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Insect immunity. Two 17 bp repeats nesting a κB-related sequence confer inducibility to the diptericin gene and bind a polypeptide in bacteria-challenged *Drosophila*

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The *Drosophila* diptericin gene codes for a 9 kDa antibacterial peptide and is rapidly and transiently expressed in larvae and adults after bacterial challenge. It is also induced in a tumorous *Drosophila* blood cell line by the addition of lipopolysaccharide (LPS). The promoter of this gene contains two 17 bp repeats located closely upstream of the TATA-box and harbouring a decameric κB-related sequence. This study reports that the replacement of the two 17 bp repeats by random sequences abolishes bacteria inducibility in transgenic fly lines. In transfected tumorous blood cells, the replacement of both or either of the 17 bp motifs reduces dramatically LPS inducibility, whereas multiple copies significantly increase the level of transcriptional activation by LPS challenge. A specific DNA–protein binding activity is evidenced in cytoplasmic and nuclear extracts of induced blood cells and fat body. It is absent in controls. It is proposed that induction of the diptericin gene mediated by the two 17 bp repeats occurs via a mechanism similar to that of mammalian NF-κB.

Key words: diptericin/insect immunity/NF-κB/transfection/transgenesis

Introduction

One of the facets of the strong antibacterial defence reactions observed in higher insects is the rapid and transient synthesis of a battery of potent bactericidal peptides (reviewed in Boman *et al.*, 1991; Hoffmann and Hetru, 1992). These are mostly small-sized cationic molecules with a broad spectrum of activity against Gram-positive and/or Gram-negative bacteria. It has been noted that several characteristics of the antibacterial host-defence of insects are evocative of the acute phase response in mammals (Kanai and Natori, 1990; Hoffmann and Hetru, 1992; Hoffmann *et al.*, 1992; Reichhart *et al.*, 1992). The recent cloning of some of the genes encoding inducible antibacterial peptides in *Hyalophora* and *Drosophila* has highlighted the presence of response elements previously identified in mammalian acute phase response genes as *cis*-regulatory sequences (Sun *et al.*, 1991a; Sun and Faye, 1992a; Reichhart *et al.*, 1992). In particular, this is the case for binding motifs for NF-κB and for proteins conferring IL-6 inducibility (NF-IL-6 and IL-6 RE-BP, see Hocke *et al.*, 1992). These observations have kindled interest in the analysis of the regulation of the expression of the genes encoding the inducible antibacterial peptides in insects and have prompted the hypothesis that a common ancestral control mechanism governs the expression of these first line host-defence genes in insects and mammals.

This study focuses on the expression of the diptericin gene in *Drosophila*. Diptericins are a family of 9 kDa anti-Gram negative polypeptides initially isolated from bacteria-challenged larvae of the fleshfly *Phormia terranovae* (Dimarcq *et al.*, 1988). *Drosophila* contains a single intronless diptericin gene which is expressed predominantly in the fat body shortly (1–2 h) after injection of low doses of bacteria (Wicker *et al.*, 1990; Reichhart *et al.*, 1992). Recent experiments with transformed flies have established that 2.2 kb of upstream sequences of the diptericin gene are sufficient to confer to a reporter fusion gene inducibility by bacterial injection and tissue specific expression identical to those of the resident diptericin gene (Reichhart *et al.*, 1992). As illustrated in Figure 1a, these upstream sequences contain several motifs homologous to mammalian immune gene response elements. Our attention was attracted by two 17 bp repeats located closely upstream of the TATA-box and harbouring a decameric κB related sequence. The potential role of these repeats in the expression of the diptericin gene during the immune response of *Drosophila* was investigated. Two sets of experiments were undertaken: (i) an inducible *Drosophila* blood cell line was transfected with reporter plasmids containing wild-type or mutated 17 bp repeats in their upstream sequences; and (ii) transgenic fly lines were generated which carry a reporter transposon with normal or mutated 17 bp repeats. In both cases the inducibility by bacteria or lipopolysaccharide (LPS) was examined. Finally the binding of these putative regulatory sequences to proteins in induced blood cells and insects was analysed.

Results

Transfection of Drosophila mbn-2 cells

The *mbn-2* cells are tumorous blood cells derived from the *D.melanogaster* recessive-lethal mutant *lethal (2) malignant blood neoplasm* (Gateff, 1978). In this mutant, phagocytotic blood cells, the so-called plasmatocytes, are malignant and show various stages of differentiation which reportedly are able to inhibit excessive bacterial growth when chance contaminations of the cell cultures occur (Gateff *et al.*, 1980). The *mbn-2* cells do not normally express the diptericin gene, but expression can be rapidly induced by the addition of LPS as illustrated in Figure 2. Live or heat-killed bacteria also induce the expression of this gene (data not shown). Other genes encoding antibacterial peptides, namely the cecropins, can also be induced by the addition of LPS or bacteria as shown previously by Hultmark and associates (Samakovlis, 1991; Samakovlis *et al.*, 1992).

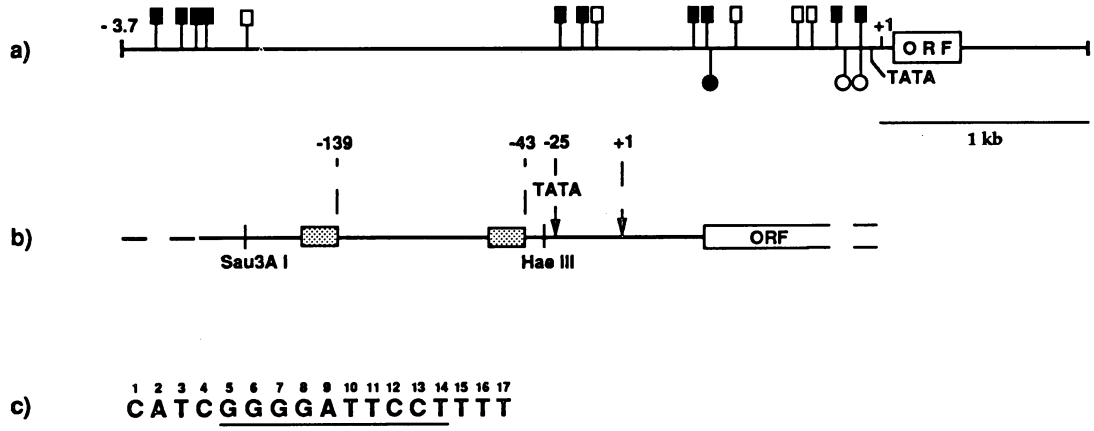

Fig. 1. Promoter elements of the *Drosophila* diptericin gene. (**a**) Distribution of sequences homologous to vertebrate immune gene reponse elements in the 3.7 kb diptericin promoter fragment. (i) Sequences related to mammalian NF-$\varkappa$B response element: ○, two 17 bp repeats harbouring a decameric $\varkappa$B-related sequence 5′-GGGGATTCCT-3′; ●, a decameric $\varkappa$B-related sequence 5′-GGGAAATTCC-3′; (ii) sequences related to mammalian NF-IL-6 response elements: ■, type I element 5′-TT/GNNGNAAT/G-3′; □, type II element 5′-CTGGGA-3′. Classification as proposed by Hocke *et al.*, 1992. (**b**) Detail of the proximal upstream sequences. The dashed boxes give the positions of the 17 bp repeats nesting a $\varkappa$B-related sequence. The numbers indicate the distance in nucleotides from the transcription start site. (**c**) Nucleotide sequence of the two 17 bp repeats numbered from 5′ to 3′ as 1 to 17. The $\varkappa$B-related decameric sequence is underlined.

This inducible tumorous blood cell line was chosen for transfections with plasmids in which upstream sequences of the diptericin gene were fused to the chloramphenicol acetyl transferase (*cat*) reporter gene. The constructions were based on the vector pBLCAT5 (Boshart *et al.*, 1992) which contains a minimal thymidylate kinase (*tk*) promoter fused to the bacterial *cat* reporter gene as shown in Figure 3. When transfected into *mbn-2* cells this plasmid does not generate CAT activity, even in the presence of LPS. Plasmid D1 was constructed in which a *Sau*3AI−*Hae*III restriction fragment (Figure 1b) was fused to the *tk* promoter sequence. This fragment corresponds to nucleotides −180 to −35 upstream of the transcription initiation site of the diptericin gene and comprises the two 17 bp repeats (Reichhart *et al.*, 1992). In cells transfected with this plasmid CAT activity was low, but was significantly (7-fold) enhanced upon the addition of LPS to the culture medium (Figure 3). However, when the two 17 bp repeats were replaced by a random sequence (plasmid D3), the LPS inducibility was lost and CAT activity was not greater than the background value observed in the absence of LPS. In fact, as illustrated in Figure 3, the replacement of either of the two repeats (plasmids D4 and D5) dramatically reduced LPS inducibility. These results establish that LPS inducibility of the diptericin gene in *mbn-2* cells is dependent on the presence of two wild-type copies of the 17 bp sequence. In reverse orientation (plasmid D2), these repeats are still able to confer LPS inducibility, although to a somewhat lower degree.

The distance observed between the two 17 bp repeats in the wild-type promoter is not mandatory for inducibility, as illustrated by the experiments performed with plasmid D6 in which the repeats were put into close proximity (20 bp as compared with 79 bp in the wild-type sequence): the inducibility was maintained, even when the two repeats were in reverse orientation (plasmid D7). Finally, when several copies (eight) were fused in tandem upstream of the reporter cassette (D9), a 40-fold enhanced CAT activity was observed in LPS-induced cells. This inducibility was reduced to 30-fold when the eight copies were in reverse orientation (plasmid D10). However, mutated multiple copies (D8, D11) did not confer inducibility.

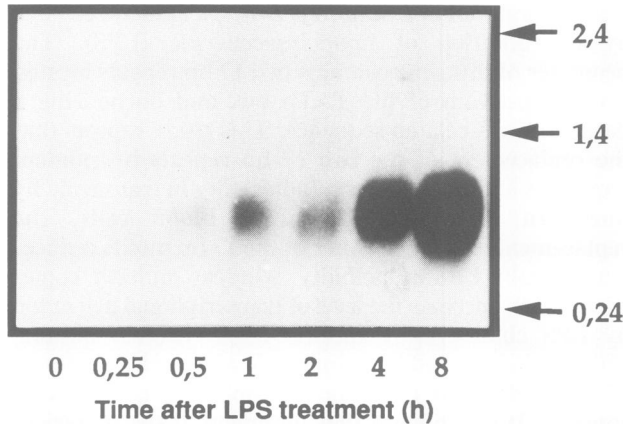

Fig. 2. Transcriptional profiles of the diptericin gene following LPS treatment in *mbn-2* cells.
Poly(A)-enriched RNA (5 μg) was isolated from *mbn-2* cells at different times after LPS treatment and subjected to electrophoresis on a 1.5% agarose gel, blotted onto a nitrocellulose filter and hybridized with a nick-translated cDNA probe (Wicker *et al.*, 1990). Marker positions are presented on the right of the figure.

Transgenic fly lines

As mentioned in Introduction, it had been shown previously in transgenic flies that a hybrid gene consisting of a 2.2 kb diptericin promoter fragment fused to the bacterial *lacZ* reporter gene was inducible by injection of live bacteria (Reichhart *et al.*, 1992). The fusion gene was expressed in the fat body cells, although it could not be ruled out that some expression also occurred in blood cells. In the reporter transposon, used in the previous study (and referred to as C20Dipt2.2-*lacZ*), the wild-type *Sau*3AI−*Hae*III fragment of the diptericin promoter (Figure 1b) containing the 17 bp repeats has been replaced by a fragment in which these repeats have been mutated to a random sequence (the same fragment as in plasmid D3, Figure 3). The mutated transposon, labelled C20Dipt2.2mut-*lacZ*, was injected into ry^{506}*C.S.* embryos. Twenty independent insertion lines were obtained out of which 15 were homozygous (on chromosomes 2, 3 or X) and five were heterozygous balanced (on chromosomes 2 and 3).

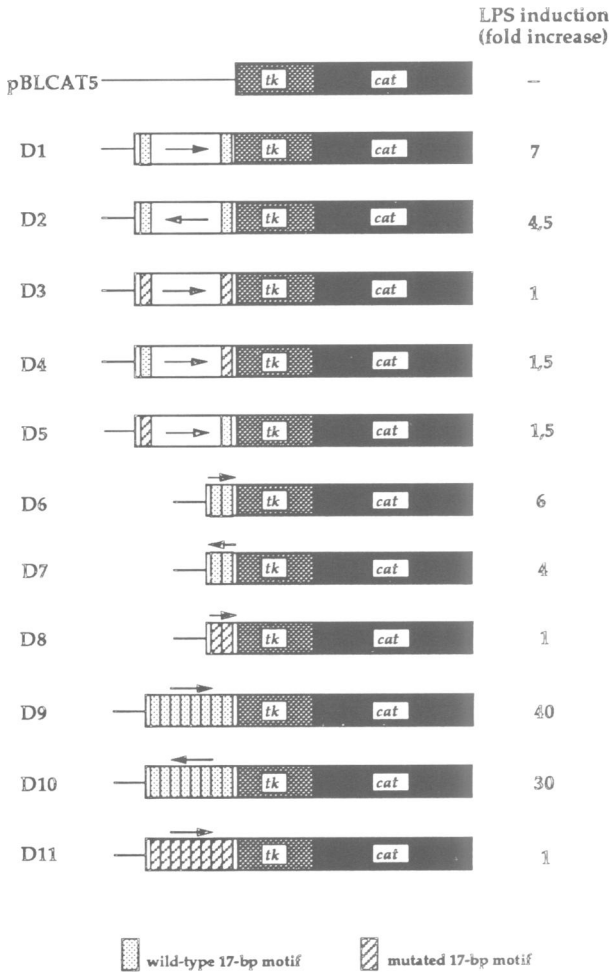

Fig. 3. Effect of LPS treatment on diptericin *cat* hybrid genes expression. Cells were cotransfected with the indicated *cat* reporter plasmid and an internal control, the β-galactosidase expression vector pACH (Thisse *et al.*, 1991), as described in Materials and Methods. CAT activity was measured in extracts from uninduced and LPS treated cells (20 μg/ml, 19 h). Results were normalized for β-galactosidase activity and expressed as the mean fold increase in CAT activity (three independent experiments). Fold increase indicates the ratio of the per cent conversion (chloramphenicol to its acetylated forms) of a LPS treated sample to per cent conversion of the untreated sample.

The effects of bacterial challenge in transgenic fly lines carrying the fusion gene with the wild-type promoter (Dipt2.2-*lacZ*) have been compared with those carrying the mutated sequences (Dipt2.2mut-*lacZ*). Injections of live bacteria were performed on late third instar larvae and adults. The expression of the reporter gene was assessed by dissection of the insects in a β-galactosidase staining solution. In all transgenic insects with wild-type promoter sequences, a deep blue colour was observed in the fat body cells, as described for this fusion gene previously (Reichhart *et al.*, 1992). In contrast, none of the 20 lines carrying the mutated promoter sequences showed a positive staining (data not shown).

In an additional series of experiments, the inducibility of the resident diptericin gene in both types of transgenic animals was compared with that of the fusion gene. For this, oligonucleotide primers were used which were complementary to the transcripts of the endogenous diptericin and fusion genes and which, after elongation, gave rise to

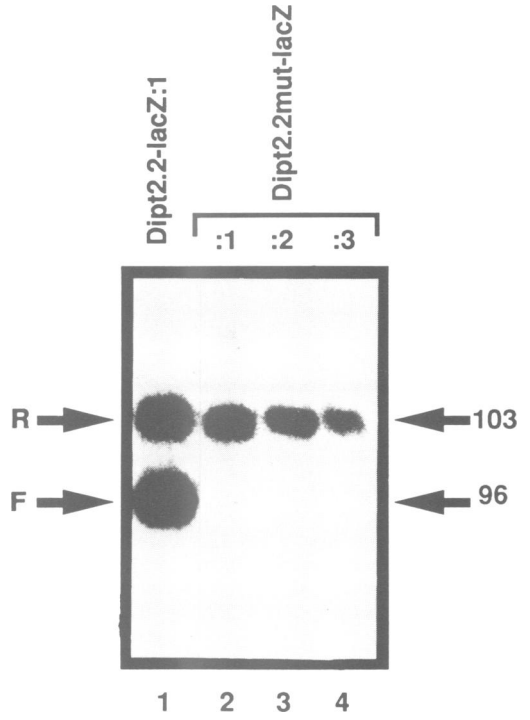

Fig. 4. Primer extension analysis of diptericin and Dipt-*lacZ* transcripts. Total RNA (5 μg) from Dipt2.2-*lacZ*:1 (lane 1) or Dipt2.2mut-*lacZ*:1−3 (lanes 2−4) wandering larvae injected for 3 h with *E.coli* were hybridized with the diptericin (R) and the Dipt-*lacZ* (F) primers and treated as described in Materials and Methods. The size (in bases) of the elongation products for the resident (R) and the fusion (F) gene transcripts are indicated.

products of 103 and 96 bp respectively (Reichhart *et al.*, 1992). Both primers were used together to analyse aliquots of RNA from bacteria-challenged late third instar larvae (Figure 4). The resident gene was induced in all transgenic fly lines whereas the fusion gene was only expressed when the 17 bp repeats were of wild-type sequence. In none of the lines obtained with mutated 17 bp sequences was induction of the transgene observed after bacterial challenge.

Southern blotting analysis confirmed that in five of the Dipt2.2mut-*lacZ* transformed lines no detectable rearrangement of the transgene had occurred. The β-galactosidase reading frame was preserved in the construct as evidenced by the blue colouration which served for the selection of the C20Dipt2.2mut-*lacZ* plasmid. By sequencing a PCR-amplified fragment of a region comprising the two mutated 17 bp motifs and the beginning of the β-galactosidase reading frame, the identity of the integrated sequence in one of the transformed strains was confirmed, to insure it contained only the expected mutation.

Interaction of proteins with the 17 bp motifs of the diptericin promoter

Having demonstrated that the 17 bp repeats within the proximal upstream region of the diptericin gene are mandatory for the LPS- or bacteria-induced expression of this gene (both in *mbn-2* cells and in transgenic fly lines), investigations were undertaken as to whether responsive tissues contained an activity that could bind to the 17 bp sites. A radioactively labelled double-stranded oligonucleotide containing a single 17 bp site was used as a probe in this analysis, which was based on electrophoretic mobility shift

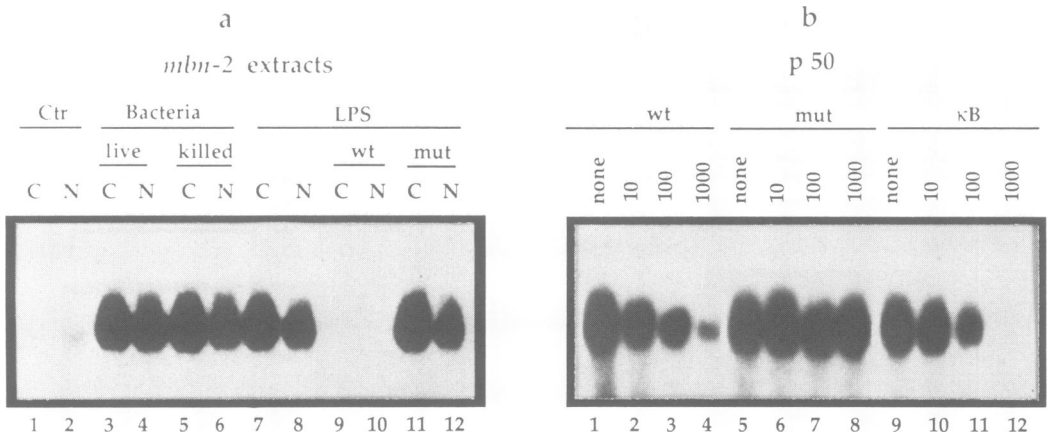

Fig. 5. DNA−protein binding activity of (**a**) a *mbn-2* cell extract and (**b**) recombinant p50. (**a**) Cytoplasmic (C) or nuclear (N) protein extracts (5 μg) of *mbn-2* cells were incubated in the presence of 2 fmol of the radioactive probe. The cells were used untreated (Ctr, lanes 1 and 2) or treated for 2 h with live bacteria (lanes 3 and 4), with heat-killed bacteria (lanes 5 and 6) or with LPS (lanes 7−12). Binding reactions contained no unlabelled oligonucleotide competitors (lanes 1−8), a 100-fold excess (200 fmol) of wild-type 17 bp probe (wt, lanes 9 and 10) or 200 fmol of mutated probe (mut, lanes 11 and 12). (**b**) Recombinant human **p50** (10 ng) (gift from P.Baeuerle) was incubated in the presence of 2 fmol of the radioactive probe without poly(dI-dC). Binding reactions contained no unlabelled competitor (lanes 1, 5 and 9), increasing amounts of wild-type probe (wt, lanes 2−4), mutated probe (mut, lanes 6−8) or mammalian κB consensus probe (5′-ATCGGGGATTTTCCTT-3′, see Lenardo and Baltimore, 1989) (κB, lanes 10−12).

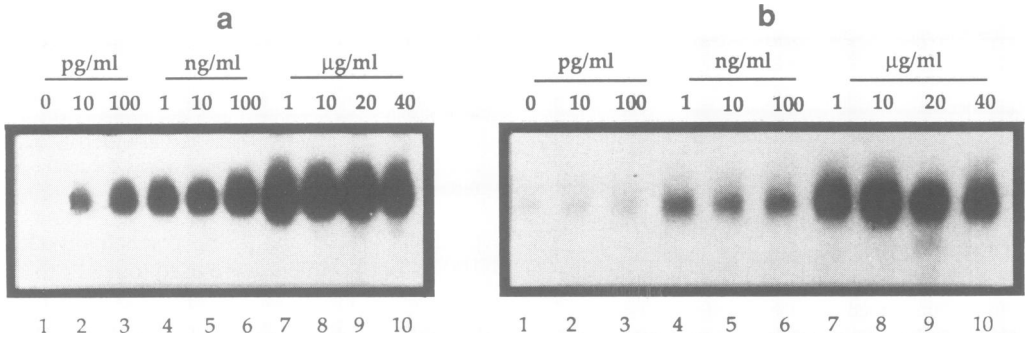

Fig. 6. Induction of binding activity: dependence on the dose of LPS. Increasing amounts of LPS were added to the *mbn-2* cell culture 2 h before harvesting and preparation of the cytoplasmic (**a**) and nuclear (**b**) extracts. Binding reactions were as in Figure 5.

assay (EMSA) with cytoplasmic and nuclear extracts. The 5′ extremity of the 17-mer is a C followed by an A. As C is a poor substrate for labelling with kinase, this nucleotide was omitted in the probe, which therefore, in effect, is a 16 bp double-stranded oligonucleotide. However, to avoid any confusion in the text and legends, the probe will be referred to as the 17 bp oligonucleotide. Figure 5a shows that the extracts of *mbn-2* cells contain a major protein−DNA complex 2 h after being induced by live or heat-killed bacteria or by LPS. In LPS-induced cells, the wild-type oligonucleotide competed effectively at a 100-fold molar excess, whereas a mutant oligonucleotide did not compete in the same conditions. In this experiment the signals obtained for the DNA−protein complex were slightly more intense in the cytoplasmic extracts than in the nuclear extracts. Most importantly, only a very faint signal was observed in uninduced cells (see Discussion). In Figure 6 it is apparent that very low doses of LPS are sufficient to induce the appearence of binding activity within 2 h. As in Figure 5a, the signal of the DNA−protein complex is more intense in the cytoplasmic extracts than in the nuclear extracts; the intensity reaches plateau levels in both extracts at a concentration of 1 μg/ml. The data presented in Figure 7

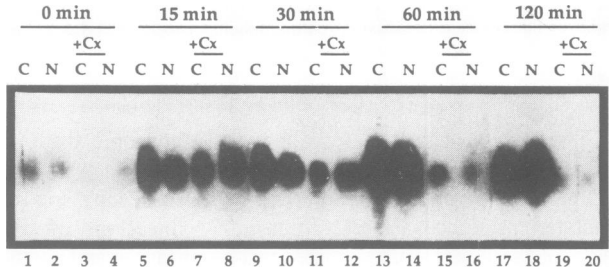

Fig. 7. Time-course of appearence of binding activity in *mbn-2* cells following cycloheximide and/or LPS treatment. *mbn-2* cells were treated with 20 μg/ml of LPS at different times (as indicated) before harvesting and preparation of cytoplasmic (C) and nuclear (N) extracts. Cycloheximide (Cx, 5 μg/ml) was added at a concentration of 5 μg/ml 1 h prior to the LPS treatment. Binding reactions were as in Figure 5.

give a time course of induction of a DNA−protein complex in cytoplasmic and nuclear extracts of *mbn-2* cells after the addition of LPS. Induction is evidenced at 15 min and reaches a maximum after 1 h. The same experiments were performed in the presence of cycloheximide. The rapid induction of the complex observed after 15 min of LPS treatment was not affected by this drug. However, a marked

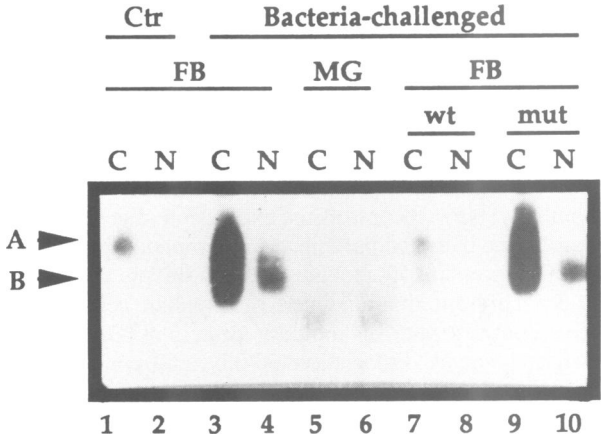

Fig. 8. DNA−protein binding activity in *Drosophila* fat body and gut extracts. *Drosophila* larval fat body (FB, lanes 1−4 and 7−10) or midgut (MG, lanes 5 and 6) were carefully dissected from control (Ctr, lanes 1 and 2) or 2 h bacteria-challenged larvae (lanes 3−10) and used for the preparation of the extracts. Cytoplasmic (C) or nuclear (N) extracts (5 μg) were incubated with 2 fmol of radioactive probe. Binding reactions contained no unlabelled competitor (lanes 1−6), nor a 100-fold excess (200 fmol) of wild-type 17 bp probe (wt, lanes 7 and 8) nor 200 fmol of mutated probe (mut, lanes 9 and 10).

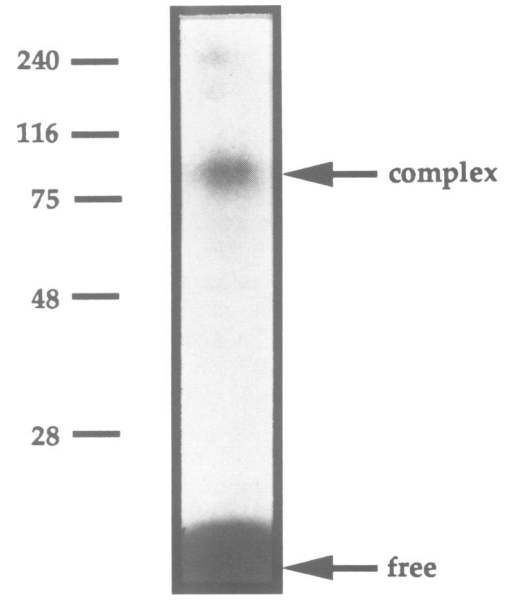

Fig. 9. UV cross-linking of a *mbn-2* nuclear extract to the 17 bp motif. Nuclear extract (20 μg) from *mbn-2* cells treated for 4 h with 1 μg/ml of LPS was incubated in the presence of 10 fmol of the radioactive probe. The DNA−protein complex was irradiated *in situ* by UV (30 min at 254 nm; see Gray *et al.*, 1990), resolved by EMSA, excised and finally analysed in a second dimension on 10% SDS−PAGE under reducing conditions. Standard proteins were prestained high molecular weight markers (Gibco BRL). Arrows indicate the positions of the cross-linked complex (complex) and of the free probe (free).

depressive effect was observed after longer time intervals and no DNA−protein complex could be seen after 2 h of LPS induction in the presence of cycloheximide. This result indicates that the initial activation is cycloheximide insensitive but that maintenance of a binding activity requires ongoing protein synthesis.

The results presented so far were obtained with *mbn-2* cells. Subsequent studies investigated the presence of a DNA−protein complex in fat body extracts. Depending on the experiment, two retarded bands were observed, referred to as A and B (Figure 8); band B has the same electrophoretic mobility as the induced band in *mbn-2* cells. Nuclear extracts from fat body of unchallenged *Drosophila* showed no complex, whereas in cytoplasmic extracts a faint signal was seen for band A. A dramatic increase in signal was noted for both bands in cytoplasmic extracts 2 h after bacterial challenge. In nuclear extracts, only band B appeared. A 100-fold molar excess of wild-type oligonucleotide totally competed off the nuclear complex in challenged *Drosophila*; in the cytoplasmic extracts, the signals for A and B became very faint upon competition. An excess of mutated probe did not affect the intensity of the induced signals in cytoplasmic (A and B) and nuclear extracts (B). Midgut of bacteria-challenged larvae only gave a faint fast-migrating retardation band different from the bands induced in the fat body and the *mbn-2* cells. The other characteristics obtained using EMSA of cytoplasmic and nuclear extracts of fat body were, within the limits of experimental error, similar to those obtained with *mbn-2* cells (data not shown).

In an additional series of experiments, the 17 bp probe containing the κB-related sequence was incubated with pure mammalian protein p50 (recombinant human protein) which binds to the native κB sequence in mammals. As shown in Figure 5b, a DNA−protein complex is observed which is competed by excess of wild-type *Drosophila* 17 bp probe but not of the mutated form. Conversely, when nuclear extracts from fat body of bacteria-challenged *Drosophila* were incubated with the wild-type 17 bp sequence, the signal obtained could be competed with a 100-fold molar excess of a probe containing a mammalian κB consensus sequence (5′-ATCGGGGATTTTCCTT-3′, see Leonardo and Baltimore, 1989) nearly as efficiently as by an excess of wild-type *Drosophila* probe (data not shown). Finally, the size of the protein binding to the 17 bp probe in nuclear extracts of induced *mbn-2* cells after UV cross-linking was estimated at 75−80 kDa, as shown in Figure 9.

Discussion

The data presented in this study provide the first experimental demonstration that κB-related binding motifs play a pivotal role in the expression of an immune gene in a non-mammalian organism. Three such motifs are present in the *Drosophila* diptericin promoter, as shown in Figure 1a, two of which are nested within two 17 bp repeats. As demonstrated here in transfected tumorous blood cells and in transgenic fly lines, the immune response, as evidenced by LPS (or bacteria) inducibility of reporter genes, is totally abolished when the 17 bp repeats are replaced by random sequences. In other words, in promoter positions, these repeats function as *cis*-acting elements that confer inducible transcriptional activity. It is apparent from the transfection experiments that the two 17 bp repeats must act in synergy because the inducibility is dramatically reduced when only one native copy is present. Conversely, multiple copies significantly increase the level of transcriptional activation, as compared with the wild-type promoter. Reverse orientation of the 17 bp repeats does not abolish the LPS inducibility, although it lowers the level of CAT activity.

Decreasing the distance between the two motifs from 79 to 20 bp does not markedly affect the level of *cat* inducibility in these experiments. Therefore, it will be of interest to generate transgenic fly lines with similarly trimmed promoter constructs and to compare the level of expression of the fusion genes in which the 17 bp elements are in near proximity with those with wild-type positions.

As expected from these results, the 17 bp repeats in the diptericin promoter actually bind to protein(s) present in induced cells and in fat body of bacteria-challenged *Drosophila*. It is apparent that no protein−DNA binding activity is present prior to induction. Faint signals in supposedly uninduced *mbn-2* cells were interpreted as resulting from minimal induction of some cells by trauma; strong shaking of the culture flasks, for instance, can induce some binding activity (data not shown). The activation of protein binding to the 17 bp motif takes minutes and can be induced by very low doses of LPS (see Figure 6). The activation is initially cycloheximide insensitive, but maintenance of activity is abolished by cycloheximide, i.e. it requires protein synthesis. Binding activity is present both in cytoplasmic extracts, where it often appears strongest, and in nuclear extracts. The data could be explained by the translocation of the binding activity from the cytoplasm to the nucleus. The binding affinity is high and is comparable in cytoplasmic and nuclear extracts. In fat body extracts, but not in *mbn-2* cells, two binding proteins can be observed using EMSA, one (band B, Figure 8) migrating as the single band observed with *mbn-2* cell extracts. It is believed that the second band represents a modified form of the first band; both bind specifically to the motif. A detailed analysis of the relevance for binding activity of the various nucleotides within the 17 bp repeats is underway. First results indicate that single mutations of G6, G7, C12 and C13 (numbering as in Figure 1c) abolish totally the binding of proteins to the probe. The necessity of nucleotides G6 and G7 is, in part, corroborated by some of the transfection experiments in which the replacement of G6, G7 and G8 resulted in the loss of LPS inducibility (Figure 3, plasmids D8 and D11, see also Materials and Methods). As underlined in Introduction, the 17 bp repeats harbour a decameric κB-related sequence. Interestingly, nucleotides G6, G7, G8, C12 and C13 are positioned within this sequence.

A considerable amount of information has accumulated over the past six years on the mammalian NF-κB transcription factor since the original report by Sen and Baltimore (1986). In essence it appears that the biological function of mammalian NF-κB is to rapidly induce gene expression upon extracellular stimulations that signal distress and pathogen invasion (reviewed in Baeuerle, 1991; Baeuerle and Baltimore, 1991). One of the established signals is LPS, and κB enhancers can serve as response elements for LPS. In mammals, NF-κB controls the expression of acute phase proteins in the liver and participates in lymphoid cells in the inducible expression of cytokines and cell surface receptors. NF-κB is present in uninduced mammalian cells as an inactive complex with an inhibitor protein, IκB, and the activation involves dissociation of this inhibitor from its cytoplasmic complex with NF-κB and nuclear translocation of the active transcription factor.

The data presented in this study are compatible with the idea that the control of the diptericin gene during the immune response of *Drosophila* occurs via a mechanism similar to that of the mammalian NF-κB system. This is corroborated by the experiment showing that the mammalian NF-κB subunit p50 forms a DNA−protein complex with the 17 bp *Drosophila* motif and that this complex can be competed by the *Drosophila* wild-type sequence. In keeping with this result is the observation that the complex formed between proteins from bacteria-challenged *Drosophila* and the 17 bp probe is competed by an excess of mammalian κB consensus probe.

NF-κB is a member of a transcription factor family comprising the products of the *rel* oncogenes and the *Drosophila* morphogen *dorsal*. The protein binding to the 17 bp motifs in the LPS-stimulated cell line of *Drosophila* has an apparent molecular weight of 75−80 kDa, comparable with that of the *dorsal* protein (Steward, 1987). These cells contain a 2.8 kb transcript hybridizing with a probe corresponding to the DNA binding domain of *dorsal* and the signal in Northern blots is enhanced upon LPS stimulation (J.N.Reichhart, M.Meister, C.Kappler, B.Lemaître, P.Georgel and J.A.Hoffmann, unpublished results). The transcript of the *dorsal* gene is also 2.8 kb (Steward, 1987) and our current working hypothesis is that the gene coding for the protein binding to the 17 bp repeats is related or identical to *dorsal*.

The involvement of κB-related binding motifs in the regulation of immune gene expression in insects may be a relatively widespread phenomenon (Table I). Indeed, Sun

Table I. κB-related sequences in promoters of immune genes in insects

Immune gene	Species	Sequence	Position	Reference
Acidic attacin	*H.cecropia*	GGGGATTCCT	−49	Sun *et al.*, 1991a
Basic attacin	*H.cecropia*	GGGGATTCCT	−59	Sun *et al.*, 1991a
Lysozyme	*H.cecropia*	(G)AGGATTCCCC	−100	Sun *et al.*, 1991b
Cecropin A	*H.cecropia*	GGGAGTCCCC	−188	Sun *et al.*, 1991a
Cecropin B	*H.cecropia*	GGGAATTTAC	−178	Sun *et al.*, 1991a
Cecropin A2	*D.melanogaster*	GGGGTTTCCT	−214	see Sun *et al.*, 1991a
Cecropin B	*D.melanogaster*	GGGATTTCTT	−656	see Sun *et al.*, 1991a
Diptericin[a]	*D.melanogaster*	GGGGATTCCT	−46 and −142	Reichhart *et al.*, 1992
Diptericin[b]	*D.melanogaster*	GGGAAATTCC	−794	Reichhart *et al.*, 1992
Mammalian NF-κB binding consensus		GGGRNTYYCC		Lenardo and Baltimore, 1989
Insect consensus		GGGRNTYYYY		

[a] κB sites within the 17 bp repeats.
[b] Unique κB site upstream of the 17 bp repeats.

et al. (1991a) reported the presence, in *Hyalophora cecropia* (Lepidoptera), of a κB-related sequence in the promoters of the genes encoding two different antibacterial peptides, namely acidic and basic attacin. Expression studies revealed that both genes are strongly induced by phorbol esters, LPS and bacteria. It was subsequently found that the genes encoding other immune peptides or polypeptides, namely lysozyme and some cecropins in this species, also contained a κB binding motif (Sun *et al.*, 1991a,b). In *Drosophila*, the genes which encode the LPS-inducible antibacterial peptides cecropin A2 and B similarly contain a κB sequence (Kylsten *et al.*, 1990; Sun *et al.*, 1991a). As discussed here, the situation observed in the diptericin promoter is particular in as far as it contains three copies of κB-related sequences and that the inducibility is dependent on the presence of at least two such copies. A strong point for the role of the κB-related binding motif in the regulation of the expression of the inducible immune gene in *H.cecropia* was recently made by Sun and Faye (1992a,b) who showed that a 65 kDa protein specifically binds to this motif. The DNA binding activity of the protein correlates well with the transcriptional induction of the immune genes and the binding protein shares some of the characteristics of NF-κB.

In contrast with the above mentioned genes encoding immune peptides or polypeptides, the promoters of other inducible immune genes (e.g. cecropin D of *Hyalophora*; Sun *et al.*, 1991a) do not contain κB-related sequences. There are reported differences in the profiles of expression of these genes and the comparative analysis of the regulatory sequences and the cognate binding proteins, which has only just begun in a few systems, may turn out to be particularly rewarding for our understanding of this phylogenetically ancient immune response.

Materials and Methods

RNA analysis

Northern blotting. Poly(A)-enriched RNA was isolated from *mbn-2* cells and analysed as described (Reichhart *et al.*, 1989).

Primer extension. Total RNA (5 μg) was extracted from 5–10 animals (from four of the transformed strains) using a LiCl/urea based protocol (Reichhart *et al.*, 1992) and coprecipitated with 10^5 c.p.m. of the appropriate ^{32}P-labelled oligonucleotide. The pellet was resuspended in 15 μl of annealing buffer (250 mM KCl, 10 mM Tris–HCl, pH 8.3) and placed in a 80°C water bath which was allowed to cool to 30°C in 45 min. 15 μl of reverse transcriptase (RTase) buffer (24 mM Tris–HCl, pH 8.3, 16 mM $MgCl_2$, 8 mM DTT, 0.4 mM of each deoxynucleotide and 100 μg/ml actinomycin D) were added together with 50 U M-MLV RTase (Superscript, Gibco BRL). The reaction was incubated for 45 min at 45°C and the products were analysed on a denaturing 8% polyacrylamide gel. Primers were as follows: Dipt, 5′-GATAAGCCAAAGTAGAA-3′ (+103 to +87); and Dipt-*lacZ*, 5′-GTTTTCCCAGTCACGAC-3′ (+96 to +80).

Construction of plasmids

Diptericin-lacZ reporter gene fusion. The 2.2 kb diptericin promoter fragment was subcloned in M13mp18 as a *Pst*I–*Hin*dIII fragment (Reichhart *et al.*, 1992). The two 17 bp motifs were replaced respectively by 5′-GTGAAG-CAGTGCGTGGG-3′ (−139) and 5′-ATGAAGCAGTGCGTATG-3′ (−43) by site-directed mutagenesis (oligonucleotide-directed *in vitro* mutagenesis system, Amersham) following the supplier's instructions. This fragment was subsequently fused to a 3.4 kb *Hin*dIII–*Xho*I fragment which contains the complete *lacZ* coding sequence and the *hsp70* termination signal. The fusion was inserted in the appropriate orientation into the *Sal*I site of the Carnegie 20 transformation vector (Reichhart *et al.*, 1992) to yield C20Dipt2.2mut-*lacZ*. The fragment subjected to mutagenesis was fully sequenced before cloning.

Diptericin-tk-cat reporter gene fusion (Figure 3). Plasmid pBLCAT5 containing the *Escherichia coli cat* gene under the control of the thymidilate kinase promoter was obtained from Dr Lukow (Boshart *et al.*, 1992). For the construction of plasmids D1 and D2, a *Sau*3AI–*Hae*III fragment containing the two 17 bp repeats from the 2.2 kb diptericin promoter (Figure 1b, see also Reichhart *et al.*, 1992) was inserted into the *Bam*HI site of pBLCAT5 in wild-type (D1) or reverse (D2) orientations by blunt-end ligation after filling up the sticky ends with the Klenow polymerase (Gibco BRL). For plasmids D3–D5, this restriction fragment was first subcloned into M13mp18 and both (D3) or either the proximal (D4) or the distal (D5) 17 bp motifs were replaced by the same random sequences as described above. The constructions of D6–D11 were as follows: wild-type 16-mer oligonucleotides (5′-ATCGGGGATTCCTTTT-3′) or mutated 16-mer oligonucleotides (5′-ATCG**A**TTATTCCTTTT-3′; mutated nucleotides are in bold) were annealed and blunt-end ligated into the *Sal*I site of M13TG131 (Kieny *et al.*, 1983) after filling up the sticky ends. The oligonucleotides were oligomerized in direct head-to-tail orientation (Eisenberg *et al.*, 1990). *Bam*HI–*Bgl*II fragments containing either two wild-type repeats in direct (D6) or reverse (D7) orientation, or two mutated repeats (D8) were cloned into the *Bam*HI site of pBLCAT5. The strategy was identical for the construction containing eight copies (D9–D11). Constructs D6–D11 were all sequenced before transfection experiments.

Cell culture and transfection

mbn-2 cells (Gateff *et al.*, 1980) were grown to 80% confluent monolayers (110 mm dishes) for 24 h prior to transfection in Schneider's medium (Sigma) supplemented with 10% fetal calf serum (Gibco BRL), 50 U/l penicillin and 100 mg/l streptomycin. The cells were cotransfected by the transfection reagent DOTAP (Boehringer-Mannheim) using 3 μg of test plasmid and 2 μg of the β-galactosidase expression vector pACH (Thisse *et al.*, 1991) as internal control for the transfection efficiencies. After 20 h, the cells of each dish were washed twice and equally distributed on two 60 mm dishes which were incubated for 48 h. Twenty hours before harvesting, one of the two dishes received an addition of 20 μg/ml of LPS (Sigma, *E.coli* 55:B5) to induce the immune reaction. Cells were lysed by the freeze–thaw procedure in 150 μl of 0.25 M Tris–HCl, pH 7.5, 5 mM DTT, 15% glycerol. β-galactosidase activity in the cell lysates was measured using *O*-nitro-phenol-β-D-galactoside as substrate (Herbomel *et al.*, 1984) and the values were used to normalize variability in the efficiency of transfection. The CAT assay was performed according to the method of Gorman *et al.* (1982).

Transgenic flies: the β-galactosidase histochemical assay

The transposon C20Dipt2.2mut-*lacZ* (300 μg/ml) was co-injected with an integration defective helper plasmid into ry^{506}*C.S.* embryos (see Giangrande *et al.*, 1987 for technical details). Transformants were recovered as G1 ry^+ flies. The chromosome carrying the insertion and stable homozygous (15 insertions) or heterozygous (five insertions) lines was established by crosses to appropriate balancers. Six lines (including the lines analysed by reverse transcriptase experiments) were examined by Southern blotting to verify the presence of a single non-rearranged insertion of the transposon (data not shown).

The transformed lines (Dipt2.2mut-*lacZ*:1–20) and the control Dipt2.2-*lacZ*:1 line (Reichhart *et al.*, 1992) were maintained at 25°C on a standard cornmeal medium. Wandering larvae and adult flies were pierced with a tungsten needle dipped in a concentrated culture of *E.coli*. For β-galactosidase histochemical assays, animals were dissected in individual droplets 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) and scored after overnight incubation.

Sequencing of the diptericin proximal promoter in the transgene of transformed flies

DNA for PCR was prepared from single flies according to the rapid protocol of Gloor and Engels (Engels *et al.*, 1990). Exponential amplification was performed in 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% gelatine, 200 μM of each deoxynucleotide, 0.15 μM of each primer, with 1 μl of the DNA-prep and 1 U of *Taq* polymerase (Boehringer-Mannheim). Thirty cycles (1 min at 95°C, 2 min at 55°C and 3 min at 72°C) were performed in a PREM Thermal cycler (LEP-Scientific). The reaction products were purified and sequenced after subcloning into M13mp19. The primers were as follows: sense primer in the diptericin promoter, 5′-ATAAGCTGGGAGGTTGGGATA-3′ (−323 to −303); antisense primer complementary to the β-galactosidase transcript, 5′-CCAGGGTTTTCCCAGTCACGA-3′ (+101 to +81).

Preparation of extracts and EMSA

Cells were collected by centrifugation for 10 min at 400 *g*. Cell pellets were resuspended in ice cold PBS and collected again by centrifugation. All the following steps were carried out at 4°C. Washed cells were resuspended

in two packed cell volumes of a hypotonic lysis buffer (buffer A; Dignam *et al.*, 1983). After 10 min, cells were homogenized by 20 strokes with a loose fitting Dounce homogenizer. Nuclei were collected by centrifugation for 10 min at 4000 *g*, resuspended in 5 volumes of buffer A and washed once by centrifugation. Proteins were extracted from washed nuclei by high salt (buffer C; Dignam *et al.*, 1983), followed by centrifugation of the nuclear extracts and dialysis against buffer D (20 mM HEPES, pH 7.8, 25 mM NaCl, 25 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 20% glycerol and 0.05% Nonidet-P40). Protein concentrations were determined by the Bio-Rad Protein Assay (based on the Bradford principle).

The standard DNA binding reaction was performed as follows: 5 μg of protein extract in binding buffer (buffer D where glycerol is replaced by 4% Ficoll) were mixed with 1 μg of poly(dI-dC) (Pharmacia) during 10 min on ice and incubated at room temperature with 20 000 c.p.m. of labelled DNA probe. The mixture was analysed on a native 5% polyacrylamide gel as described by Sen and Baltimore (1986). The oligonucleotides used as radioactive DNA probe (5′-ATCGGGGATTCCTTTT-3′ and 5′-AAAAGGAATCCCCGAT-3′) were annealed, labelled with γ-^{32}P ATP (6000 Ci/mmol) by the T4 polynucleotide kinase (Gibco BRL) and purified on a native 15% polyacrylamide gel. The mutated oligonucleotides used for competition (5′-ATCGATTATTCCTTTT-3′ and 5′-AAAAGGAAT-AATCGAT-3′) had the same length as the radioactive probe.

For measurement of DNA binding activity, the binding assays were carried out under probe saturation (i.e. 5 fmol of radioactive probe). Increasing known amounts of non-radioactive homologous probe were added to the binding reaction as competitor. After gel electrophoresis and autoradiography, radioactive bands corresponding to free and to protein-bound DNA probe were excised and quantitated by liquid scintillation counting.

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