The immune response in *Drosophila*: pattern of cecropin expression and biological activity

Christos Samakovlis, Deborah A. Kimbrell,
Per Kylsten, Åke Engström¹ and Dan Hultmark

Department of Microbiology, University of Stockholm, S-106 91 Stockholm and ¹Department of Immunology, University of Uppsala, Biomedicum, Box 582, S-751 23 Uppsala, Sweden

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Cecropins are antibacterial peptides, induced in *Drosophila* as part of the humoral immune response to a bacterial invasion. We have used the cloned *Drosophila* cecropin genes *CecA1*, *A2* and *B* as probes to study the developmental and tissue specific regulation of this response. The genes are strongly expressed in fat body and hemocytes after injection of bacteria, the *CecA* genes being much more active than *CecB* in the fat body. All parts of the fat body and 5–10% of the hemocytes are involved in this response. *CecA1* and *A2* are most active in larvae and adults; *CecB* is preferentially active in early pupae. A small peak of constitutive cecropin expression in early pupae appears to be caused by bacteria in the food. Cecropin A, the common product of the *CecA1* and *A2* genes, was identified in the hemolymph of immunized flies at a concentration of 25–50 μ M, enough to kill all tested bacteria except *Serratia*, a *Drosophila* pathogen. A useful *in vitro* system to study the immune response has been found in Schneider's line 2 cells which respond to lipopolysaccharide and laminarin by cecropin expression.

Key words: antibacterial peptides/cecropin/*Drosophila*/insect immunity

Introduction

Drosophila and many other insects activate a remarkable defense mechanism when challenged by microbial infections. This complicated immune reaction can be separated into two parts: a cellular response in which the blood cells encapsulate or phagocytose the invader (reviewed in Götz and Boman, 1985; Ratcliffe *et al.*, 1985) and a humoral response that comprises a number of effector molecules that are secreted into the hemocoel. The humoral immune response has been best characterized in higher insects such as Lepidoptera and Diptera (reviewed in Dunn, 1986; Boman and Hultmark, 1987). It is most efficiently induced by living bacteria but it can also be triggered by the injection of other foreign substances, or to some extent even by a sterile wound (see Götz and Boman, 1985). In a few cases, the same system has also been shown to be activated during development in embryos or early pupae, without any external stimuli (Bakula, 1970; Nanbu *et al.*, 1988). The presence of an inducible immune defense in *Drosophila* was first demonstrated by Boman *et al.* (1972), and further

characterized by Robertson and Postlethwait (1986) and Flyg *et al.* (1987).

Perhaps the most potent of the induced peptides are the cecropins, first found in the cecropia moth *Hyalophora cecropia* (Hultmark *et al.*, 1980; Steiner *et al.*, 1981), where they constitute a major part of the induced antibacterial activity. The *Hyalophora* cecropins are a family of strongly amphipathic peptides, 35–37 amino acids long, that efficiently kill and lyse bacteria by attacking the cell membrane (Steiner *et al.*, 1988). Cecropins with similar properties have also been isolated from other insects, mainly moths (reviewed in Boman and Hultmark, 1987), but also from the flesh fly *Sarcophaga peregrina* (Okada and Natori, 1985). Recently a cecropin was even found in pig intestine (Lee *et al.*, 1989), showing an interesting link between the nonspecific immune systems in insects and mammals.

An important part of the immune response is the initial recognition of the invading microbes, and the mechanism of induction of the antibacterial proteins. To address these questions we have started to investigate the immune response in *Drosophila melanogaster*, and recently cloned the cecropin locus from this species (Kylsten *et al.*, 1990). It was found to contain three functional cecropin genes that we propose to call *CecA1*, *A2* and *B*, and two pseudogenes, all arranged as a compact cluster in the genome (Figure 1). These genes are strongly induced when bacteria are injected into the hemocoel, and they are thus convenient markers for the humoral immune response in the fly. We have now used the cloned genes as molecular probes for an extensive characterization of the developmental and tissue-specific patterns of expression of the three cecropin genes. We have also used chemically synthesized peptides as references to study the accumulation of cecropins in the hemolymph as well as their biological activity. Finally, we have identified a tissue culture system where the activation of the cecropin genes can be studied.

Results

Cecropin gene expression during development

We followed the expression of the three cecropin genes during development, before and after injection of bacteria (Figure 2). Although the sequences of the cecropin genes are very similar, especially those of *CecA1* and *A2*, it was possible to detect the transcripts of the genes separately by RNase protection assay, using the short 3' probes shown in Figure 1. All three genes were strongly induced by bacteria, not only in adults, but also in pupae and third instar larvae (Figure 2). For technical reasons we have not injected earlier stages. We could not detect cecropin transcripts in untreated animals except for a small but marked mRNA peak in early pupae, and sometimes a variable and usually very low expression in adults. The *CecB* gene differed from the *CecA* genes in being relatively less inducible in larvae and adults. Instead it was preferentially induced in early

Fig. 1. Maps of the cecropin transcripts and probes. **Panel a** shows the organization of the cecropin locus (Kylsten *et al.*, 1990), and **panel b** illustrates the location in the transcripts of the probes for the three cecropin genes. Coding regions are shown as black boxes. The RNA probes are shown as dashed arrows. They were transcribed *in vitro* from T3 promoters in the vectors, and the size of the hybridizing fragments are indicated.

pupae. The higher activity of *CecB* at this stage was also reflected in the relatively high basal levels in the untreated pupae. However, it should be noted that the developmental specificity of *CecB* for the early pupal stage is only in relative terms. The *CecB* gel in Figure 2 had to be exposed for longer than the others, and we estimate that even at this stage expression of *CecB* does not exceed that of the *CecA* genes. *CecA1* and *A2* encode identical peptides, and they appear to be expressed in parallel.

It has been suggested that the peak of antibacterial activity in the early pupae is there to protect the animal from bacteria that might be released during the metamorphosis of the gut (Bakula, 1970). We turned this argument around, and asked whether the increased cecropin mRNA levels at this stage may in fact be a result of induction by gut bacteria, especially since we had observed that certain bacteria in the food can induce cecropin transcription in adults (Kylsten *et al.*, 1990). Indeed, when we investigated axenically grown animals, we found that the early pupal expression was practically absent (Figure 2), and we conclude that even in the untreated animals, most of the cecropin expression is induced by bacteria.

Site of cecropin gene expression

In order to get an overview of the site of cecropin expression in *Drosophila*, and also to get detailed information about what cell types are involved, we probed whole-body tissue sections by *in situ* hybridization to *CecA* and *B* probes. Figure 3 shows that the *CecA* genes are extensively expressed in the fat body of immunized adults (panels a–b), pupae (panel d) and larvae (panel e). As negative controls, we used uninduced animals. No significant labeling was observed in the controls, either in adults (panel c) or in other stages (data not shown). Maximal expression in adults appears to involve all fat body cells of the head, thorax and abdomen. In young adults, larval fat body cells persist together with the adult fat body (see Rizki, 1978b), and in this case, both types of

Fig. 2. Expression of the cecropin genes during development, assayed by RNase protection. Early and late third instar larvae refer to feeding and wandering stages, respectively; white: white prepupae; early pupae: up to 49 h after pupariation, and late: 49 h until eclosion. Twenty μ g of total RNA from untreated or vaccinated animals (6 h after injection) were used for each hybridization. Fifty μ g tRNA were used for the negative control lanes. **Panels A1 and A2** were probed with short 3' *CecA1* and *A2* probes (Figure 1) and exposed overnight, **panel B** was probed with *CecB* probe and exposed for 3 days with intensifying screens. **Right hand part** shows *CecB* expression in axenic and normal early pupae. One axenic culture became contaminated with bacteria and shows increased *CecB* expression. The integrity and quantity of the RNA in each lane were checked by Northern blotting, where the same volumes of RNA were blotted and hybridized to actin 5C (data not shown).

fat body cells express *CecA* (panel b). In some individuals, we observed a more patchy pattern of expression, where only a fraction of the fat body cells were activated, apparently at random (data not shown). The *CecA2* probe used covers the conserved coding region and does not discriminate between the *CecA1* and *A2* genes. However, no differences in the expression of the two genes were seen when the short gene-specific probes from the 3' non-coding regions were used (data not shown). The *CecB* gene is also expressed in the fat body, although at a considerably lower level (Figure 4). In the pupal sections, very intense spots of hybridization were often seen within the region of the fat body, both for *CecA* (Figure 3d) and *CecB* probes (Figure 4b).

Invading bacteria are directly attacked by hemocytes (Götz and Boman, 1985), and in a few cases, hemocytes have also been shown to take part in the production of immune proteins (Dickinson *et al.*, 1988; Matsuyama and Natori, 1988b). Since it is difficult to identify circulating hemocytes in tissue sections, we analyzed hemocyte spreads for cecropin expression by *in situ* hybridization. Figure 5 shows that a fraction of the hemocytes do indeed hybridize to the cecropin probes. Approximately 5–10% were positive for cecropin expression, with similar values for *CecA* and *B*, although these figures hide the fact that there is a continuous gradation of responses, from strongly positive to completely negative cells. Many of the positive cells appeared to be more flattened than the rest, and may correspond to lamellocytes (Rizki, 1978b). In contrast to the situation in fat body, the *CecB* probe gave signals as strong as the *CecA* probe in the hemocytes.

Accumulation of processed cecropin in the hemolymph

The processing of the primary translation products of the cecropin genes to mature products cannot be deduced unambiguously from their primary sequences. The *CecA* genes could potentially give rise to a cecropin identical to the major form in *Sarcophaga*, sarcotoxin IA, and *CecB* could also give a similar product. Signal peptidase is likely to cleave at a position two residues upstream of the predicted amino terminals, and we suggested an additional processing step that removes the extra dipeptides (Kylsten *et al.*, 1990), in analogy to the situation in *Hyalophora*. We also predicted the carboxy terminals to be amidated, like those of sarcotoxin IA and most other cecropins described. In order to test these predictions, we prepared the anticipated products by chemical synthesis and separated them on native acidic polyacrylamide gels along with samples of hemolymph from immunized flies. The positions of antibacterial components were recorded with a bacterial overlay (see Materials and methods). Figure 6a shows that at least six different antibacterial components accumulate in the hemolymph after immunization, one of them with the mobility of synthetic cecropin A. Since the mobility in this type of gel is sensitive to charge differences due to amide groups or extra dipeptides (Boman *et al.*, 1989), we conclude that at least cecropin A appears to be processed to a form identical to *Sarcophaga* cecropin, and exported to the hemolymph. The sensitivity and resolution of this technique is probably not sufficient for the detection of cecropin B, although a prominent antibacterial factor with a slightly lower mobility than expected for this form was observed. At least two induced proteins could be observed in a stained gel (Figure 6b), one of them in the cecropin region.

A crude estimate of cecropin A concentration in the hemolymph can be obtained by comparison with the dilution series of the synthetic cecropin in Figure 6a. From this and similar experiments, we estimate that in vaccinated flies, the hemolymph contains 25–50 μM cecropin A. The total antibacterial activity in the hemolymph, as judged by the inhibition zone assay (Hultmark *et al.*, 1982), corresponds to a cecropin concentration of $\sim 100 \mu\text{M}$ (data not shown).

Bactericidal activity of *Drosophila* cecropins

We also used the inhibition zone assay to compare the bactericidal spectra of the synthetic *Drosophila* cecropins to that of *Hyalophora* cecropin A, a well studied lepidopteran cecropin (Table I). Like their lepidopteran counterparts, the *Drosophila* cecropins show a wide spectrum of antibacterial activity, killing both Gram-negative and Gram-positive bacteria. *Drosophila* cecropin A is at least as potent as the *Hyalophora* cecropin in killing Gram-negative bacteria, but is less active on the Gram-positives. The activity of the major cecropin in *Sarcophaga*, which is identical in structure to *Drosophila* cecropin A, has previously been assayed with some of the bacterial strains we used, with very similar results (Li *et al.*, 1988). Synthetic *Drosophila* cecropin B was difficult to handle, as it tended to form a gel in water solution. This behavior was accentuated under the ionic conditions in the antibacterial plates, and is likely to have affected the antibacterial assay negatively. Thus, although cecropin B appears to be somewhat less potent than the A form, this may not reflect the situation *in vivo*. In order to avoid the diffusion problems of a gel-forming peptide, we

also compared the activities in a miniaturized MIC assay with bacteria in suspension (see Materials and methods). However, very similar values were obtained with the two methods (data not shown). Among the bacteria tested, we also included the *Drosophila* pathogen *Serratia marcescens* Db11 and its non-pathogenic derivative Db1140 (Flyg and Xanthopoulos, 1983). With these bacteria, very small and diffuse zones of growth inhibition were obtained, and we were unable to calculate the lethal concentrations. Instead, the results of the MIC assay are shown (Table I). Obviously, *Serratia* is very resistant to the cecropins. Unexpectedly this is also true of the non-pathogenic mutant Db1140, although this strain is known to be sensitive to immune hemolymph (Flyg and Xanthopoulos, 1983).

Induction of the cecropin genes in tissue culture

We were interested to identify different inducers of the *Drosophila* immune response. However, the results of these experiments were inconclusive because of a variable and often very strong transcription of the cecropin genes in the saline-injected controls (data not shown). As an alternative approach, we investigated whether *Drosophila* cell lines might also express the cecropin genes. Figure 7 shows that for Schneider SL2 cells this is normally not the case, but that expression of the cecropin genes can be induced in these cells by the addition of microbial products. Both lipopolysaccharide, a component of the outer membrane of Gram-negative bacteria, and laminarin, an algal β -1,3-glucan related to fungal cell wall components, stimulate the Schneider cells to transcribe the cecropin genes.

Discussion

Our results clearly identify the fat body as the major site of cecropin gene expression in *Drosophila*. Furthermore, there are no specialized cell types or lobes of the fat body set aside for this function, but all parts of the fat body participate. The importance of the fat body in the immune response was first suggested by Faye and Wyatt (1980), who showed that fat body isolated from *Hyalophora* pupae exported immune proteins into the medium. Later, cecropin expression in fat body tissue was demonstrated (Matsumoto *et al.*, 1986; Trenczek and Faye, 1988), but the possible involvement of other tissues has usually not been addressed. Only Dickinson *et al.* (1988) have investigated different tissues in *Manduca* for cecropin expression. By Northern blotting they found some cecropin mRNA in all tissues investigated, fat body being by far the most active. However, in such experiments it is difficult to rule out contamination by fat body or hemocytes in other tissues.

Besides the fat body, some hemocytes appear to be synthesizing cecropin mRNA very actively. Because of their small number, the hemocytes probably contribute little to the total production of cecropin. However, it is possible that activated hemocytes can give rise to high local concentrations of cecropin at the site of infection.

Although the different cecropin genes can be induced throughout development and are expressed in both fat body and hemocytes, expression of *CecB* is in several respects very different from that of the others. Transcripts of this gene are not as abundant, and in contrast to the *CecA* genes, *CecB* is preferentially expressed in early pupae. Expression of *CecB* in the fat body is much lower than for the *CecA*

Fig. 4. Localization of *CecB* transcripts. Tissue sections of immunized and control larvae, pupae and adults were hybridized with the ^{35}S -labeled RNA probe for *CecB*. As shown in Figure 3 for *CecA*, *CecB* transcripts also localize to the fat body only after immunization. **Panel a:** abdomen of immunized adult. Dark field illumination. **Panel b:** immunized pupa. **Panel c:** immunized third instar larva. Intensity of labeling with *CecB* probe is reproducibly less than with *CecA* probe. **b** and **c:** bright field on left, dark field of the same section on right. Scale bar 0.1 mm.

genes, whereas in hemocytes the transcription of the genes is comparable. Furthermore, some transcription of *CecB* could also be detected in various other tissues, and to a larger extent than for the *CecA* genes (Kimbrell, D.A. unpublished). The antibacterial spectra of cecropins A and B are not

Fig. 5. *In situ* hybridization to hemocyte spreads. Blood cells from immunized (right half) and control larvae (left half) were hybridized to [^{35}S]UTP labeled RNA probes on the same slide and exposed for 9 days. **Panel A:** cells hybridized to the *CecA2* long probe (see Figure 1). **Panel B:** cells hybridized to the *CecB* probe.

Fig. 6. Separation of antibacterial proteins in immune hemolymph on acidic polyacrylamide gels. **Panel a:** known amounts of synthetic cecropin A and immune hemolymph (I.H.) from different numbers of immunized flies were electrophoresed, and the antibacterial components were detected with a bacterial overlay. The mobilities of synthetic cecropin markers are indicated by small arrows to the right of the gel. **Panel b:** a Coomassie stain of the same type of gel as in panel a, without the bacterial overlay. Immune (I.H.), and control (N.H.) hemolymph from 20 flies was loaded in the respective lanes.

dramatically different, and the biological significance of their different patterns of expression is still unclear.

A special situation exists in the early pupae when the *CecB*

Fig. 3. Localization of *CecA* transcripts. Tissue sections of immunized and control larvae, pupae and adults were hybridized with the ^{35}S -labeled insert of the *CecA2* cDNA clone k-15. **Panel a:** immunized adult showing labeling of fat body in head, thorax and abdomen. Bright field illumination. **Panel b:** abdomen of an immunized adult showing labeling of the fat body, showing specifically that fat body cells of both larval (l) and imaginal (a) origin are labeled. **Panel c:** abdomen of a control adult showing only background labeling. **Panel d:** immunized pupa showing unequal distribution of label within the region of the fat body. **Panel e:** immunized third instar larva showing complete labeling of the fat body. Control pupae and larvae also show no specific labeling. **a:** scale bar 0.2 mm; **b–e:** the same section is shown in bright field on the left and dark field on the right. Scale bar 0.1 mm.

Table I. Comparison of the antibacterial activity of *Drosophila* and *Hyalophora* cecropins

	Lethal concentrations (μ M)		
	<i>Drosophila</i> cecropin A	<i>Drosophila</i> cecropin B	<i>Hyalophora</i> cecropin A
<i>Escherichia coli</i> D21	0.3	1.8	0.4
<i>Enterobacter cloacae</i> β 12	0.4	1.0	0.4
<i>Pseudomonas aeruginosa</i> OT97	2.2	4.2	2.5
<i>Serratia marcescens</i> Db11	93 ^a	> 149 ^a	> 127 ^a
<i>Serratia marcescens</i> Db1140	> 93 ^a	> 149 ^a	> 127 ^a
<i>Bacillus megatherium</i> Bm11	2.6	3.7	0.6
<i>Bacillus subtilis</i> Bs11	16	3.2	6.4
<i>Micrococcus luteus</i> MI11	8.5	59	1.5

^aValues obtained by MIC assay. **Fig. 7.** Induction of the cecropin genes in Schneider SL2 cells by lipopolysaccharide and laminarin, 100 μ g/ml. Twenty μ g of total RNA from untreated or induced cells (4 h after addition of inducers) were assayed by RNase protection. Fifty μ g tRNA were used for the negative control lanes. The **left panel** shows the induction of *CecA1* transcription. Exposure was overnight. The **right panel** shows the induction of the *CecB* gene. In this case exposure was for 3 days with intensifying screens.

gene is most active. At this time, the basal membrane around the fat body is dissolved and the tissue is infiltrated by hemocytes (Whitten, 1962; Rizki, 1978b; Kurata *et al.*, 1989). The expression in the pupal fat body of both *CecA* and *B* is very patchy at this stage, and it is difficult to tell whether the expression comes mainly from fat body cells or possibly from infiltrating hemocytes. This is also a period when the larval gut is being replaced by the adult structure, from primordia in the gut wall (Bodenstein, 1950). The peak in cecropin expression observed at this developmental stage correlates well with the increased antibacterial activity in the hemolymph reported by Bakula (1970). This peak appears to depend on the presence of bacteria in the food, and is probably directly induced by bacteria from the metamorphosing gut.

Cecropin A was found to be exported to the hemolymph, and to accumulate to a concentration sufficient to kill all tested bacteria, both Gram-positive and Gram-negative, except *S. marcescens*. In addition to cecropin A we were able to detect at least five different inducible antibacterial factors in *Drosophila* hemolymph (Figure 6a). A second major spot in the cecropin region moved just behind the position of synthetic cecropin B, and probably does not correspond to

this weakly expressed cecropin. The mobility of this second spot in the acidic gel is similar to that of the non-amidated form of cecropin A (Li *et al.*, 1988). Evidence for non-amidated variants of the major cecropins have previously been reported for *Hyalophora* pupae (Hultmark *et al.*, 1982) and *Sarcophaga* cell culture (Matsuyama and Natori, 1988a), and it is possible that a considerable fraction of the cecropin is non-amidated in *Drosophila*. The other antibacterial factors remain to be identified, although one of the upper bands on the gel is probably due to dipterocin. This antibacterial protein is abundantly transcribed in immunized *Drosophila* (C. Wicker, J.-M. Reichhart, D. Hoffmann, D. Hultmark, C. Samakovlis and J. Hoffmann, in preparation), and its larger mol. wt and less basic nature should give a lower mobility than cecropins in the acidic gel.

It is unlikely that the fat body cells interact directly with invading bacteria, and the nature of the signal that triggers the immune response in this tissue is unknown. De Verno *et al.* (1984) suggested that activated hemocytes release a diffusible factor that induces the fat body response, and Dunn *et al.* (1985) hypothesized that peptidoglycan fragments from bacterial cells that have been processed by hemocytes may be the diffusible factor. However, other signals must exist, at least to mediate the response to sterile wounds. Indeed, it appears likely that the immune response is regulated through a complex interaction between microbes, hemocytes, fat body cells and damaged cells in other tissues. The molecular study of such a complicated system *in vivo* is very difficult since the different components involved cannot be isolated and investigated separately. In this connection it is interesting to note that microbial products such as lipopolysaccharide and β -1,3-glucan can induce the cecropin genes in the embryonic SL2 cell line, and one may speculate that a subpopulation of these cells is related to hemocytes. Constitutive production of antibacterial proteins has previously been observed in cell lines from *Periplaneta* (Landureau and Jolles, 1970) and *Sarcophaga* (Matsuyama and Natori, 1988a), but the Schneider cells are the first to show an inducible immune response *in vitro*. In this system much of the complexity of the *in vivo* situation is eliminated, and it should have considerable potential for studies of the structures responsible for the recognition of invading microorganisms and the activation of the immune response.

Materials and methods

Flies, immunization and antibacterial assays

Canton S flies were kept on autoclaved corn meal/yeast food at 25°C with a 10 h light/14 h dark cycle. Axenic flies were raised from dechorionated eggs on sterile medium as described (Roberts, 1986). The animals were immunized by injections of frozen *Enterobacter cloacae* β 12 as described (Flyg *et al.*, 1987). In later experiments we tried different dilutions of stationary phase bacteria in anticoagulant Ringer (Trenczek and Faye, 1988). The best response was obtained with a concentration of 8×10^6 /ml in the vaccine.

For the detection of antibacterial components in the hemolymph, flies were surface sterilized in ethanol 24 h after immunization, and $\sim 0.1 \mu$ l hemolymph was bled and harvested with a glass capillary. Alternatively, flies were bled in pools of five in 2.5 μ l anticoagulant Ringer on Parafilm. The immune hemolymph was kept on ice over a few crystals of phenylthiourea. Hemocytes were removed by centrifugation. Hemolymph components were separated by electrophoresis in a 0.75 mm polyacrylamide gel, pH 4.0 (Hultmark *et al.*, 1980), and detected with a bacterial overlay. Approximately 5 ml log phase *Escherichia coli* D21 (10^6 /ml) was applied on the surface of the neutralized gel and after 1 min the excess was removed. The gel was incubated at 30°C overnight.

Antibacterial activity was determined by the inhibition zone assay; serial

dilutions of the synthetic cecropins were applied in wells on bacterial plates, and the lethal concentrations were calculated from the concentration dependence of the diameter of zones of growth inhibition (Hultmark *et al.*, 1982). The plates contained 0.8% agarose in LB medium and they were incubated at 30°C overnight, or until bacterial growth was visible. To determine the activity in free suspension, a miniaturized MIC (minimal inhibitory concentration) assay was used. Serial 2-fold dilutions of the cecropins (1 µl) were added to ~100 bacterial cells in 20 µl LB medium, and incubated in multititer plates in a humidified box at 30°C. The lowest final concentration that prevented bacterial growth was recorded.

The *Serratia* strains are described in Flyg and Xanthopoulos (1983), and references for the other bacterial strains used are given in Hultmark *et al.* (1982).

Probes

Probes were prepared from the Bluescript plasmids k-5, k-7 and k-15 containing the cDNA inserts for *CecB*, *A1* and *A2*, respectively (Kylsten *et al.*, 1990). The insert of k-7 extends between the coordinates 872–1155 given in Kylsten *et al.* (1990), k-15 between 2056 and 2452, and k-5 between 3506 and an *EcoRI* site 0.7 kb upstream of the major transcript. Short RNA probes were prepared by *in vitro* transcription from the T3 promoter as described by Gilman (1987). The templates were linearized with *BglII*, *HincII* and *XhoI*, as indicated in Figure 1. For the long *CecA2* probe, k-15 was linearized by cleavage with *HindIII* in the cloning cassette of the vector. The probes contained ~77 nucleotides of vector sequence in addition to the hybridizing fragment.

DNA probes were prepared from gel purified inserts of plasmids k-15 and k-5, and from a 1.6 kb fragment from the 5C actin locus (Fyrberg *et al.*, 1980), using a Pharmacia kit based on the random oligo-priming method of Feinberg and Vogelstein (1983).

RNA preparation and analysis

RNA was prepared from flies according to Kylsten *et al.* (1990) and from cultured cells as described by Gilman (1987). RNase protection assays were as in Gilman (1987) with ³²P-labeled probes; hybridizations were done overnight at 30°C for *CecA1* and *A2* and at 45°C for *CecB*. RNase digestions were done at 30°C for 45 min. The protected fragments were loaded onto a 6% sequencing gel together with sequencing ladders as markers. Northern blots were as in Kylsten *et al.* (1990).

In situ hybridization

Larvae, pupae and adults for tissue sections were quick frozen in OCT compound (Tissue Tek II). Sections (8 µm) were cut at -14°C, recovered onto subbed slides, and fixed in 4% paraformaldehyde. Immunized animals had been previously injected with bacteria and stored at 25°C for 6 h or, for adults, up to 16 h. For DNA probes, pretreatment of sections was according to Hafen *et al.* (1983), except that after post-fixation, sections were acetylated as by Akam and Martinez-Arias (1985). Hybridizations (overnight, 37°C) and washing (7 h, 45°C) were according to Akam (1983), except that dextran sulfate (to 10%) was added to the hybridization mix and dithiothreitol (up to 10 mM) to the hybridization mix and wash buffer. For RNA probes, hybridizations and washing were according to Ingham *et al.* (1985), except that washes were done in 50% formamide, 2 × SSC, 10 mM dithiothreitol. Autoradiography and staining were also according to Akam (1983). Slides were exposed for 5 days to 6 weeks. Within each experiment, intensity of labeling among different immunized animals was often variable. For each exposure time of an immunized animal, a control slide was exposed for the same length of time.

For *in situ* hybridization of hemocytes, we used poly-L-lysine covered multiwell slides. Blood cells from injected and control late third instar larvae were treated on the same slide. Cells from 3–4 larvae were allowed to settle on each well for 30 min on ice. After fixing with 50 µl 4% paraformaldehyde in PBS, each well was washed five times with 50 µl PBS. The slides were dried and stored in 70% ethanol at 4°C until hybridized. Acetylation and hybridization were done as described in Ingham *et al.* (1985) except that hybridizations were done for 3 h at 50°C with 2 mg/ml bovine serum albumin instead of Denhardt's, and the washes were as follows: 3 × 5 min and 2 × 20 min in 50% formamide, 2 × SSC at 55°C, and four rinses in 2 × SSC. After RNase digestion, the slides were further washed in 50% formamide, 2 × SSC at 55°C for 20 min and finally in 2 × SSC for 10 min at room temperature. Slides were dehydrated for 1 min in 70%, 80% and 90% ethanol baths. After drying, the slides were dipped in Kodak NTB2 emulsion. Exposure times were 6–12 days.

Chemical synthesis of cecropins

Cecropins were synthesized by automated solid-phase synthesis (Merrifield, 1963) on an Applied Biosystems (Foster City, CA) Model 430 A peptide

synthesizer. Symmetrical anhydrides were coupled twice and eventually, free amino groups remaining after coupling were blocked with acetic anhydride. After final cleavage with HF, the peptides were purified by reversed-phase chromatography and the purity of the final products was ascertained by ²⁵²Cf-plasma desorption time of flight mass spectroscopic analysis (Sundqvist and MacFarlane, 1985) on a BIOION Model 20 spectrometer (Bio-ion Nordic AB, Uppsala, Sweden). *Hyalophora* cecropin A, synthesized by D.Andreu, was a gift from Hans G.Boman.

Cell culture

Schneider's 'line 2' cells (SL2, Schneider, 1972) were grown in Schneider's medium (Gibco) supplemented with 10% fetal calf serum. Cells were induced by the addition of lipopolysaccharide or laminarin to a final concentration of 100 µg/ml in the culture medium. Induced cells were harvested 4 h after the addition of the inducer. Lipopolysaccharide from *E.coli* 055:B5 was obtained from the Department of Bacteriology, Karolinska Institute, Stockholm, and laminarin from Calbiochem.

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