodies contaminated with haemocytes (presumed to participate in immune response, see references in Introduction) were excised and the polyadenylated RNA was extracted. A cDNA library was prepared in bacteriophage λgt11 and screened with oligonucleotide probes, complementary to residues 30–35 of defensin A, the predominant defensin of immune *Phormia* (the isologue defensin B differs from A by the replacement of a Gly in position 32 by an Arg; see Lambert *et al.*, 1989). Because of the multiplicity of the codons, we devised four 17mer probe pools, each with 18 possibilities: these probe pools were end-labelled and separately hybridized on Northern blots with poly(A)-enriched RNA extracted from immune and control fat bodies. The probe pool 5′ AC A/G/TCC CTT A/G/TCC GTT A/GCA 3′ gave a strong signal with a 0.7 kb immune RNA, plus a signal at 1.1 kb (data not shown). This probe was used to screen the library. Five positive clones were isolated from 30 000 insert-containing phage and sequenced. Two were found to encode a defensin A precursor peptide. Sequencing of mRNA by primer extension with a probe complementary to residues 14 to 20 as primer (5′ ACA GTG TGC AGC ACA AGC CGA 3′) provided additional information on the 5′ region; the sequencing signals were unambiguous, indicating the presence of a single mRNA type.

As shown in Figure 1, an open reading frame of 282 nucleotides starting with an ATG codon is present in the sequence. It codes for a peptide of 94 residues with a calculated molecular weight of 10 098 daltons. The peptide starts with a putative signal sequence which most likely comprises the first 23, mostly hydrophobic residues (see Figure 2 for hydropathy plot) and ends with an Ala, which is conventional for this type of peptide [e.g. diptericin in *Phormia* (Reichhart *et al.*, 1989); cecropin in *Hyalophora* (Lidholm *et al.*, 1987) and *Sarcophaga* (Matsumoto *et al.*, 1986)]. If our assumption on the length of the signal peptide is correct, the rest of the molecule consists of a pro-defensin of 71 residues. After the first 29 N-terminal residues, this pro-defensin shows a potential proteolytic cleavage site Lys–Arg, which is followed by the full sequence of defensin A. Interestingly the codon of the last residue in the messenger is immediately followed by a stop codon, indicating that defensin A is not amidated, which corroborates our previous mass spectrometric data on the native peptide (Lambert *et al.*, 1989).

The protein coding region is preceded by a stretch of 35 untranslated nucleotides and followed by a 3′ non-coding

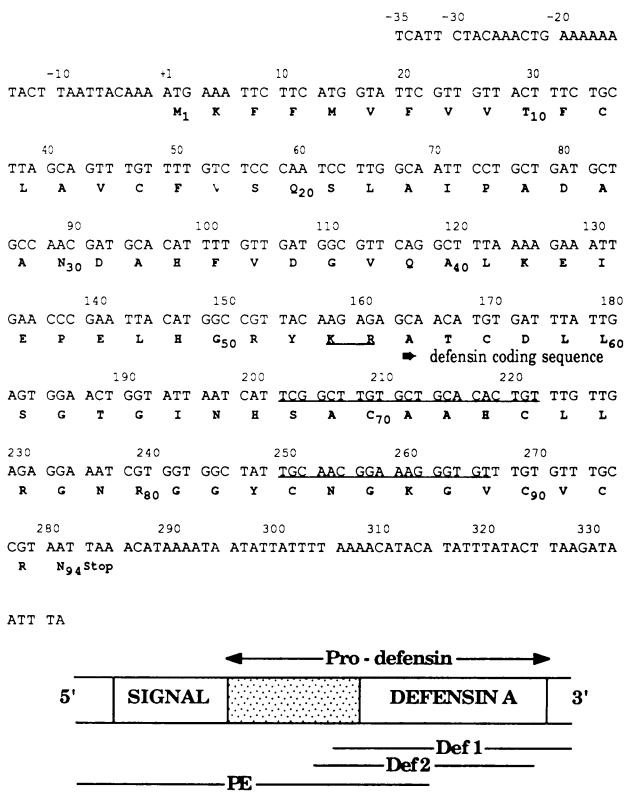

Fig. 1. Nucleotide sequence encoding prepro-defensin A of *P.terranovae*. The information is based on the sequences of two overlapping cDNAs (Def 1, Def 2) and primer extension experiments (PE). The deduced amino acid sequence is shown below the nucleotide sequence. Amino acid residues are numbered starting with the first methionine residue. Underlined regions correspond (i) to sequences complementary to the two oligonucleotides used in this study (a 21mer oligonucleotide and a pool of 17mer probes, see Materials and methods), and (ii) to the potential K-R proteolytic cleavage site.

region of 52 nucleotides which is however incomplete as it does not contain the polyadenylation consensus sequence.

The biosynthetic origin of insect defensin and diptericin detected by in situ hybridization

An oligonucleotide probe complementary to the nucleotide sequence coding for residues 14–20 of defensin A (Figure 1, underlined) was used to investigate the presence of mRNAs encoding defensin in various tissues of axenically raised immune wandering, crop-half-empty, larvae of *P. terranovae*.

Of all tissues investigated at various time intervals after a bacterial challenge (injection of 10^4 heat-killed *Escherichia coli*), only fat body cells and some blood cells gave a positive result. Cells of normal larvae never hybridized with the probe. When an anti-sense probe was applied to immune larvae, no signal was observed (data not shown).

Fat body cells. A systematic analysis of fat body cells in the head, thorax and abdomen of the larvae showed similar levels of hybridization with the probe, indicating that all of the fat body cells participate in the production of defensin. We have undertaken a similar study for diptericin, using an oligonucleotide probe complementary to the nucleotide sequence encoding residues 7 to 13 of diptericin (5′ TGG AGC AGG TGT AGG CAA TAT 3′) (Reichhart *et al.*, 1989). All fat body cells gave a distinct signal in immune

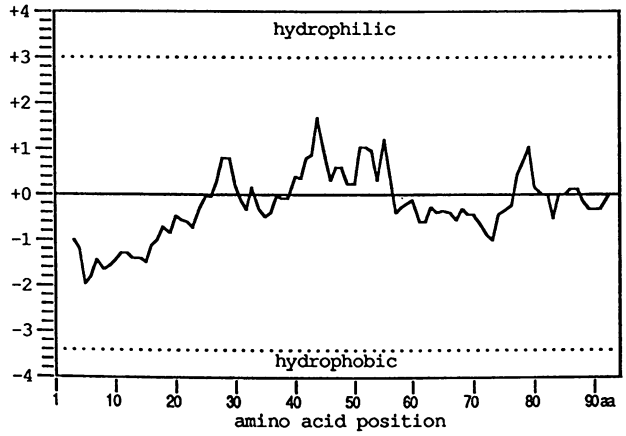

Fig. 2. Hydropathy analysis of *Phormia* prepro-defensin A. The distribution of hydrophobic and hydrophilic domains was determined by computer analysis [DNA Inspector^{TM} Ile (Textco) software]. Numbers of amino acid residues are shown at the bottom. Data presented as hydrophilic and hydrophobic portions are plotted above and below the horizontal line.

larvae but not in normal larvae of the same age. Figure 3 illustrates the results obtained with the use of the defensin and the diptericin probes in serial sections of abdominal fat body and demonstrates that both genes are transcribed in the same cells.

Blood cells. Wandering larvae of *P.terranovae*, like those of the related cyclorrhaphous dipteran *Calliphora erythrocephala*, contain in the circulating blood (haemolymph) between 10 000 and 12 000 blood cells (haemocytes) per mm^3 which are produced in an abdominal haemopoietic tissue. Three cell types are distinguished on the basis of morphological and functional criteria: plasmatocytes, oenocytoids and thrombocytoids (Zachary and Hoffmann, 1973). The plasmatocytes represent some 80% of the blood cells. They are phagocytotic cells which are primarily involved in the uptake and degradation of various debris from the blood and namely of histolysing tissue fragments. As such, they play an essential role in metamorphosis. Surprisingly, we have never detected phagocytosis of injected live or heat-killed bacteria by these cells; furthermore, plasmatocytes did not hybridize with either the defensin or the diptericin probe. Oenocytoids are rare and unusually large haemocytes of unknown function. As the plasmatocytes, they did not hybridize with either probe in our conditions.

In contrast to plasmatocytes and oenocytoids, the thrombocytoids reacted strongly with both probes (Figure 4). Thrombocytoids are remarkable blood cells in many respects (see Figure 5 for an electron micrograph of a thrombocytoid). These large cells show some dense invaginations of their cytoplasmic membrane which dissect the peripheral cytoplasm. Eventually larger cytoplasmic compartments break off from the cell. Each cell fragment is surrounded by an intact plasma membrane and its normal cytoplasmic characteristics remain unaltered. When bacteria are injected into the body cavity of larvae, they are rapidly surrounded by thrombocytoid cell fragments which agglutinate and form intricate meshes encapsulating the bacteria. Thrombocytoid fragments also seal off wounds of the integument (Zachary and Hoffmann, 1973; D.Zachary, unpublished observations).

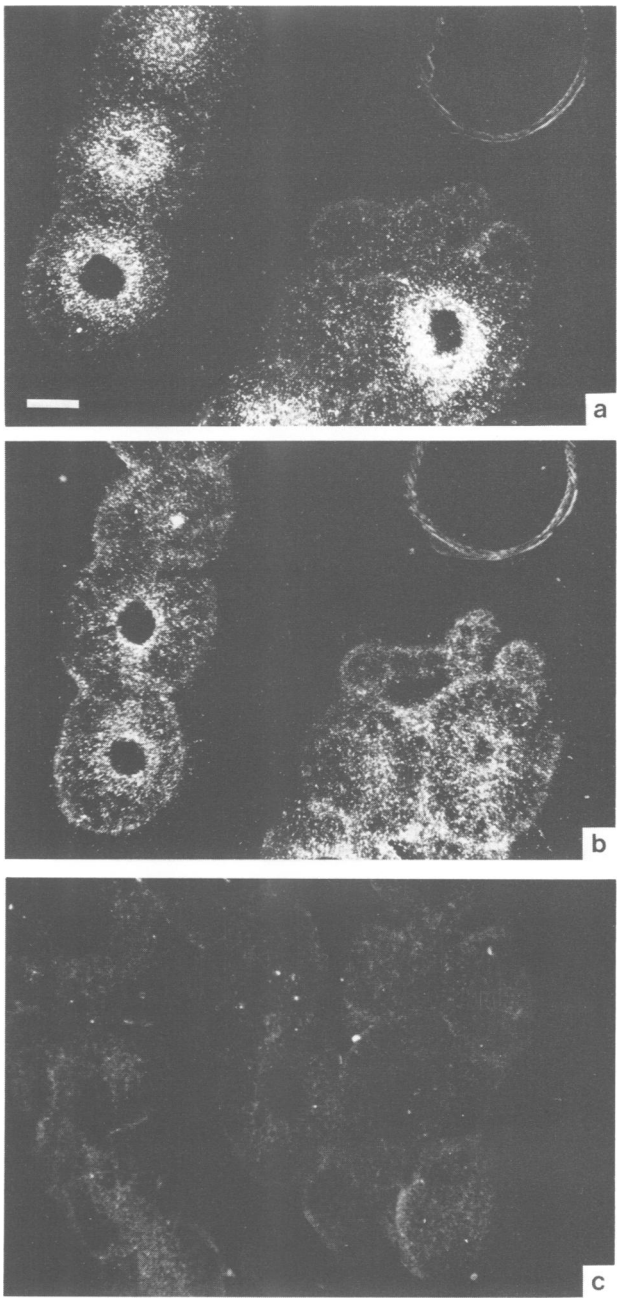

Fig. 3. Detection of defensin and diptericin mRNAs in adipocytes of axenically raised wandering larvae of *P.terranovae* using *in situ* hybridization. Paraffin sections of fat bodies from control and challenged larvae (6 h after the injection of 10^4 heat-killed *E.coli*) were hybridized with [γ-^{32}P]ATP end-labelled oligonucleotide probes, autoradiographed, stained with haematoxylin and viewed by dark field microscopy. **Panel a**: hybridization with the defensin probe. **Panel b**: hybridization with the diptericin probe [(a) and (b) are serial sections from challenged larvae]. **Panel c**: absence of hybridization in adipocytes of unchallenged larvae with the defensin probe. Negative results were also obtained when the diptericin probe was used in unchallenged larvae or an anti-sense defensin probe was used in challenged larvae (data not shown). Bar represents 40 μm.

In some respects, the thrombocytoids present a morphological and functional analogy with the mammalian megakaryocyte, the agglutination of anucleated cytoplasmic fragments recalling the formation of a white 'thrombus',

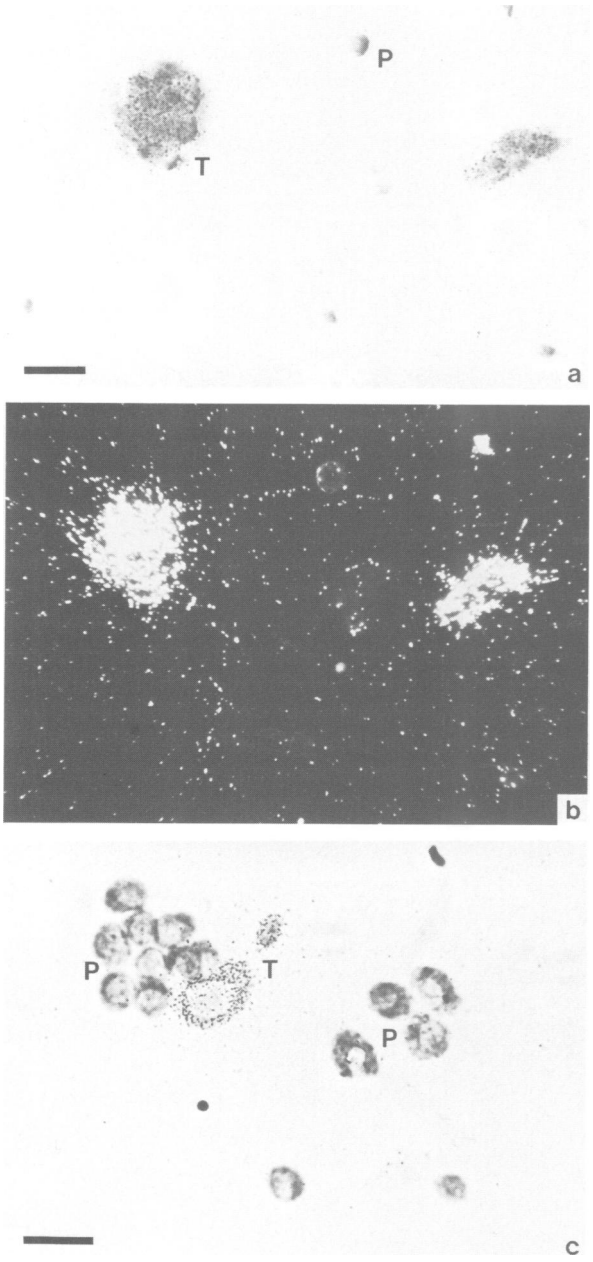

Fig. 4. Expression of defensin and diptericin genes in blood cells of *P.terranovae* using *in situ* hybridization. Haemocyte smears from challenged axenically raised wandering larvae of *P.terranovae* (6 h after the injection of 10^4 heat-killed *E.coli*). Smears were hybridized with [γ-^{32}P]ATP end-labelled oligonucleotide probes. The slides were autoradiographed, stained with haematoxylin and examined under bright or dark field. **Panels a** and **b**: hybridization with the defensin probe, bright field (a) and dark field (b). Bar represents 40 μm. **Panel c**: hybridization with the diptericin probe, bright field. Bar represents 20 μm. P, plasmatocyte; T, thrombocytoid.

which prompted the designation of this cell type as thrombocytoid (Zachary and Hoffmann, 1973). In view of these morphological and functional observations, it is of particular interest to see that thrombocytoids are the blood cell type synthesizing both antibacterial peptides, defensins and diptericins.

Transcriptional profiles for defensins and diptericins

Transcriptional profiles for defensins and diptericins were investigated using respective labelled cDNA fragments as probes on Northern blots of total RNA from immune and the normal fat body of wandering, crop-half-empty, 3rd instar larvae of *Phormia*. The insects were raised axenically to avoid uncontrolled wound infection before and after the experimental inoculation.

Three types of immunization were performed: (i) an epidermal injury produced with a sterile needle; (ii) the injection of either the Gram negative *E.coli* or the Gram positive *Micrococcus luteus*; these bacteria were heat killed before being injected at the dose of 10^4 cells/2 μl saline or 10^5 cells/2 μl saline; (iii) the injection of lipopolysaccharide, a major component of the outer membrane of Gram negative bacteria (doses of 100 ng/2 μl or 1 μg/2 μl per larva were injected). Injections of live bacteria were not used in these experiments as they frequently lead to uncontrolled individual infections when performed on axenically raised larvae. This is an obvious drawback in a study aimed at comparing the effects of various stimuli on groups of larvae. For Northern blotting experiments, total RNA was extracted from fat body of the larvae after respectively 8 h, 24 h and 48 h. Hybridization at high stringency was first performed with labelled defensin cDNA and after dehybridization of the filters, with diptericin cDNA under the same conditions.

Both probes gave clear signals in challenged insects. The defensin probe hybridized to a major band at ~0.7 kb and gave an additional signal at ~1.1 kb (a signal at 2.4 kb obtained with defensin probe is interpreted as ribosomal RNA). The diptericin gave only one signal at ~0.65 kb which corroborates our previous results (Reichhart *et al.*, 1989) on the length of the mRNA of diptericin. The results presented in Figure 6 call for the following comments. (i) In untreated axenically raised larvae of *Phormia* no transcription of either defensin or diptericin genes is detected (Figure 6, IA and IC). (ii) A sterile injury of these larvae is sufficient to turn on the transcription of both types of genes (IB and ID). (iii) The injection of heat-killed Gram negative bacteria induces a very strong response, as seen after 8 h in Figure 6 (IIA and IIC), both for defensin and diptericin genes. The signal has disappeared at 24 h (defensin) or 48 h (diptericin) after inoculation. When similar experiments are performed by injecting a 10 times higher concentration of heat-killed bacteria, the signal is obviously stronger for both types of transcripts and persists for a longer time (Figure 6, IIB and IID). Undoubtedly a more intense challenge in terms of the number of injected heat-killed bacteria results in a stronger, longer-lasting response as monitored by these hybridization experiments. Parallel inoculations of heat-killed Gram positive *M.luteus* yielded similar results (data not shown). (iv) When heat-killed bacteria are replaced by LPS (from the Gram negative *Serratia marcescens*), the transcriptional profiles for both defensin and diptericin remain roughly similar. Again, a stronger response is observed with a higher dose (Figure 6, IIIA, B, C and D).

In the 48 h tracks on Figure 6II and in the 24 h tracks on Figure 6III, a shift in mRNA size is observed. For diptericin, it has been previously demonstrated (Reichhart *et al.*, 1989) that such a shift can be fully explained by a difference in the length of the poly(A) tail. The same phenomenon could explain the decrease of the length of defensin mRNA.

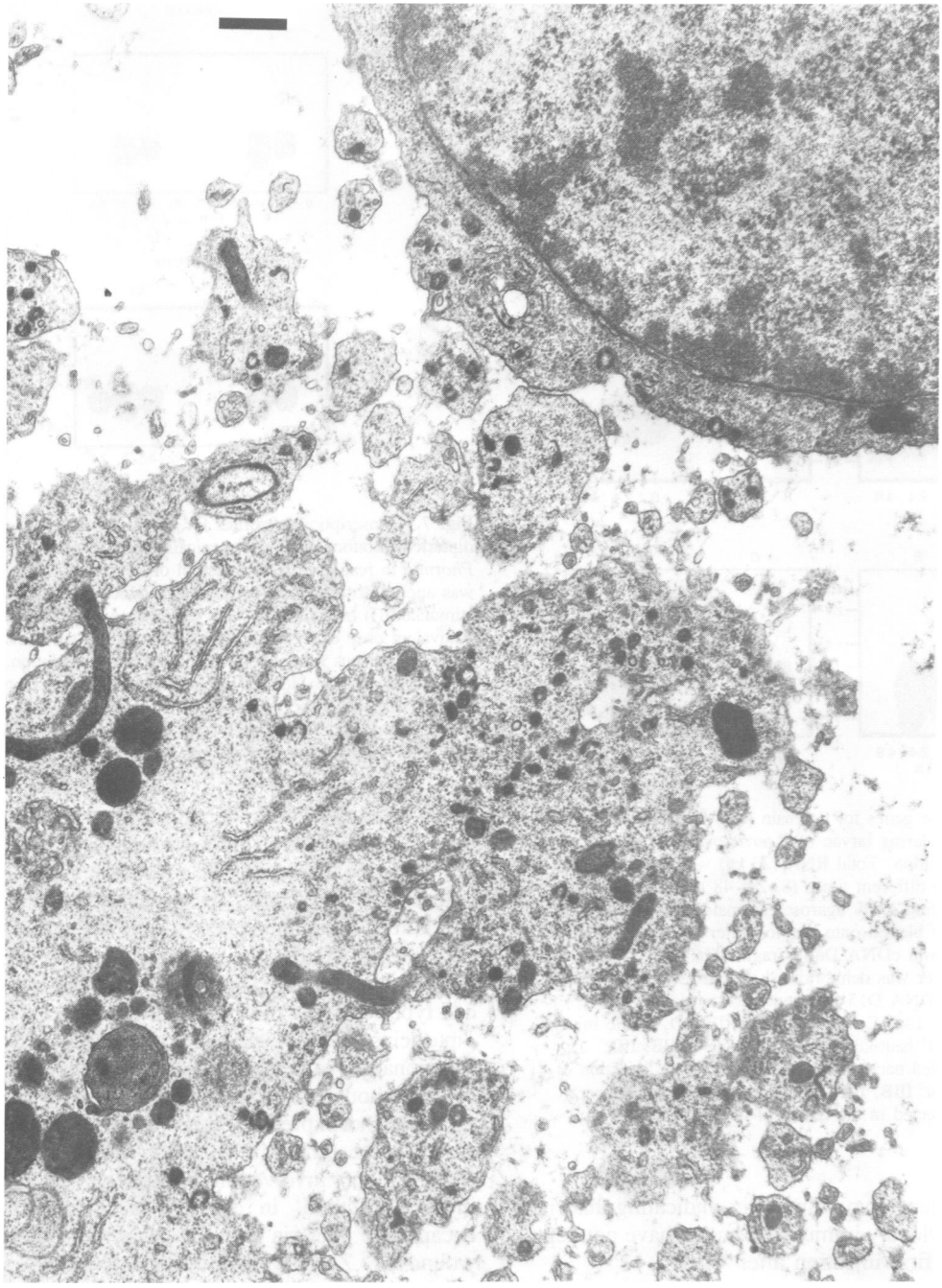

Fig. 5. Electron micrograph of a thrombocytoid after the process of fragmentation. The large nucleus appears surrounded by a thin layer of cytoplasm at this stage and numerous cytoplasmic fragments of various sizes are seen in section. Note that the cytoplasmic membranes are intact and that the normal cytoplasmic characteristics remain unaltered in the cell fragments. Rough surfaced endoplasmic cisternae, Golgi complexes and numerous lysozymal structures of various sizes are conspicuous. Bar represents 0.5 μm.

Surprisingly, defensin and diptericin transcripts are still present in significant amounts in larvae which have simply been injured under sterile conditions (IB, ID), whereas in the other types of stimulation they have either disappeared at that stage (IIA, IIC) or have markedly decreased (and undergone partial degradation) (IIB, IID, IIIA–D). This could reflect mRNA turnover and/or stability differences.

A low signal at 24 h (IB) is observed. The stimulus, consisting of a sterile injury, may be of low efficiency and possibly could explain the rather atypical transcription profile in this case.

In all these experiments, no hybridization signal was observed for either defensin or diptericin at 72 h post-inoculation (data not shown). We have submitted insects which have received an injection of 100 ng of LPS to a second injection after 48 h and compared the transcriptional profiles with those of larvae of the same age receiving a first challenge. As shown in Figure 7, these profiles are essen-

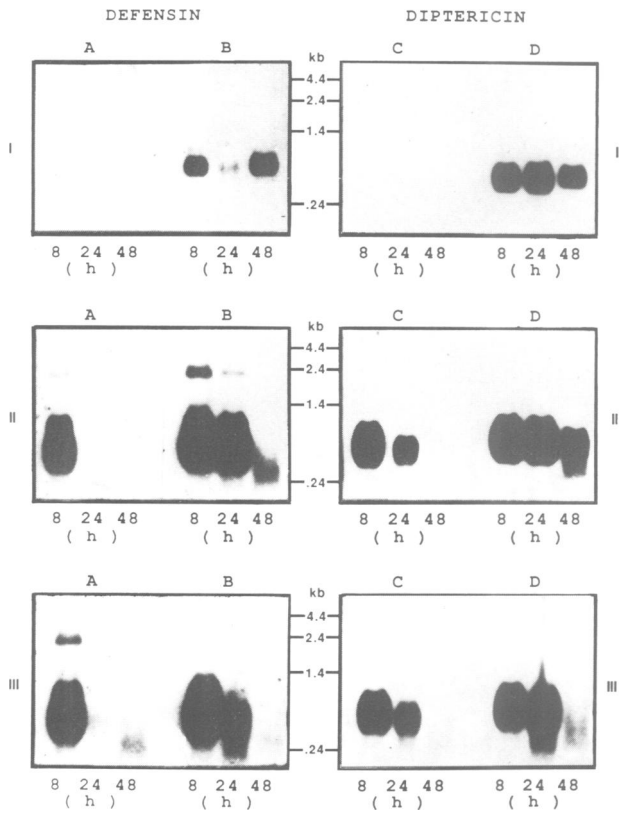

Fig. 6. Expression of the genes for defensin (left) and diptericin (right) in axenically raised wandering larvae of *P.terranovae* in response to different types of stimulation. Total RNA (20 μg) was extracted from fat bodies (10 larvae) at different times (8, 24, 48 h) after stimulation and subjected to denaturing 1.5% agarose–formaldehyde gel electrophoresis. The RNA was blotted onto a nylon filter and hybridized with the nick-translated defensin cDNA Def 1 fragment (Figure 1). After autoradiography, the filter was dehybridized and subjected to a second hybridization with the cDNA D15 fragment encoding diptericin (Reichhart *et al.*, 1989). IA, IC, normal larvae; IB, ID, sterile injury; IIA, IIC, injection of 10^4 heat-killed *E.coli* per larva; IIB, IID, injection of 10^5 heat-killed bacteria per larva; IIIA, IIIC, injection of 100 ng of LPS per larva; IIIB, IIID, injection of 1 μg of LPS per larva. Markers are presented in the centre of the figure.

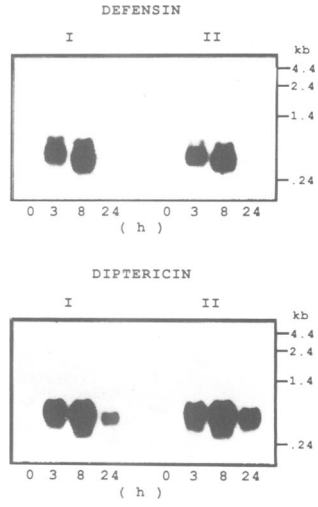

Fig. 7. Transcriptional profiles for defensin (upper panel) and diptericin (bottom panel) in axenically raised wandering larvae of *Phormia* in response to an injection of 100 ng of LPS. The challenge was applied to larvae of the same age which had either never been stimulated (I) or submitted 48 h earlier to a previous injection of an equivalent dose of LPS (II). Total RNA (20 μg) was extracted from fat bodies at various times (0, 3, 8, 24 h) after stimulation, subjected to 1.5% agarose/formaldehyde gel electrophoresis and blotted onto a nylon membrane. The filters were first hybridized with the cDNA Def 1 encoding defensin, then with the diptericin cDNA D15 as in Figure 6. Markers are presented at the right of the figure.

tially similar for defensin and diptericin, indicating that, in our conditions, previously immunized larvae have not kept a 'memory' of the first injection after 48 h.

Discussion

Although significant information has been accumulated on various aspects of the immune response in the higher insect orders over the last years, it appeared important to investigate the expression of genes coding for peptides with markedly different antibacterial spectra. *Phormia* obviously represents a favourable model system as it produces predominantly one family of anti-Gram positive molecules, the defensins, and one family of anti-Gram negative peptides, the diptericins during the response. No lysozyme is detected in these insects and cecropins account for only a minor percentage of the antibacterial activity of the blood of immune *Phormia*.

A first clear result concerns the biosynthetic origin of both peptides. Two different cell types, the adipocytes and the thrombocytoids, both of mesodermal origin (Anderson, 1972; Mori, 1979) produce defensins and diptericins. No regional specialization in the fat body cells was detected with our approach: apparently all cells participate similarly in the reaction. This excludes the possibility of the presence of specialized immunocompetent cells integrated into fat body complexes, similar to Kuppfer cells in the liver of vertebrates. We were surprised to note that among the blood cell types only the thrombocytoids produced defensins and diptericins. These cells morphologically and, in part, functionally evoke mammalian megakaryocytes. Plasmatocytes, although capable of active phagocytosis of various debris, do not express the genes for the antibacterial peptides. This is in keeping with the observation that these cells are apparently not involved in phagocytosis or encapsulation of injected bacteria, in contrast to thrombocytoids which encapsulate bacteria and play an active role in sealing off wounds (D.Zachary, unpublished observations).

Diptericins have no reported counterpart in vertebrates, but insect defensins have several homologues in mammals. In humans, defensin RNA synthesis occurs primarily in neutrophil precursor cells in normal bone marrow. A functional equivalent in *Phormia* would be the abdominal haematopoietic tissues which, however, do not synthesize insect defensins in unchallenged insects.

In the context of the structural and functional similarities between human and insect defensins, two differences have to be pointed out. (i) In humans, the defensins appear to be constitutively expressed in normal bone marrow, whereas only cells of challenged insects transcribed these genes in this study. This statement must be somewhat modified as in mammals the reaction may be increased or even induced in some cells; for instance, in the rabbit, an increased content of two defensins in alveolar macrophages can be elicited by complete Freund's adjuvant and it has been suggested that

human defensin synthesis may be inducible in macrophages and perhaps polymorphonuclears, in response to appropriate signals (Daher *et al.*, 1988). (ii) The second difference concerns the fact that in insects, the defensins are secreted into the haemolymph and account for part of the circulating antibacterial activity, whereas in mammals these molecules apparently remain within the cells where they contribute as a major group of neutrophil granule proteins to the microbicidal activity of these cells.

Finally, as regards defensins, our results are consistent with Northern blot analysis of Matsuyama and Natori (1988b) who have detected in the Dipteran *Sarcophaga peregrina* the expression of a defensin-related antibacterial peptide (referred to as sapecin) in (undefined) haemocytes. These authors stated that fat bodies gave a positive result which could be related to contaminating haemocytes. This point is clarified by our *in situ* hybridization data. Matsuyama and Natori initially observed constitutive expression of this peptide in an embryonic cell line, which raises several interesting questions as regards the control of the expression of these genes, which we will come back to in a future study (in preparation).

The second major problem addressed in this paper concerns the possible relationship between the nature of the stimulus and the expression of the anti-Gram positive and/or anti-Gram negative peptides. Again, the answer is relatively clearcut—regardless of the nature of the stimulus (including injection of Gram negative or Gram positive bacteria), both types of genes are concomitantly transcribed. Furthermore a sterile injury induces the expression of both genes in these axenically raised larvae which shows that the induction mechanism is independent of the presence of bacterial determinants and underlines the nonspecificity of this defence mechanism. The intensity of the reaction—and its duration—as judged by the hybridization signals shown in Figure 6 is actually influenced by the dose of foreign material which is injected. Similar results were obtained with larvae raised in their natural septic environment.

The third question pertains to the response of challenged insects to a second stimulus applied after sufficient time has elapsed to allow defensin and diptericin transcripts to disappear from the cells. We found that the two responses were equivalent in our conditions, which does not argue in favour of a mechanism allowing the insect to keep the 'memory' of the first injection.

Finally, our data on the structure of the messenger RNA show a marked similarity in the organization of the precursor peptide for insect defensin with that of human defensin. The domain organization, signal peptide/pro-sequence/mature defensin, is identical for both molecules. Human defensins are made as 94-amino acid precursor proteins (Daher *et al.*, 1988) (94 for insect defensin). Following signal peptide removal (putative length of the signal peptide: 19 residues in human defensin versus 23 residues in *Phormia* defensin) a 75-amino acid pro-defensin (71 for *Phormia*) is further cleaved to remove a 45 residue pro-sequence from mature defensin (30). In *Phormia*, a doublet of basic residues (Lys—Arg) is present just in front of the N-terminus of the mature defensin, whereas in human defensin an Arg—Lys doublet is located two residues upstream of the N-terminus of the mature peptide. Although the homology for the pro-sequences between *Phormia* defensin and human defensin is relatively low, their lengths are comparable which may reflect the necessity of these sequences for a correct tridimensional arrangement of the mature peptide which has three precisely ordinated disulphide bridges.

The organization of the defensin related sapecin precursor of a *S.peregrina* cell line (Matsuyama and Natori, 1988b), shows close homologies to that of *Phormia*, the major differences being in the pro-sequence, which is, however, of identical length.

In conclusion, wandering larvae of *Phormia* respond indifferently to a variety of stimuli by transcribing the genes for diptericins and defensins in the sessile fat body cells and the circulating thrombocytoids. The transcription of both types of genes occurs concomitantly in the same individual cells. The intensity of the reaction is increased when the amount of foreign material injected is higher. The synthesis of these antibacterial peptides appears to be induced by a general and non-specific mechanism which is independent of the presence of bacteria or bacterial membrane determinants. Even a sterile injury in axenic larvae is sufficient for the induction, suggesting that the disruption of the integument releases a signal or initiates a cascade of biochemical events, inducing the response. The identification of the signalling mechanism and the control of the coordinate transcription of the immune genes are obviously among the challenging questions in this field of research.

Materials and methods

Insects, immunization

The normal rearing of *P.terranovae* were as described previously (Dimarcq *et al.*, 1988; Reichhart *et al.*, 1989). Axenic raising of larvae was as follows: newly-laid eggs were first sterilized by several baths in 0.5 N NaOH (2 × 15 min) and 70% ethanol (2 × 10 min) and deposited into sterile boxes containing autoclaved food (pork kidney, yeast extract, beef extract and bactoagar). The temperature was maintained at 23°C; third instar wandering larvae (crop-half-empty) were isolated and kept in sterile dry boxes at 23°C. In our conditions, the duration of larval, pupal and adult development was similar for normal and axenically raised insects.

Immunizations were performed by various procedures: injections of *E.cloacae* β12 [5 × 10^3 live cells/2 μl saline (NaCl 0.9%)], *E.coli* (10^4 or 10^5 heat-killed cells/2 μl saline), *M.luteus* (10^4 or 10^5 heat-killed cells/2 μl saline), lipopolysaccharides from *S.marcescens* (Sigma L 4766, 100 ng or 1 μg/2 μl saline). Animals were kept at 23°C before being killed at various time intervals after the challenge.

Construction of the recombinant cDNA library

Total RNA from fat bodies of challenged larvae (6 h after the injection of live *E.cloacae* β12) was extracted by the hot guanidinium technique (Maniatis *et al.*, 1982) with slight modifications: after guanidinium—hot phenol extraction, proteinase K treatment and precipitation by ethanol, the RNA was resuspended in water and precipitated in the presence of 3 M LiCl to separate RNA from contaminating DNA. Poly(A)-enriched RNA was isolated from total RNA by chromatography on oligo(dT) cellulose (type 7, Pharmacia; two runs) (Maniatis *et al.*, 1982). Loading buffer was 0.01 M Tris—HCl, pH 7.5 containing 0.5 M LiCl and 0.1% lithium dodecyl sulphate (LDS). Elution buffer was 0.01 M Tris—HCl, pH 7.5, containing 0.05% LDS. The basic procedure for preparation of double stranded cDNA and introduction into phage λgt11 was that of Huynh *et al.* (1985) with modifications from Ausubel *et al.* (1987). The first strand was synthesized after $(dT)_{12-18}$ (Biolabs) priming on poly(A)-enriched RNA using AMV reverse transcriptase; the second strand was synthesized using T4 DNA polymerase together with RNaseH and *E.coli* DNA ligase. After *Eco*RI methylation of the double stranded cDNA, *Eco*RI linkers were added. cDNA was purified from excess linkers by size selection chromatography (AcA 54, LKB). 3 μg of polyadenylated RNA yielded 1 μg of double stranded cDNA from which 5 × 10^6 recombinant clones were obtained. For the initial screening, libraries were plated out at 30 000 p.f.u./14 cm Petri dish. Plaque lifts on nitrocellulose filters (BA85, 0.45 μm, Schleicher and Schuell) were performed as described by Mason and Williams (1985).

cDNA cloning and sequencing

A pool of synthetic oligomers (5′ AC A/G/TCC CTT A/G/TCC GTT A/GCA 3′) was used to screen the cDNA library for defensin clones. The oligomers were complementary to residues 30 to 35 of defensin A (Lambert *et al.*, 1989). Hybridization of nitrocellulose filters with [γ-^{32}P]ATP end-labelled probe was performed in 4% formamide, 6 × SSC, 5 × Denhardt's, 100 μg/ml denatured salmon sperm DNA, 0.1% SDS, 0.5% pyrophosphate, overnight at 42°C. Filters were washed in 2 × SSC, 0.1% SDS, 1 h at room temperature and once in 1 × SSC at 42°C for 30 min. Putative cDNAs for defensin were subcloned into M13mp19 (Yanisch-Perron *et al.*, 1985) and sequenced on both strands, using the dideoxy method of Sanger, with the sequencing kit Sequenase™ (US Biomedical Corporation) following the protocol of the suppliers. The products were separated on polyacrylamide gels (8% acrylamide, 7 M urea). Routine computer analyses of the sequencing data were performed on a Macintosh+ microcomputer (DNA Inspector™ IIe [Textco] software).

Primer extension

Extended cDNA fragments were prepared according to Geliebter *et al.* (1986). A DNA primer (5′ ACA GTG TGC AGC ACA AGC CGA 3′) complementary to the nucleotide sequence for amino acids 14 to 20 of defensin A (Figure 1, underlined) was end-labelled with [γ-^{32}P]ATP. 5 ng of primer were combined with 5 μg of polyadenylated RNA from immunized larvae and incubated in annealing buffer (250 mM KCl; 10 mM Tris−HCl, pH 8.3) for 3 min at 80°C followed by 45 min at 60°C. 2 μl of this solution were added to 3.3 μl of reverse transcriptase buffer (24 mM Tris−HCl, pH 8.3; 16 mM $MgCl_2$; 8 mM DTT; 0.4 mM of each dATP, dCTP, dTTP, 0.8 mM dGTP; 100 μg/ml actinomycin D) containing 5 U of AMV reverse transcriptase. For sequencing, four samples were incubated at 50°C for 45 min in the presence of 1 mM of each ddNTP. The products of the sequencing reactions were separated on polyacrylamide gels (6% acrylamide, 7 M urea).

In situ hybridization

The larvae were anaesthetized and dissected in heparin (20 U/ml) containing saline. Tissues were fixed for 2 h with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 at 4°C. After washing in 0.1 M phosphate buffer, pH 7.4, the tissues were dehydrated in ethanol, transferred in xylene and embedded in paraffin. Sections were cut 7 μm thick and deposited on glass slides coated with gelatin−chrome alum and stored at −20°C. For haemocyte smears, the integument was punctured with a thin needle and a droplet of haemolymph was collected on a gelatinized glass slide and spread out. The smears were dried for 30 s, fixed in 4% paraformaldehyde, 0.1 M phosphate buffer, pH 7.4 at 4°C for 30 min. After washing in the same saline solution, the smears were dehydrated in ethanol and stored at −20°C until further use.

Prior to hybridization, the tissue sections or the haemocyte smears were warmed at room temperature for 10 min, and then placed in 0.25% acetic anhydride in 0.1 M triethanolamine, 0.9% NaCl, pH 8, for 10 min at room temperature. After two rinses in 2 × SSC, they were dehydrated in ethanol and dried.

The slides were hybridized with [γ-^{32}P]ATP end-labelled oligonucleotide probes (specific activities: ~7 × 10^8 d.p.m./μg of DNA). Defensin mRNAs were detected with a 21mer oligonucleotide probe (5′ ACA GTG TGC AGC ACA AGC CGA 3′) complementary to the nucleotide sequence encoding the residues 14 to 20 of defensin A (Figure 1, underlined). The 21mer (5′ TGG AGC AGG TGT AGG CAA TAT 3′) used to detect diptericin mRNAs was complementary to the coding region for amino acids 7 to 13 of diptericin (Reichhart *et al.*, 1989). Hybridization was performed overnight at 42°C in 10 mM Tris−HCl, pH 8, buffer containing 4 × SSC, 1 mM EDTA, 1 × Denhardt's, 200 μg/ml BSA, 500 μg/ml yeast tRNA, 25 μg/ml denatured salmon sperm DNA and formamide (18% for diptericin probe and 20% for defensin probe). After hybridization, slides were washed in 4 × SSC (1 × 30 min at room temperature), 1 × SSC (1 × 45 min at room temperature), 1 × SSC (2 × 15 min at 42°C). After autoradiography (emulsion Kodak NTB-2), sections were stained with haematoxylin and viewed by bright and dark field microscopy.

Northern blot analysis

Total RNA was extracted from fat bodies by direct precipitation in 3 M LiCl, 6 M urea (Auffray and Rougeon, 1980). RNAs were fractionated by denaturing electrophoresis in 1.5% agarose−formaldehyde gels with MOPS buffer (Corces *et al.*, 1981). Transfer to nylon membranes (Hybond™, Amersham) was performed essentially as described by Thomas (1980). The filters were first hybridized overnight at 42°C with nick-translated [^{32}P]cDNA Def 1 encoding defensin A (Figure 1) in 50% formamide, 6 × SSC, 5 × Denhardt's, 100 μg/ml denatured salmon sperm DNA, 0.1% SDS. Then the filters were washed in 2 × SSC (3 × 15 min at room temperature), 1 × SSC (1 × 30 min at 42°C), 0.2 × SSC (1 × 30 min at 42°C). After autoradiography, the filters were dehybridized according to the manufacturer and hybridized with cDNA D15 encoding diptericin (Reichhart *et al.*, 1989). Hybridization and washing conditions were the same as those used for defensin cDNA. For both probes, the specific activity was around 5 × 10^8 d.p.m./μg of DNA.

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References

Anderson,D.T. (1972) In Counce,S.J. and Waddington,C.H. (eds), *Developmental Systems: Insects*. Vol. I. Academic Press, London, pp. 162−242.

Ando,K. and Natori,S. (1988) *Biochemistry*, **27**, 1715−1721.

Auffray,C. and Rougeon,F. (1980) *Eur. J. Biochem.*, **107**, 303−314.

Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (1987) *Current Protocols in Molecular Biology*. John Wiley and Sons, New York.

Casteels,P., Ampe,C., Jacobs,F., Vaeck,M. and Tempst,P. (1989) *EMBO J.*, **8**, 2387−2391.

Corces,V., Pellicer,A., Axel,R. and Meselson,M. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 7038−7042.

Daher,K., Selsted,M.E. and Lehrer,R.I. (1986) *J. Virol.*, **60**, 1068−1074.

Daher,K., Lehrer,R.I., Ganz,T. and Kronenberg,M. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7327−7331.

Dickinson,L., Russell,V. and Dunn,P.E. (1988) *J. Biol. Chem.*, **263**, 19424−19429.

Dimarcq,J.L., Keppi,E., Dunbar,B., Lambert,J., Reichhart,J.M., Hoffmann,D., Rankine,S.M., Fothergill,J.E. and Hoffmann,J.A. (1988) *Eur. J. Biochem.*, **171**, 17−22.

Faye,I. and Wyatt,G.R. (1980) *Experientia*, **36**, 1325−1326.

Ganz,T., Selsted,M.E., Harwig,S.S.L., Szlarek,D., Daher,K., Bainton,D.F. and Lehrer,R.I. (1985) *J. Clin. Invest.*, **76**, 1427−1435.

Ganz,T., Selsted,M.E. and Lehrer,R.I. (1986) *Sem. Resp. Infect.*, **1**, 107−117.

Geliebter,I., Zeff,R.A., Melvod,R.W. and Nathenson,S.G. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 3371−3375.

Hultmark,D., Engström,A., Andersson,K., Steiner,H., Bennich,H. and Boman,H.G. (1983) *EMBO J.*, **2**, 571−576.

Huynh,T.V., Young,R.A. and Davis,R.W. (1985) In Glover,D.M. (ed.), *Constructing and Screening cDNA Libraries in λgt10 and λgt11. DNA Cloning. Vol. 1. A Practical Approach*. IRL Press, Oxford, pp. 1−49.

Keppi,E., Zachary,D, Robertson,M., Hoffmann,D. and Hoffmann,J.A. (1986) *Insect Biochem.*, **16**, 395−402.

Klebanoff,S.J. (1988) In Gallin,J.I., Goldstein,I.M. and Snyderman,R. (eds). *Inflammation: Basic Principles and Clinical Correlates*. Raven, New York, pp. 391−444.

Lambert,J., Keppi,E., Dimarcq,J.L., Wicker,C., Reichhart,J.M., Dunbar,B., Lepage,P., van Dorsselaer,A., Hoffmann,J.A., Fothergill,J. and Hoffmann,D. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 262−266.

Lehrer,R.I., Daher,K., Ganz,T. and Selsted,M.E. (1985) *J. Virol.*, **54**, 467−472.

Lidholm,D.A., Gudmundsson,G.H., Xanthopoulos,K.G. and Boman,H.G. (1987) *FEBS Lett.*, **226**, 8−12.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Mason,P.J. and Williams,J.G. (1985) In Hames,B.D. and Higgins,S.J. (eds). *Nucleic Acids Hybridization: A Practical Approach*. IRL Press, Oxford, pp. 113−138.

Matsumoto,N., Okada,M., Takahashi,H., Qu,X.M., Nakajima,Y., Nakanishi,Y., Komano,H. and Natori,S. (1986) *Biochem. J.*, **239**, 717−722.

Matsuyama,K. and Natori,S. (1988a) *J. Biol. Chem.*, **263**, 17112−17116.

Matsuyama,K. and Natori,S. (1988b) *J. Biol. Chem.*, **263**, 17117−17121.

Mori,H. (1979) In Gupta,A.P. (ed.) *Insect Hemocytes−Development, Forms, Functions and Techniques*. Cambridge University Press, Cambridge, pp. 3−27.

Okada,M. and Natori,S. (1985) *J. Biol. Chem.*, **260**, 7174–7177.
Reichhart,J.M., Essrich,M., Dimarcq,J.L., Hoffmann,D., Hoffmann,J.A. and Lagueux,M. (1989) *Eur. J. Biochem.*, **182**, 423–427.
Seldsted,M.E., Brown,D.M., DeLange,R.J,. Harwig,S.S.L. and Lehrer,R.I. (1985a) *J. Biol. Chem.*, **260**, 4579–4584.
Seldsted,M.E., Harwig,S.S.L., Ganz,T., Schilling,J.W. and Lehrer,R.I. (1985b) *J. Clin. Invest.*, **76**, 1436–1439.
Seldsted,M.E. and Harwig,S.S.L. (1987) *Infect. Immun.*, **55**, 2281–2286.
Steiner,H., Hultmark,D., Engström,A., Bennich,H. and Boman,H.G. (1981) *Nature*, **292**, 246–248.
Thomas,P.S. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 5201–5205.
Trenczek,T. (1988) *Insect Biochem.*, **181**, 299–312.
Yanisch-Perron,C., Vieira,J. and Messing,J. (1985) *Gene*, **33**, 103–119.
Zachary,D. and Hoffmann,J.A. (1973) *Z. Zellforsch.*, **141**, 55–74.

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