The EMBO Journal vol.11 no.4 pp.1469 - 1477, 1992

Insect immunity: developmental and inducible activity of the *Drosophila* diptericin promoter

Jean-Marc Reichhart[1], Marie Meister[1,2], Jean-Luc Dimarcq[1], Daniel Zachary[1], Danièle Hoffmann[1], Claude Ruiz[2], Geoff Richards[2] and Jules A.Hoffmann[1]

[1]Laboratoire de Biologie Générale de l'Université Louis Pasteur, Unité Associée au CNRS no. 672 Endocrinologie et Immunologie des Insectes, 12 rue de l'Université, 67000 Strasbourg and [2]Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, Unité 184 de Biologie Moléculaire et de Génie Génétique de l'INSERM, Institut de Chimie Biologique, Faculté de Médecine, 11 Rue Humann, 67085 Strasbourg Cedex, France

Communicated by J.-P.Lecocq

Diptericins are 9 kDa inducible antibacterial peptides initially isolated from immune haemolymph of *Phormia* (Diptera). Following the isolation of a *Drosophila* cDNA encoding a diptericin homologue, we have now cloned a genomic fragment containing the *Drosophila* diptericin gene. To dissect the regulation of this gene, we have transformed flies with a fusion gene in which the reporter β-galactosidase gene is under the control of 2.2 kb upstream sequences of the diptericin gene. We show that such a fusion gene is inducible by injection of live bacteria or complete Freund's adjuvant and respects the tissue specific expression pattern of the resident diptericin gene. Our analysis reveals at least four distinct phases in the regulation of this gene: young larvae, late third instar larvae, pupae and adults. This complexity may be related to the presence in the upstream sequences of multiple copies of response elements previously characterized in genes encoding acute phase response proteins in mammals (e.g. NK-κB, NF-κB related, NF-IL6 response elements).

Key words: acute phase response/antibacterial peptide/diptericin/insect immunity/transgenic *Drosophila*

Introduction

The antibacterial defence reactions in the higher, endopterygote insect orders, namely the Lepidoptera, Diptera, Hymenoptera and Coleoptera, are characterized by the synthesis of a battery of potent bactericidal peptides. These are mostly small cationic molecules exhibiting a broad spectrum of activity against Gram-positive and/or Gram-negative bacteria (Steiner *et al.*, 1981; Hultmark *et al.*, 1983; Okada and Natori, 1985; Ando and Natori, 1988; Dimarcq *et al.*, 1988; Casteels *et al.*, 1989; Lambert *et al.*, 1989; Bulet *et al.*, 1991). The peptides are produced within a few hours after injury or the injection of bacteria, the main site of synthesis being the fat body (Faye and Wyatt, 1980; Dickinson *et al.*, 1988; Trenczek, 1988; Dimarcq *et al.*, 1990; Samakovlis *et al.*, 1990), a functional equivalent of the mammalian liver. Several characteristics of the immune response of insects are clearly reminiscent of the mammalian acute phase response (see Kushner, 1982; Koj, 1985, for reviews on this response). In mammals, acute phase protein genes (e.g. C-reactive protein, haptoglobin, haemopexin, α2-macroglobulin) are induced in the liver during the acute phase response by transcriptional activation of their promoters (Morrone *et al.*, 1988; Hattori *et al.*, 1990. Isshiki *et al.*, 1991). Several distinct activators appear to be involved in this process which is at present the centre of intense investigations. Our current interest is the regulation of expression in Diptera of genes encoding inducible antibacterial peptides. We have initiated our studies using the diptericins, a family of 9 kDa, glycine-rich anti-Gram-negative peptides, which we initially isolated from the fly *Phormia terranovae* (Dimarcq *et al.*, 1988). In *Drosophila*, cDNA cloning studies showed that larvae and adults injected with bacteria produce transcripts encoding an 83-residue diptericin (Wicker *et al.*, 1990).

We report the isolation of a *Drosophila* genomic fragment encoding diptericin. Interestingly, analysis of the upstream sequences reveals the presence of several putative transcription regulatory sequences identical or similar to consensus motifs present in promoters of genes encoding acute phase proteins. We have constructed a chimeric gene in which sequences upstream of the *Drosophila* diptericin coding sequence are fused to the bacterial β-galactosidase gene. Using P-element mediated germ-line transformation, we have generated fly lines carrying the chimeric construct. We have addressed basic questions concerning the tissue specific and the developmental expression of the transgene as well as its inducibility. We show that the inducible response is complex and must involve both stage and stimulus specific elements.

Results

Isolation of the Drosophila diptericin gene

Southern hybridization of *Drosophila* genomic DNA using the cDNA encoding diptericin revealed one hybridization band (Wicker *et al.*, 1990). Using this cDNA as a probe we isolated a 15 kb fragment from an Oregon-R genomic library. The restriction map of this fragment was determined and a 6 kb *Eco*RI−*Bam*HI subfragment, which hybridized with the cDNA probe, was subcloned into the phagemid pTZ 18R. The 3203 nucleotides between a *Cla*I and a *Pst*I site were sequenced after subcloning of various restriction fragments into M13 phages (Figure 1). This region consists of 2.2 kb of 5′ sequence upstream of an open reading frame of 106 codons which is identical to the sequence of the cDNA. The colinearity of the genomic DNA and cDNA indicates that the *Drosophila* diptericin gene is intronless. The cap site, as determined by primer extension (data not shown, but see Figure 6), is located 41 nucleotides upstream of the initiating Met codon. The cap site sequence

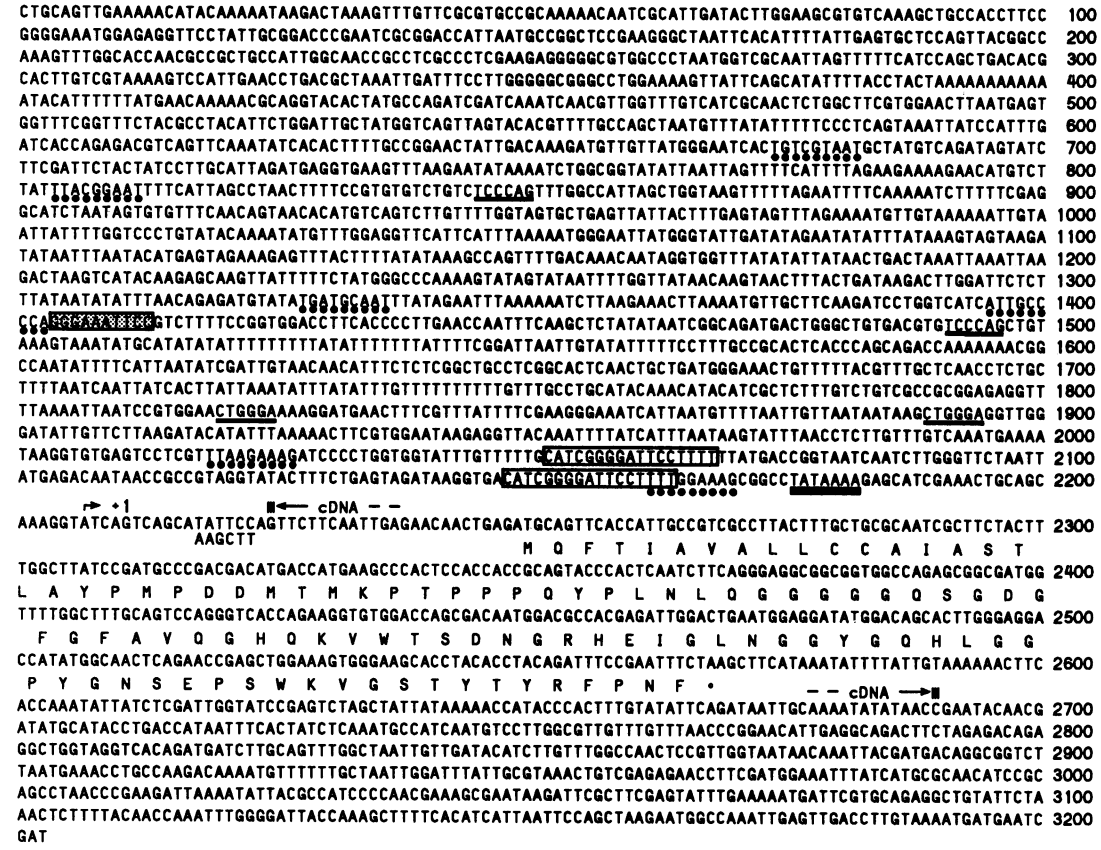

Fig. 1. The 3203 bp genomic fragment containing the *Drosophila* diptericin gene. The extent of the previously published cDNA sequence (Wicker *et al.*, 1990) is shown together with the preditericin protein sequence (single letter amino acid code). The startsite (arrow, +1) and TATA box (underlined) are indicated. The synthetic *Hin*dIII site is shown under the genomic sequence that has been replaced. In the 2.2 kb upstream sequences, potential binding sites (on either strand) for regulatory factors are highlighted as follows: i, NF-IL6 (dots); ii, NF-$\varkappa$B (shaded box); iii, NF-$\varkappa$B-related (open box); iv, hexameric acute phase gene promoter motif (underlined). See Figure 7 and Discussion for further details.

(ATCAGT) corresponds to a *Drosophila* consensus cap site sequence described by Hultmark *et al.* (1986). Upstream of the cap site, the genomic sequence contains a TATA box (TATAAAA, −31 to −25). No polyadenylation consensus signal was found in the 600 nucleotides following the stop codon. A computer-aided search of the 5′ sequences revealed homologies (indicated in Figure 1) to a number of established transcription regulatory sequences associated with genes encoding immune response proteins in mammals (see Discussion).

Construction of a diptericin−lacZ reporter gene

For the dissection of tissue and stage specific expression of promoters, *lacZ* reporter constructs offer the advantage that they facilitate large scale studies on individual animals. However, they present the disadvantage that the β-galactosidase product is extremely stable and this must be borne in mind when interpreting developmental profiles of expression (see below).

The reporter transposon is shown in Figure 2. It consists of the 2.2 kb diptericin promoter fragment fused in the leader sequence to a bacterial *lacZ* gene which is followed by *Drosophila hsp70* termination sequences (see Materials and methods for details of construction). Whereas diptericin gene products are secreted into the haemolymph following cleavage of the signal peptide (see Dimarcq *et al.*, 1988; Wicker *et al.*, 1990), the reporter gene product will remain localized in the cells where it is synthesized. This transposon

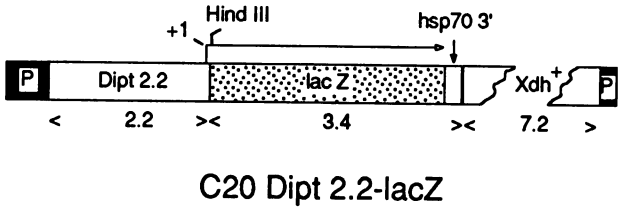

Fig. 2. The elements of the Carnegie 20 diptericin−*lacZ* fusion construct C20 Dipt2.2−*lacZ* that are inserted in transformed lines. Source of DNA fragments: P-element sequences (solid box segments), diptericin DNA (open box) from −2.2 kb from the starsite to the synthetic *Hin*dIII site at +12 in the leader sequence, bacterial *lacZ* (dotted box) and *hsp70* 3′ termination sequences (open box) (see Simon *et al.*, 1985) and the Xdh$^+$ (ry^+) fragment (broken box) that serves as a marker gene in Carnegie 20 (Rubin and Spradling, 1983). Fragment sizes below are in kilobase pairs.

was injected into ry^{506} *C.S.* embryos and five independent insertions were obtained: Dipt2.2−*lacZ*:1 to 5. For detailed developmental studies, one of these, Dipt2.2−*lacZ*:1, was grown in mass culture, and all critical results were confirmed by experiments using the other four insertions.

Fusion gene activity following injection

In pilot experiments, we investigated the effect of injection of live bacteria (*Escherichia coli*) or complete Freund's adjuvant (CFA) into late third instar larvae. Recent experiments in this laboratory with various insect species have shown that CFA is a potent inducer of diptericins and

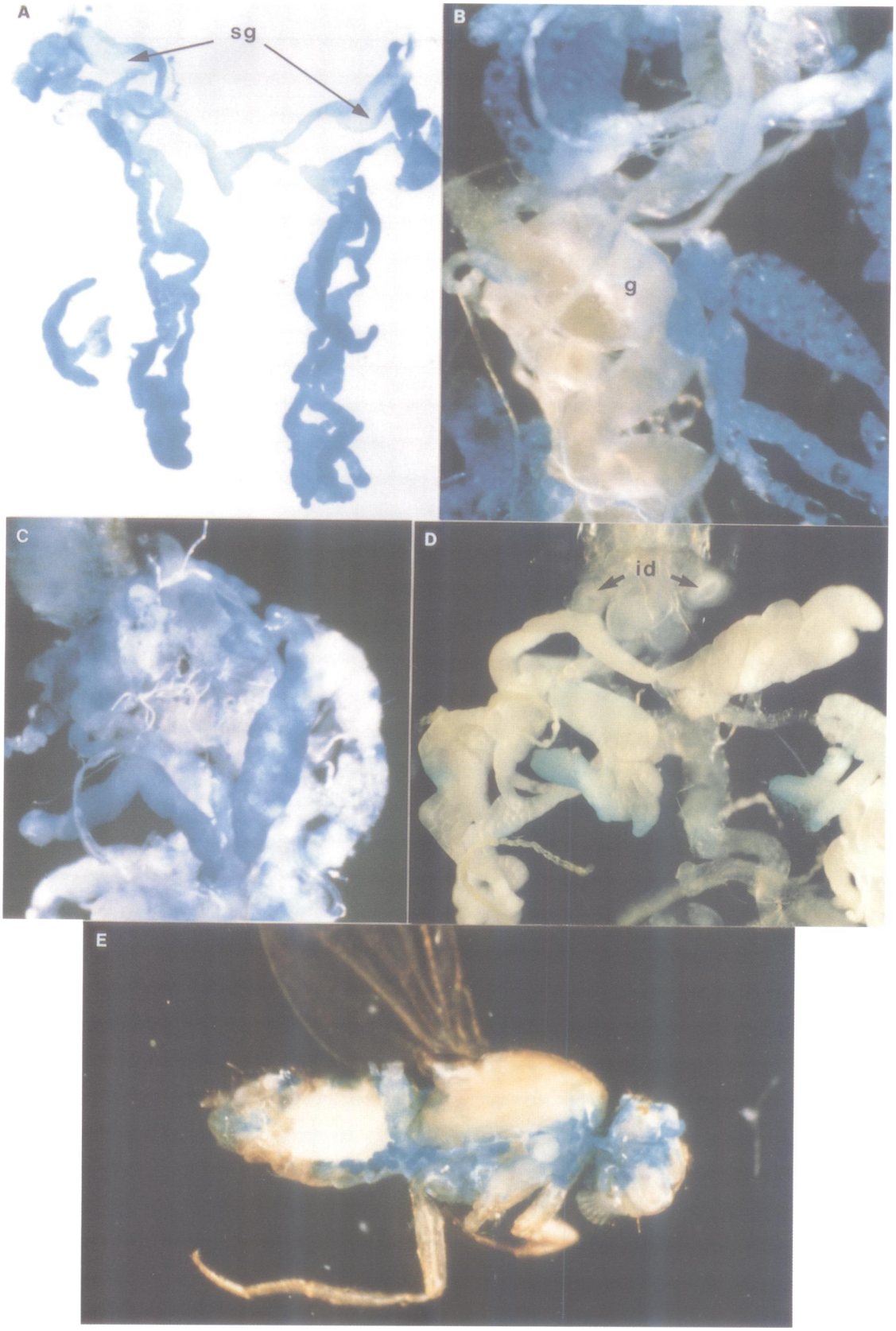

Fig. 3. Histochemical staining of β-galactosidase activity in Dipt2.2−*lacZ*:1 transformants. (**A**) Whole fat body dissected from a wandering stage third instar larva injected for 3 h with *E.coli*. sg, salivary glands; (**B**) detail from animal similar to that shown in (A) g, gut; (**C**) mosaic expression in fat body of a 96 h larva injected for 6 h with CFA; (**D**) spontaneous expression in a restricted number of fat body cells of a late third instar larva (non-injected) id, imaginal discs; (**E**) 4-day-old adult injected for 6 h with *E.coli*: fat body cells are stained in the head, thorax and abdomen. Note that following extended staining periods (>2 h) there is a tendency for coloration by contact of tissues in the proximity of intensely stained fat body cells.

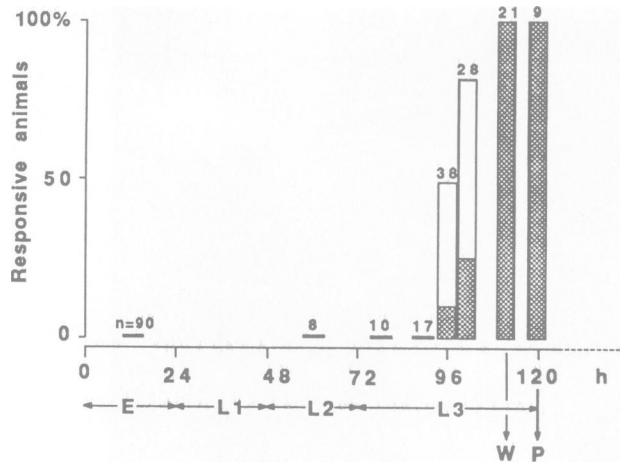

Fig. 4. The response to injection of embyros and larvae of the Dipt2.2−*lacZ*:1 line as a function of developmental stage in hours after egg laying. Animals were staged and injected with LPS (embryos) or CFA (larvae) as described in Materials and methods and dissected and stained 6 h after injection. Solid columns represent the percentage of larvae showing a response of all fat body cells; open columns represent those showing a mosaic response (see Figure 3 and text). Sample sizes (*n*) are given above each column. Developmental stages are marked below: E, embryos; L1, L2 and L3, first, second and third instar larval instars; W, beginning of wandering stage (110 h) and P, pupariation.

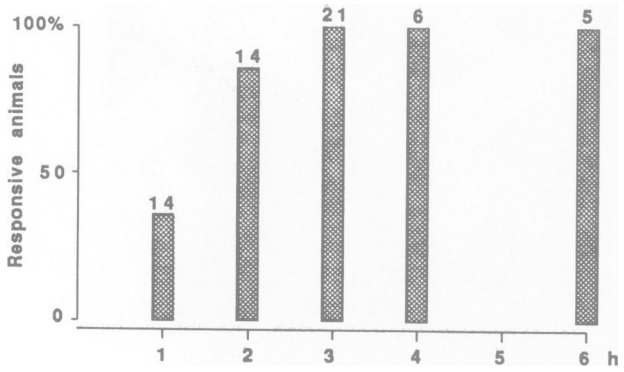

Fig. 5. The response to injection of late third instar (110−120 h) larvae of the Dipt2.2−*lacZ*:1 line. Larvae were pierced with a needle dipped in CFA at t_0 and dissected 1−6 hours afterwards into β-galactosidase staining solution. The response is expressed as a percentage of animals responding and the size of each sample is given above the corresponding column.

defensins (J.-L.Dimarcq, unpublished). We observed an intense coloration in all cells of the fat body of animals dissected 3−6 h after inoculation (Figure 3A and B). This reflects the distribution of induced endogenous diptericin transcripts as detected by *in situ* hybridization (data not shown). Note that some control animals dissected directly into staining solution show a weak spontaneous expression of the fusion gene (see below).

We used a standard CFA stimulus for a developmental study (Figure 4). Unexpectedly, we observed that, unlike late third instar wandering larvae, embryos, first, second and early (72−84 h) third instar larvae did not express the fusion gene in response to the injection. To determine the precise time of acquisition of competence for this response, we staged animals at the second/third instar moult (72 h) and challenged them at different ages by injection followed by a 6 h delay before dissection and staining. Up to 90 h, all animals remained negative; by 96 h, a few animals gave a full response (indistinguishable from that of late wandering stage larvae) while in some only a few fat body cells stained (Figure 3C); some clearly did not respond. From 110 h onwards until pupariation at 120 h, all animals were fully competent, i.e. all fat body cells reacted positively (Figure 3A and B).

Table I. Induction of the Dipt2.2−*lacZ* gene in adult flies

		CFA		*E.coli*	
	t (h)	*n*	Positive response	*n*	Positive response
Adults (0−24 h)	1	7	1		
	2	5	1	10	10
	3	5	2		
	4	8	2	10	10
	5	6	1		
	6	5	2	12	12
	24	21	3	11	11
Adults (>7 days)	2	8	3*	10	10*
	4	7	3*	10	10
	6	6	2*	5	5
	24	9	2*	9	9

Young (0−24 h) or old (>7 days) adults from the Dipt2.2−*lacZ*:1 line were injected with CFA or *E.coli* for the number of hours (*t*) shown before dissection and staining for β-galactosidase activity. *n* = sample size. A positive response is the staining of some or all of larval or adult fat body cells. Asterisks denote samples where staining was noticeably weaker.

We studied this response in detail by dissecting and staining competent animals 1, 2, 3, 4 or 6 h after injection (Figure 5). The rapidity of the response varied between individuals but by 3 h all animals showed a maximal staining. This staining is rapid, the majority of cells being stained 30 min after addition of the chromophore. With a longer staining period, all fat body cells showed a positive reaction. We did not observe any differential zones of reaction although the response is clearly cell autonomous in that there was a mosaicism of reactivity throughout the fat body. This mosaicism was reflected in animals dissected <2 h after injection where we obtained only a partial response with positive cells scattered throughout the fat body. We did not detect the activity of the fusion gene in other tissues, including haemocytes (see Discussion).

We investigated the stability of the fusion protein in competent larvae by dissecting animals 24 or 48 h after injection. In some cases the injury caused a considerable delay in development so that larvae were present in both groups. However, all animals, both larvae and pupae, showed a full response.

From pupariation (120 h), there is a gradual decrease in the response to challenge and in late pupae (72−96 h after pupariation), some animals did not respond at all (data not shown). In contrast to late third instar larvae, young adults (0−24 h) responded poorly to CFA injection (Table I). At maximum, 40% responded by an intense staining throughout the fat body, both with short (1−6 h) or longer (24 h) periods between inoculation and dissection. In older adults (after histolysis of the larval fat body), the proportion of animals which reacted was similar to that seen in young adults (Table I). The overall staining in adult fat body cells was markedly lower than in larval cells. The transgene in lines Dipt2.2−*lacZ*:2 to 5 showed the same developmental

Table II. Spontaneous and induced expression in Dipt2.2−*lacZ*:1 animals raised axenically

	n	Fat body staining				% spontaneous expression
		>90%	50−5%	<5%	Negative	
a. Non-stimulated						
Late third instar larvae	24	−	7	−	17	29
Prepupae/pupae 0−24 h	30	1	7	6	16*	47
Pupae 3−4 days	10	−	−	5	5*	50
Adults 0−2 days	9	−	−	3	6	33
b. Stimulated late third instar larvae						
CFA injection	20	10	6	4	−	
Sterile injection	22	−	4	13	5	

See Materials and methods for experimental details. *n* = sample size. Fat bodies from individuals were assigned to one of the four staining classes defined by the percentage of cells staining. Asterisks in pupal samples denote the presence of some individuals with high levels of endogenous galactosidase activity (see text) which may mask low levels of expression of the fusion gene and may lead to an underestimation of the percentage of individuals showing spontaneous expression (last column).

Table III. Spontaneous expression of the Dipt2.2−*lacZ* gene in Dipt2.2−*lacZ*:1 animals at various stages as shown

Stage	*n*	Fat body staining				% spontaneous expression
		>90%	50−5%	<5%	Negative	
Embryo (0−24 h)	200	−	−	−	200	0
First instar larva	20	−	−	−	20	0
Second instar larva	21	−	−	−	21	0
Early third instar larva	20	−	−	−	20	0
Late third instar larva	64	7	25	−	32	50
White prepupa (0 h)	14	−	5	1	8	43
Prepupa (1−6 h)	54	3	11	13	27	50
Prepupa (9−12 h)	20	3	2	1	14*	30*
Pupa (15−24 h)	30	−	5	10	15*	50*
Pupa (48 h)	10	2	−	2	6*	40*
Pupa (72 h)	10	4	−	−	6*	40*
Pupa (96 h)	15	1	2	4	8*	46*
Adult (0−24 h)	19	−	5	5	9	53
Adult (>3 days)	45	1	−	−	44	2

All symbols are as for Table II.

profile as regards its inducibility as that described here for Dipt2.2−*lacZ*:1.

The above results were obtained with animals raised under standard culture conditions. To investigate the potential importance of commensal microorganisms in the response, we raised axenic animals under sterile conditions (see Materials and methods). The development of these animals was delayed considerably, larvae taking 12−14 days to reach wandering stage (instead of the normal 5 days). In the axenic larvae and pupae we observed a similar frequency and level of spontaneous expression (see below) as in normal animals (Table II). By CFA injection, we observed that competence was acquired later, in that, although some larvae showed a full response, many wandering stage larvae responded still in a mosaic fashion. Aseptic injury somewhat surprisingly gave little or no response in competent axenic larvae (Table II).

When we compared expression of the fusion gene with that of the endogenous diptericin gene (Wicker *et al.*, 1990), we were surprised to note that the latter was significantly higher in adult insects. As in the previous study we had used a bacterial stimulus, we undertook a second series of experiments using live *E.coli*. Under these conditions, all adults showed a full response (Table I and Figure 3E). In earlier stages, we now detected a low frequency (10−20%) of second instar larvae showing faint staining in a few fat body cells. In keeping with this result, the acquisition of competence to respond to live bacteria in third instar larvae occurred several hours before that observed with CFA (see Discussion). The pupal response to the two stimuli was essentially similar in that many late pupae did not respond (data not shown).

Fusion gene activity in non-stimulated animals

In certain untreated larvae dissected directly into staining solution, we observed a low level of spontaneous expression of the fusion gene, normally in <100 of the >2000 cells of the fat body (Figure 3D). In a detailed study of this spontaneous expression using the Dipt2.2−*lacZ*:1 line (Table III) we detected no expression in embryos, first, second and early third instar larvae. In late third instar, ~50% of animals showed activity, the majority in a few cells, although occasionally individuals were indistinguishable from injected larvae (i.e. these scored as >90%, Table III, see Discussion). This result was confirmed with the other insertions, the frequency of animals that stain ranging from 25 to 45%. With the exception of those individuals showing staining throughout the fat body, we estimate that spontaneous expression rarely exceeds 5% of induced expression, given the low number of cells involved and the weakness of the staining reaction in those cells. There were no obvious differences in the frequency of staining in the

various regions of the fat body. Similar data were obtained for early prepupae (0−6 h after pupariation); thereafter a variable endogenous galactosidase activity (see Materials and methods) tends to mask the spontaneous expression of the fusion gene, although coloration in transgenic animals is clearly more important than in the control line (ry^{506} *C.S.*). The endogenous galactosidase activity in adults is localized in specific structures (see Materials and methods) which enables us to distinguish it from the product of the fusion gene which is specifically expressed in larval and/or adult fat body cells. In young adults, the expression of the fusion gene in larval fat body may reflect persistence of activity during the pupal period.

As the above results show that the diptericin promoter responds rapidly to the injury stimulus, we asked whether it might not equally contain elements able to respond to stress conditions, i.e. heat shock. Competent third instar larvae were subjected to a 1 h exposure at 36°C followed by a 1 or 2 h recovery period at 25°C, conditions in which larvae carrying an *hsp70−lacZ* construct treated in parallel showed a maximum response [CHBΔ-89 (19A), Simon *et al.*, 1985]. No staining, other than the low level of spontaneous expression seen in control animals (see above), was observed in the Dipt2.2−*lacZ*:1 animals (data not shown). This indicates that there are two distinct responses to two different forms of aggression.

Reverse transcriptase analyses of diptericin and fusion gene expression

These histochemical analyses detect qualitative aspects of the expression of the fusion gene. We have extended our study by using primer extension analysis of transcripts. We chose oligonucleotide primers complementary to transcripts of the endogenous diptericin gene and the fusion gene (see Materials and methods), which, after elongation, give rise to products of 103 and 96 bases respectively (Figure 6a, lanes 1 and 2); the latter result shows that the fusion gene is indeed transcribed using the diptericin start site. We then used these primers together (10^5 c.p.m. of each) to analyse aliquots of RNA from injected animals (Figure 6a, lanes 3−7). Bearing in mind that the efficiency of radio-labelling, hybridization and elongation may be primer and/or transcript specific, we can nonetheless conclude that the expression of the fusion gene in injected Dipt2.2−*lacZ*:1 larvae is comparable to that of the endogenous diptericin gene. Fusion gene expression in transgenic lines 2, 4 and 5 is similar to that of line 1, whilst in the heterozygous balanced line 3, the activity is less than that of the endogenous gene. A similar quantitative variability in transgene expression due to position effects has been observed for different insertions of *Sgs-3* constructs (see Giangrande *et al.*, 1987). In histochemical experiments we detected the same temporal and spatial distribution of β-galactosidase activity and inducibility of the transgene in all five lines (data not shown), implying that this promoter fragment is resistant to enhancer-trap type position effects.

When using this technique to analyse transcripts from second instar larvae (Figure 6b, lane 1), we were surprised to observe an induction of the endogenous diptericin gene: in contrast, the fusion gene was not transcribed, which is consistent with the results of the staining experiments. As expected, in late third instar larvae (lanes 2, 2′ and 3) both genes are similarly expressed after injection of bacteria or CFA. However, in adults there is again a striking difference between the activity of the two genes, which is even more pronounced when the stimulus is CFA (lanes 4 and 5). Note that these analyses show that both genes present low levels of spontaneous expression (see above) in non-injected larvae and pupae (lanes 6 and 7).

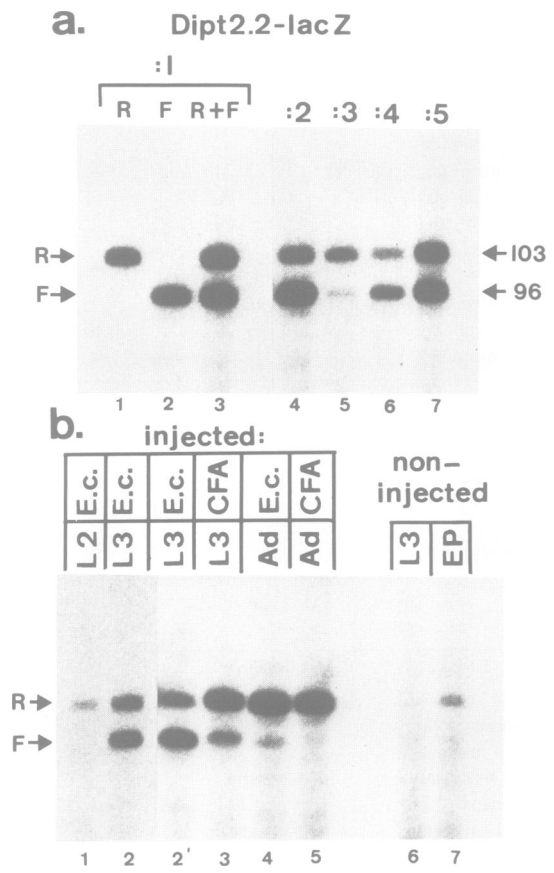

Fig. 6. Primer extension analyses of diptericin and Dipt2.2−*lacZ* transcripts. (**a**) 5 μg of total RNA from Dipt2.2−*lacZ*:1 larvae injected for 3 h with *E.coli* were hybridized with the diptericin (R, lane 1), the Dipt2.2−*lacZ* (F, lane 2) or both (lane 3) primers and treated as described in Materials and methods. In lanes 4−7, 5 μg total RNA from larvae of the Dipt2.2−*lacZ*:2 to 5 lines, injected for 3 h with *E.coli*, were analysed with both primers. The size (in bases) of the elongation products for the resident (R) and fusion gene (F) transcripts are indicated. (**b**) 5 μg of total RNA from Dipt2.2−*lacZ*:1 animals were analysed with both primers (see panel a): second instar larvae injected for 6 h with *E.coli* (lane 1); late third instar larvae injected for 3 h with *E.coli* (lanes 2 and 2′, two independent experiments) or CFA (lane 3); 5 day-old adults injected for 6 h with *E.coli* (lane 4) or CFA (lane 5); spontaneous expression in late third instar larvae (lane 6) and in early pupae (lane 7).

Discussion

The diptericin gene, which is present in a single copy per haploid genome (mapping at 56A, see Wicker *et al.*, 1990) is devoid of intronic sequences. This relatively simple situation contrasts with that of cecropins, the only other inducible antibacterial peptides so far cloned in *Drosophila*. Three cecropin genes, each containing a single intron of 58−61 nucleotides, and two pseudogenes, are clustered within <4 kb of DNA (mapping at 99E, see Kylsten *et al.*, 1990). To dissect the regulation of the diptericin gene, we have transformed flies with a fusion gene in which the reporter β-galactosidase is under the control of 2.2 kb

upstream sequences of the diptericin gene. Our results demonstrate that such a fusion gene is inducible and respects the tissue specific pattern of the resident diptericin gene. Indeed, the responsiveness of the reporter gene during immune challenge has provided us with an efficient experimental tool for a detailed analysis at the level of individual insects and cells.

As regards the tissue specific pattern, the examination of several hundred transgenic animals showed that the expression of the fusion gene is restricted to the cells of the fat body. In late third instar larvae, all cells of the fat body are responsive and we did not detect differences between the various regions of the fat body. However, the reaction is cell autonomous as illustrated by the mosaicism of reactivity throughout the fat body. *In situ* hybridization studies in another dipteran insect, *Phormia terranovae*, revealed expression of the diptericin genes in fat body and in one of the circulating blood cell types, the thrombocytoid (Dimarcq *et al.*, 1990). This cell type is absent in *Drosophila* (Brehélin and Zachary, 1983). In *Drosophila*, the cecropin genes are expressed in the fat body cells and in 5−10% of the haemocytes (Samakovlis *et al.*, 1990). We did not detect activity either of the endogenous diptericin gene in blood cells by *in situ* hybridization or of the fusion gene by staining or by using an anti-β-galactosidase antibody (not shown) and although we cannot exclude the possibility that *Drosophila* blood cells express the fusion gene or the endogenous diptericin gene, their contribution to the overall synthesis of diptericin, if it exists, is certainly minimal.

Before considering the complexity of the inducibility of the fusion gene by an injection, we wish to discuss briefly the perplexing problem of spontaneous expression. Wicker *et al.* (1990) noted that untreated pupae as well as young adults of *Drosophila* contained low levels of diptericin transcripts. Samakovlis *et al.* (1990) did not detect cecropin transcripts in untreated *Drosophila* except for a small but marked mRNA peak in early pupae, and sometimes a variable and usually very low expression in adults. Constitutive expression of two other inducible antibacterial peptides, insect defensin and a cecropin-related peptide, have also been detected in pupae of *Sarcophaga* (Matsuyama and Natori, 1988; Nanbu *et al.*, 1988). These observations were mainly based on Northern blot analyses of RNAs extracted from pools of insects. Our primer extension analyses confirm the presence of low levels of expression in larvae and pupae which are similar for the endogenous gene and our fusion gene (Figure 6b). However, by following the expression of the fusion gene in individual insects, we were able to refine the image of spontaneous expression which is not a general phenomenon among a given group of insects, but usually affects less than half of the animals, with expression limited to a few cells. This is in contrast to the expression pattern induced by injection of bacteria in which all the fat body cells of all the treated insects are reactive.

The spontaneous reaction which we observe in normal rearing conditions occurs only in late larvae, pupae and young adults. As at least half of the insects within a population do not express the diptericin fusion gene spontaneously, it is difficult to believe that the endogenous diptericin plays a specific role in normal development, as has been proposed earlier (see e.g. Wicker *et al.*, 1990). It should be kept in mind that the period of development when spontaneous expression is observed, is centred around

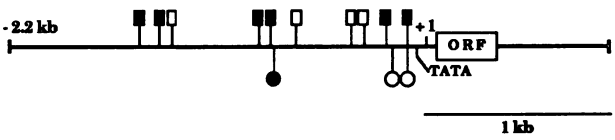

Fig. 7. The distribution of sequences homologous to vertebrate immune gene response elements in the 2.2 kb diptericin promoter fragment. ■, the NF-IL6 motif TT/GNNGNAAT/G; ●, the NF-κB motif GGGRNNYYCC; ○, an NF-κB-related heptadecameric motif CATCGGGGATTCCTTTT; and □, a hexameric motif, CTGGGA, present in acute phase protein gene promoters (see Locker and Buzard, 1990, for references).

the process of metamorphosis. It is possible that erratically occurring lesions both in those control larvae in which we detect intense staining and in some of the insects undergoing metamorphosis induce the expression of immune genes. At least as far as diptericin gene expression is concerned, this phenomenon is independent of the presence of bacteria, as it is also observed in axenically raised animals.

The complexity of the response to injection is such that we will discuss it in relation to development, both in terms of endogenous and fusion gene expression and the nature of the stimulus. In young larvae (L2 and early L3), the endogenous gene is clearly induced, in contrast to the fusion gene which remains silent. This suggests that during early development, induction requires remote regulatory sequences (5′ and/or 3′) other than those contained in the 2.2 kb diptericin promoter fragment. During the third larval instar, the fusion gene promoter becomes increasingly responsive and in the wandering stage the level of fusion gene transcripts is similar to those of the endogenous gene, suggesting that the more remote sequences are no longer necessary.The responsiveness to the injection of live bacteria occurs a few hours earlier in the instar than that to CFA. In wandering larvae the response of both genes to the two stimuli appear similar both in rapidity and intensity. While the response of early pupae to both stimuli resembles that of late larvae, it decreases by 3−4 days of pupal development and indeed many animals fail to respond.

Following adult emergence, a new pattern of response is evident: whereas the endogenous diptericin gene is strongly induced by both stimuli, the fusion gene only weakly responds to CFA. While the response to bacteria is stronger, it remains inferior to that of the endogenous gene. As is the case in young larvae, this result suggests that the regulatory sequences in the 2.2 kb construct are insufficient to support a maximal response in adults. Thus, the analysis of this first fusion gene construct has revealed at least four distinct phases in diptericin gene regulation, i.e. young larvae, late third instar larvae, pupae and adults.

At present the transcriptional activators which regulate the expression of the immune genes have not been characterized in insects. To direct our dissection of the complexity of the response in future experiments, we have used a computer-assisted analysis of the 2.2 kb upstream sequences. This analysis reveals several putative transcription control sequences associated with the mammalian immune response (Figure 7, see also Figure 1). (i) The binding motif for NF-IL6. The diptericin promoter contains several copies of the consensus motif for the binding site of a mammalian nuclear factor conferring IL-6 inducibility (Isshiki *et al.*, 1991) to several acute phase protein genes in the liver (Oliviero and Cortese, 1989; Poli and Cortese, 1989). (ii)

A hexameric sequence CTGGGA which has been identified as an interleukin response element (IL-6RE) by functional assays in the acute phase gene α2-macroglobulin (Northemann *et al.*, 1988; Hattori *et al.*, 1990). Similar sequences have been found in other acute phase genes (Fowlkes *et al.*, 1984; Isshiki *et al.*, 1991). The 2.2 kb diptericin upstream sequences contain four copies of this motif. (iii) A binding motif identical to the consensus binding sequence of NF-$\varkappa$B. Nuclear factor $\varkappa$B (NF-$\varkappa$B) inducibly initiates the transcription of a wide variety of genes by binding to decameric sequence motifs in enhancer and promoter elements (see Baeuerle and Baltimore, 1991; Baeuerle, 1991, for reviews). Most of its target genes fall into three classes encoding immuno-modulatory cytokines, immunologically important cell surface receptors and acute phase response proteins (Urban *et al.*, 1991). The 2.2 kb diptericin upstream sequences contain a single NF-$\varkappa$B motif but in addition harbour two identical NF-$\varkappa$B related decameric sequences which diverge from the NF-$\varkappa$B consensus sequence only by the replacement of the 3′ C by a T (see Figures 1 and 7). Single decameric nucleotide sequences identical or similar to those present within the two heptadecamers of the diptericin promoter have been noted upstream of several immune genes of *Hyalophora cecropia* (cecropin B, lysozyme, acidic and basic attacins), as well as in one of the cecropin genes of *Drosophila* (see Sun *et al.*, 1991). As for the diptericin decameric consensus sequence, they also diverge from the mammalian NF-$\varkappa$B binding motif by the replacement of the 3′ C by a T. A functional analysis of these and other putative regulatory sequences in the diptericin promoter is in progress.

Our results give a first insight into the complexity of the regulation of the immune response in insects and suggest that it may not be significantly simpler than that of vertebrates. The experimental advantage of insect systems is clearly demonstrated by our transgenic approach and we are constructing transposons to investigate the different phases of regulation we have defined here. One particularly intriguing aspect is the mechanism by which our 2.2 kb promoter becomes fully inducible during the third instar. This follows a time course resembling those recently reported for ecdysone regulated changes in larval gene activity (Murtha and Cavener, 1989; Georgel *et al.*, 1991) and we are currently investigating the possibility that the hormone is involved directly or indirectly in the maturation of the diptericin promoter or a signal transduction pathway.

Materials and methods

Isolation and sequencing of the diptericin genomic fragment

We screened 100 000 p.f.u. of an amplified *Drosophila* Oregon-R genomic library constructed in λ EMBL4 (gift of Dr V.Pirrotta) with a nick-translated diptericin cDNA probe (Wicker *et al.*, 1990) and obtained four positive plaques shown by restriction mapping to be identical. DNA sequences were determined on both strands by the dideoxyoligonucleotide chain termination method, after subcloning appropriate restriction fragments into M13 phages by standard methods (Sambrook *et al.*, 1989). Computer analysis of transcription control sequences was performed using Laser gene DNAstar™ software.

Construction of the diptericin–lacZ reporter gene fusion and isolation of transformed strains

The *hsp70–lacZ* fusion construct p8970ZT (kindly provided by John Lis, see Simon *et al.*, 1985) was modified (C.Gerst and G.Richards, unpublished) by the creation of a *Hin*dIII site by oligonucleotide directed mutagenesis at position 306, which is at +214 in the *hsp70* leader, some 25 bp before the ATG which initiates protein synthesis and the insertion of a *Xho*I linker in the *Bgl*II site at 3678. The resulting 3.4 kb *Hin*dIII–*Xho*I fragment which contains the entire *lacZ* coding sequence and *hsp70* transcription termination sequences was fused to the 2.2 kb diptericin promoter fragment which had been modified by the insertion of a *Hin*dIII site at +12 in its leader sequence (see Figure 1). The fusion was inserted in the *Sal*I site of the Carnegie 20 transformation vector in the orientation shown in Figure 2.

The resulting transposon, C20 Dipt2.2–*lacZ* (300 μg/ml), was co-injected with an integration defective helper plasmid into ry^{506} *C.S.* embryos. Transformants were recovered as G1 ry^+ flies. The chromosome carrying the insertion and stable homozygous (insertions 1, 2 and 4) or heterozygous (3 and 5) lines were established by crosses to appropriate balancers. All lines were examined by Southern analysis to verify the presence of a single non-rearranged insertion of the transposon (data not shown). Chromosome locations were: line 1 (X chromosome), 2 (3), 3 (3), 4 (2) and 5 (2).

Culture conditions and inoculations

The control (ry^{506} *C.S.*) and transformed lines were maintained at 25°C on a standard cornmeal medium. In these conditions, the first embryos hatch ~22 h after egg laying, the second to third larval moult is at 72 h, pupariation occurs at 120 h and adults emerge at 240 h. Control and injected embryos were mixed populations recovered from overnight (0–16 h) egg collections. First and second instar larvae were from 24–48 and 48–72 h after egg laying respectively. For the third instar, larvae were reselected at the second to third moult by size and anterior spiracle morphology, placed in 1.5 cm diameter tubes (~30 larvae per tube) and returned to 25°C until they reached the desired age. Larvae synchronized in this way leave the food at ~110 h and pupariate in a 4–8 h period at ~120 h. For later stages animals were selected as white prepupae, a stage which lasts ~15 min, and then aged at 25°C in fresh food tubes.

Embryos were injected using our standard transformation injection apparatus with glass needles, containing a 0.25% lipopolysaccharide solution; LPS is an established inducer of the immune response in insects (e.g. Dimarcq *et al.*, 1990) and was preferred because of the narrow gauge of the needles. Embryos from CHBΔ-89(19A), an *hsp70–lacZ* transformed line (kindly supplied by John Lis), were heat shocked and treated in parallel as a control for galactosidase staining. For later stages, animals were pierced with a sodium nitrite sharpened tungsten needle dipped in a saturated culture of *E.coli* or in complete Freund's adjuvant (Sigma). Routine survival rates (3, 6, 24 or 48 h after injury) were at least 90%.

Axenic animals were produced by treating an overnight egg collection for 3 min with Chlorox and transferring the dechorionated embryos onto standard medium which had been sterilized by reautoclaving. Under these conditions development was slower, with pupariation occurring on day 12–15 rather than at the end of day 5. All sterile manipulations used a laminar flow hood and all samples were held between inoculation and dissection on sterile Petri dishes containing LB agar medium. These were placed at 37°C overnight at the end of the experiment to verify that no bacteria or yeast were present.

The β-galactosidase histochemical assay

Animals were rinsed in distilled water and dissected in individual droplets (50 μl) of staining solution (0.3% X-gal, 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide, 50 mM sodium phosphate pH 8.0, 25% Ficoll-400; see Vijay Raghavan *et al.*, 1986). Animals were scored in the period 15 min to 2 h after dissection and again after overnight incubation. Staining of a number of tissues, notably regions of the gut, pupal and adult Malpighian tubules, adult pericardial cells, the male sperm pump as well as a generalized coloration of variable intensity in mid-pupae was observed in the control line as described by Glaser *et al.* (1986). However, in agreement with their findings, this endogenous *Drosophila* galactosidase activity is weaker than that of the transgene and, with the exception of the gut, is only seen after overnight staining.

RNA analysis

For primer extension assays, 5 μg of total RNA [extracted from 5–20 animals using a LiCl–urea based protocol (Richards *et al.*, 1983)] were co-precipitated with 10^5 c.p.m. of the appropriate ^{32}P-labelled oligonucleotide. The pellet was resuspended in 10 μl of hybridization buffer (250 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 0.2% SDS and 10 mM ribonucleoside–vanadyl complex) and placed in a 65°C water bath which was allowed to cool to 33°C overnight. After sodium acetate–ethanol precipitation, the pellet was resuspended in 30 μl reverse transcriptase buffer (200 mM Tris HCl, pH 8.3, 200 mM KCl, 20 mM MgCl2, 20 mM DTT, 0.5 mM of each deoxynucleotide) with 5 U AMV RTase (Pharmacia). The reaction was incubated for 45 min in a water bath at 42°C and then reprecipitated as above. The pellet was resuspended in 2 μl TE (10:1, pH 8),

3 μl of formamide loading buffer were added and, after heating for 4 min at 90°C, the products were analysed on a denaturing 8% polyacrylamide gel, in parallel with the diptericin DNA sequence as size marker. Primers were as follows: Dipt, 5′-GATAAGCCAAAGTAGAA-3′ (+103 to +87); and Dipt−*lacZ*, 5′-GTTTTCCCAGTCACGAC-3′ (+96 to +80).

Acknowledgements

Marie Meister thanks Professor P.Chambon for providing research facilities that enabled us to pursue this collaboration. We thank John Lis for the *hsp70−lacZ* construct and the transformed strain used in control experiments. A.Meunier provided excellent technical assistance. We thank A.Staub and F.Ruffenach for the synthesis of oligonucleotides. We are grateful to C.Hetru for his help in the preparation of the figures. This work was supported by institutional grants from the CNRS and the INSERM.

References

Ando,K. and Natori,S. (1988) *Biochemistry*, **27**, 1715−1721.
Baeuerle,P.A. (1991) *Biochim. Biophys. Acta*, **1072**, 63−80.
Baeuerle,P.A. and Baltimore,D. (1991) In Cohen,P. and Foulkes,J.G. (eds), *Molecular Aspects of Cellular Regulation, Hormonal Control Regulation of Gene Transcription*. Elsevier/North Holland Biomedical Press, Amsterdam, pp. 409−432.
Brehélin,M. and Zachary,D. (1983) *Dev. Comp. Immunol.*, **7**, 683−686.
Bulet,P., Cociancich,S., Dimarcq,J.-L., Lambert,J., Reichhart,J.M., Hoffmann,D., Hetru,C. and Hoffmann,J.A. (1991) *J. Biol. Chem.*, **266**, 24520−24525.
Casteels,P., Ampe,C. Jacobs,F., Vaeck,M. and Tempst,P. (1989) *EMBO J.*, **8**, 2387−2391.
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.
Dimarcq,J.-L., Zachary,D., Hoffmann,J.A., Hoffmann,D. and Reichhart,J.M. (1990) *EMBO J.*, **9**, 2507−2515.
Faye,I. and Wyatt,G.R. (1980) *Experientia*, **36**, 1325−1326.
Fowlkes,D.M., Mullis,N.T., Comeau,C.M. and Crabtree,G.R. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 2313−2316.
Georgel,P., Ramain,P., Giangrande,A. Dretzen,G., Richards,G. and Bellard,M. (1991) *Mol. Cell. Biol.*, **11**, 523−532.
Giangrande,A., Mettling,C. and Richards,G. (1987) *EMBO J.*, **6**, 3079−3084.
Glaser,R.L., Wolfner,M.F. and Lis,J.T. (1986) *EMBO J.*, **5**, 747−754.
Hattori,M., Abraham,L.J., Northemann,W. and Fey,G.H. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 2364−2368.
Hultmark,D., Engström,A., Andersson,K., Steiner,H., Bennich,H. and Boman,H.G. (1983) *EMBO J.*, **2**, 571−576.
Hultmark,D., Klemenz,R. and Gehring,W.J. (1986) *Cell*, **44**, 429−438.
Isshiki,H., Akira,S., Sugita,T., Nishio,Y., Hashimoto,S., Pawlowski,T., Suematsu,S. and Kishimoto,T. (1991) *New Biol.*, **3**, 63−70.
Koj,A. (1985) In Gordon,G. and Koj,A. (eds) *The Acute-phase Response to Injury and Infection*. Elsevier, Amsterdam Vol. 10, pp. 139−232.
Kushner,I. (1982) *Ann. N.Y., Acad. Sci.*, **389**, 39−48.
Kylsten,P., Samakovlis,C. and Hultmark,D. (1990) *EMBO J.*, **9**, 217−224.
Lambert,J., *et al.* (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 262−266.
Locker,J. and Buzard,G. (1990) *DNA Seq. Map.*, **1**, 3−11.
Matsuyama,K. and Natori,S. (1988) *J. Biol. Chem.*, **263**, 17117−17121.
Morrone,G., Ciliberto,G., Oliviero,S., Arcone,R., Dente,L. Content,J. and Cortese,R. (1988) *J. Biol. Chem.*, **263**, 12554−12558.
Murtha,M.T. and Cavener,D.R. (1989) *Dev. Biol.*, **35**, 66−73.
Nanbu,R., Nakajima,Y., Ando,K. and Natori,S. (1988) *Biochem. Biophys. Res. Commun.*, **150**, 540−544.
Northemann,W., Shiels,B.R., Braciak,T.A., Hanson,R.W., Heinrich,P.C. and Fey,G.H. (1988) *Biochemistry*, **27**, 9194−9203.
Okada,M. and Natori,S. (1985) *J. Biol. Chem.*, **260**, 7174−7177.
Oliviero,S. and Cortese,R. (1989) *EMBO J.*, **8**, 1145−1151.
Poli,V. and Cortese,R. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 7547−7551.
Richards,G., Cassab,A., Bourouis,M., Jarry,B. and Dissous,C. (1983) *EMBO J.*, **2**, 2137−2142.
Rubin,G.M., and Spradling,A. (1983) *Nucleic Acids Res.*, **11**, 6341−6351.
Samakovlis,C., Kimbrell,D.A., Kylsten,P., Engström,A. and Hultmark,D. (1990) *EMBO J.*, **9**, 2969−2976.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, second edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Simon,J.A., Sutton,C.A., Lobell,R.B., Glaser,R.L. and Lis,J.T. (1985) *Cell*, **40**, 805−817.
Steiner,H., Hultmark,D., Engström,A., Bennich,H. and Boman,H.G. (1981) *Nature*, **292**, 246−248.
Sun,S.C., Lindström,I. and Faye,I. (1991) *Eur. J. Biochem.*, **196**, 247−254.
Trenczek,T. (1988) *Insect Biochem.*, **181**, 299−312.
Urban,M.B., Schreck,R. and Baeuerle,P.A. (1991) *EMBO J.*, **10**, 1817−1825.
Vijay Raghavan,K., Crosby,M.A., Mathers,P.H. and Meyerowitz,E.M. (1986) *EMBO J.*, **5**, 3321−3326.
Wicker,C., Reichhart,J.M., Hoffmann,D., Hultmark,D., Samakovlis,C. and Hoffmann,J.A. (1990) *J. Biol. Chem.*, **265**, 22493−22498.

Received on November 4, 1991; revised on December 13, 1991

Note added in proof

The sequence data reported here have been deposited in the EMBL Sequence Data Library under the accession number Z11728.