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Insect immunity. A transgenic analysis in *Drosophila* defines several functional domains in the diptericin promoter

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Diptericins are antibacterial polypeptides which are strongly induced in the fat body and blood cells of dipteran insects in response to septic injury. The promoter of the single-copy, intronless diptericin gene of *Drosophila* contains several nucleotide sequences homologous to mammalian *cis*-regulatory motifs involved in the control of acute phase response genes. Extending our previous studies on the expression of the diptericin gene, we now report a quantitative analysis of the contribution of various putative regulatory elements to the bacterial inducibility of this gene, based on the generation of 60 transgenic fly lines carrying different elements fused to a reporter gene. Our data definitively identify two κB-related motifs in the proximal promoter as the sites conferring inducibility and tissue-specific expression to the diptericin gene. These motifs alone, however, mediate only minimal levels of expression. Additional proximal regulatory elements are necessary to attain some 20% of the full response and we suspect a role for sequences homologous to mammalian IL6 response elements and interferon-γ responsive sites in this up-regulation. The transgenic experiments also reveal the existence of a distal regulatory element located upstream of −0.6 kb which increases the level of expression by a factor of five.

Key words: diptericin promoter/insect immunity/κB-related/transgenic *Drosophila*

Introduction

Bacterial challenge or septic injury induce in larvae and adults of *Drosophila* the rapid and transient transcription of a battery of genes encoding antibacterial peptides (for recent reviews, see Boman, 1991; Hoffmann *et al.*, 1993; Hultmark, 1993; Cociancich *et al.*, 1994). We are interested in the control of expression of these genes and have chosen the diptericin gene as a model system. *Drosophila* contains a single copy of an intronless diptericin gene which encodes an 83 residue anti-Gram negative polypeptide (Reichhart *et al.*, 1992). A previous study based on RNA analysis suggested that a transgene carrying 2.2 kb of upstream sequences of the diptericin gene fused to a *lacZ* reporter responded to bacterial challenge in third instar wandering larvae similarly to the resident diptericin gene, but was expressed at lower rates than the resident gene at other stages of development (Reichhart *et al.*, 1992). The proximal upstream sequences of this gene contain a remarkably high number of motifs related to nucleotide sequences involved in the regulation of mammalian acute phase response genes. This is in particular the case for κB-related motifs and sequences conferring interleukin-6 (interleukin-6 response element, IL6 RE) or interferon-γ (interferon consensus response element, ICRE) inducibility during the inflammatory response in mammals. The functionality of these sequences in the proximal diptericin promoter in *Drosophila* during the immune response has been suggested by DNase I footprinting and electrophoretic mobility shift assays (Georgel *et al.*, 1993). Prominent among the various immune response elements in the diptericin promoter are two 17 bp repeats nesting a decameric κB-related sequence (Figure 1a). When both of these repeats were replaced by random sequences in transgenic experiments, the bacterial inducibility of the reporter gene was lost (Kappler *et al.*, 1993). The significant role of these 17 bp repeats was also evident from transfection experiments with a tumourous blood cell line from *Drosophila* (*mbn-2* cells; Gateff, 1978) in which the diptericin gene is inducible upon lipopolysaccharide (LPS) treatment: when a 145 bp restriction fragment containing the proximal promoter sequences of the diptericin gene was fused to a *CAT* reporter gene and transfected into these cells, expression of CAT was induced by LPS only when the two 17 bp motifs within this fragment had the wild-type sequence (Kappler *et al.*, 1993). Mutated sequences did not confer inducibility in these cells. Conversely, a reporter gene under the control of several copies of the 17 bp motifs could be induced by lipopolysaccharide when transfected into the tumorous blood cells, in the absence of any other upstream sequences. Again, mutated motifs were unable to direct expression of the reporter gene.

In the present study we have investigated quantitatively the relative contribution of various regions of the upstream sequences to the normal induction of the diptericin gene. Our study is based on transgenic experiments with a bacterial *lacZ* reporter and subsequent quantitative determination of β-galactosidase activity after bacterial challenge of the insects. Although, as shown below, some blood cells express the transgene, our results derive essentially from the induction of the transgene in the larval or adult fat body, which probably accounts for 99% of diptericin expression.

We confirm the role of the 17 bp repeats in the bacterial inducibility of the transgene, but show that alone they are able to sustain only a minimal response. Additional sequences within the proximal 0.6 kb are necessary to reach up to 20% of a full response and our data point to

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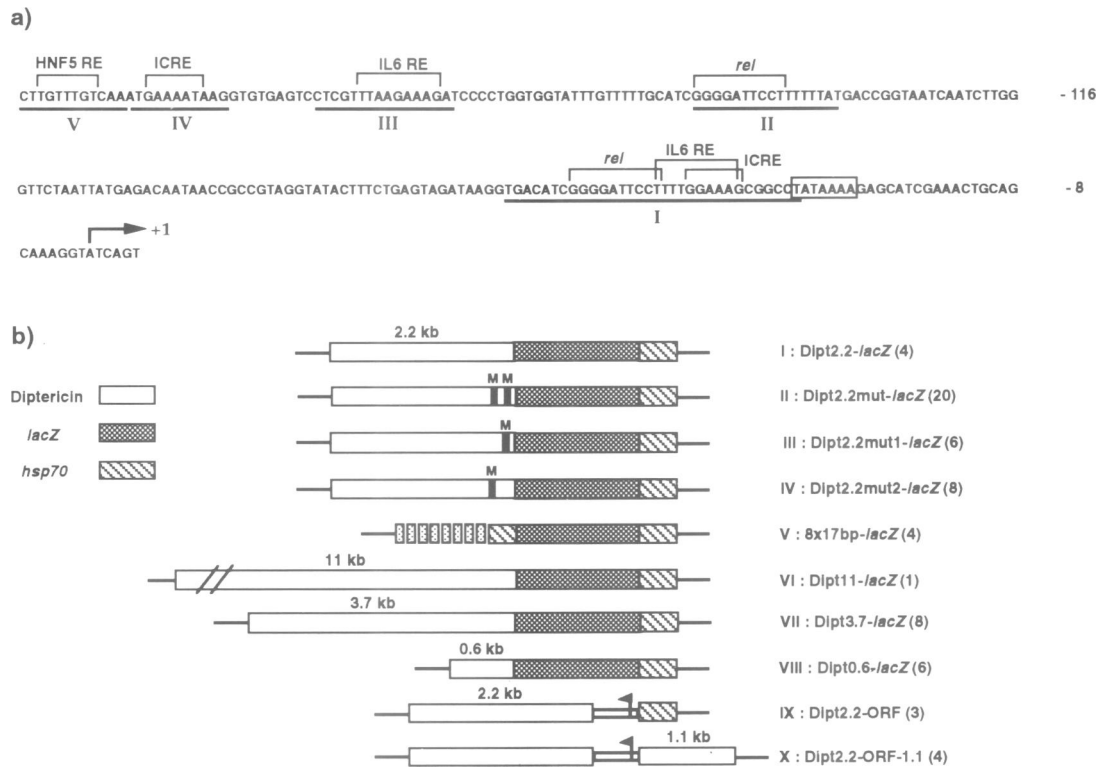

Fig. 1. Promoter elements of the *Drosophila* diptericin gene. (**a**) Nucleotide sequence of the diptericin proximal promoter. The regions (I–V) protected in DNase I footprinting experiments with protein extracts from bacteria-challenged *Drosophila* (Georgel *et al.*, 1993) are underlined. The start site (arrow, +1) and the TATA box (boxed) are indicated. The potential binding sites for regulatory factors are overlined. ICRE, interferon consensus response element (see Williams, 1991); IL6 RE, interleukin-6 response element (see Hocke *et al.*, 1992); HNF5 RE, hepatic nuclear factor 5 response element (Grange *et al.*, 1991). (**b**) Constructs used to establish transgenic *Drosophila* fly lines. In the first eight constructs, various diptericin promoter fragments were fused upstream of a reporter *lacZ* gene followed by an *hsp70* transcription termination sequence. Constructs I–IV contain 2.2 kb of diptericin upstream region in which the two proximal 17 bp repeats nesting the κB-related sites were either native (I) or mutated (M in II–IV). In construct V, the *lacZ* gene was under the control of eight repeats of the 17 bp motif and a minimal *hsp70* promoter. Constructs VI–VIII contain various lengths of diptericin upstream regions. In constructs IX and X, 2.2 kb of the diptericin promoter were placed upstream of the diptericin coding sequence with an additional 45 bp stretch (black flags) followed either by the *hsp70* terminator (IX) or by 1.1 kb of diptericin downstream regions (X). The numbers in parentheses give the number of fly lines obtained for each construct.

the existence of a distal regulatory element, located between −2.2 and −0.6 kb which increases the level of expression by a factor of five. Additional upstream or downstream sequences do not affect the response.

Results

The present study is based on the establishment of transgenic fly lines by P element-mediated germline transformation with the constructs illustrated in Figure 1b. In constructs I–VIII, the bacterial *lacZ* gene served as reporter gene and was fused 3′ to the following sequences: (i) wild-type upstream regions of the diptericin promoter of various lengths (I, 2.2 kb; VI, 11 kb; VII, 3.7 kb; VIII, 0.6 kb); (ii) 2.2 kb of the promoter region in which either both 17 bp motifs (II) or the proximal (III) or distal (IV) motif had been replaced by random sequences; (iii) eight copies of the 17 bp motif upstream of an *hsp70* minimal promoter (V). Constructs IX and X were devised to investigate a potential role for the downstream sequences (X) or the coding region of the diptericin gene in up-regulating the level of bacteria-induced expression. Both constructs contained the diptericin coding sequence fused to an additional 45 bp sequence immediately upstream of the stop codon, which served as a reporter (tag) for the expression of the corresponding transgenes. In constructs IX and X the diptericin coding sequence was preceded by 2.2 kb of wild-type upstream region. In construct IX, as in constructs I–VIII, the reporter sequences were followed by the *Drosophila hsp70* termination region, while in construct X the diptericin coding sequence was followed by 1.1 kb of homologous downstream elements of the diptericin gene.

In the experiments described below we have primarily used third instar wandering larvae, which have been shown to be particularly responsive to bacterial challenge (Reichhart *et al.*, 1992). The larvae were pricked with a bacteria-soaked needle (see Materials and methods) and sacrificed after 6 h, which corresponds to the time of maximum induction of the resident diptericin gene (M.Meister, unpublished results). The majority of the constructs contained the *lacZ* reporter gene and in the corresponding transgenic fly lines β-galactosidase activity was measured after bacterial challenge.

For each of the Dipt–*lacZ* transgenic experiments we have generated several independently derived fly lines and subsequently analysed three to seven pools of five individuals for each line. As shown below (see Figure 2), a certain variability was observed for β-galactosidase activity between fly lines carrying the same transgene.

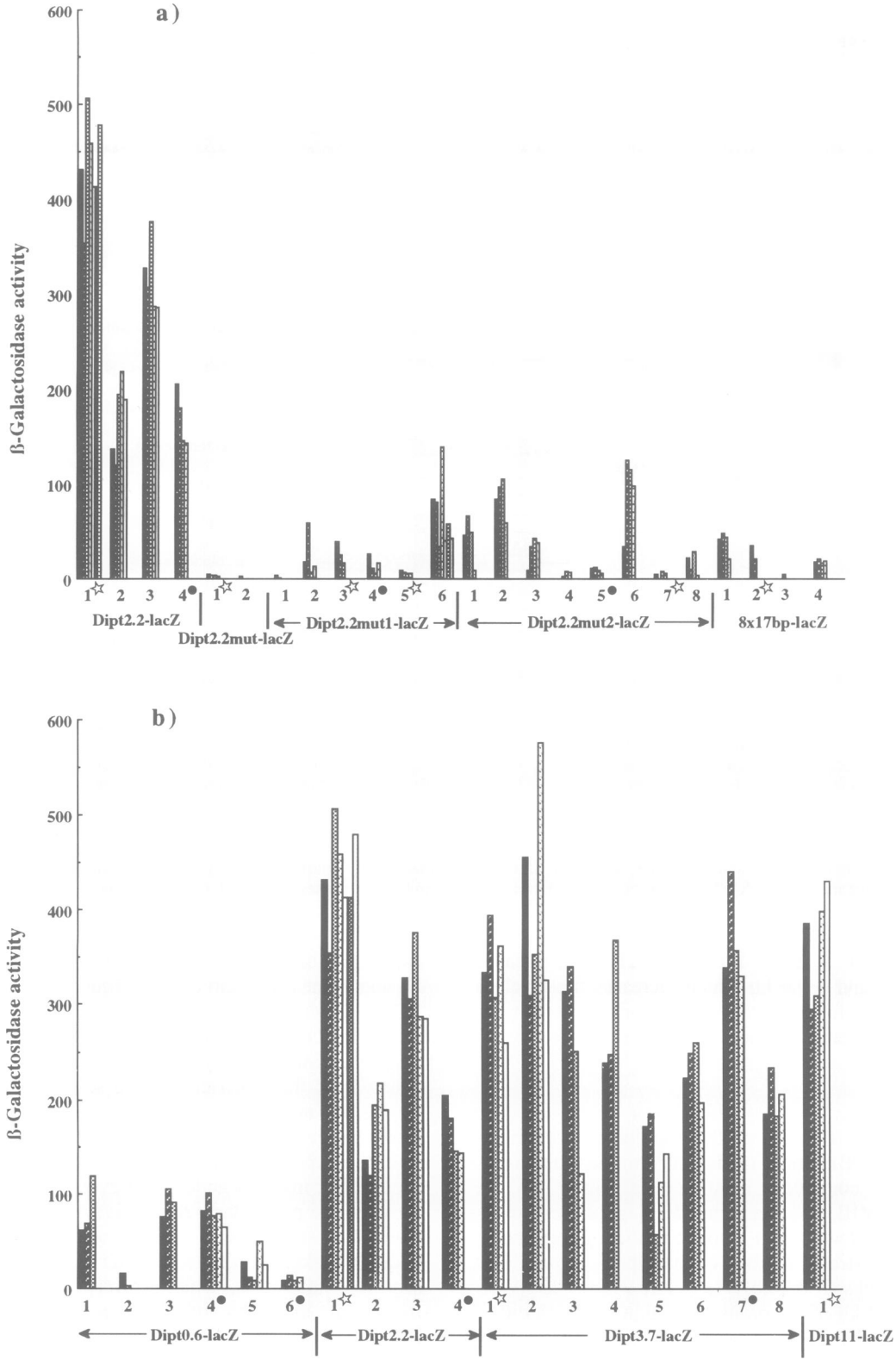

Fig. 2. β-Galactosidase activity in bacteria-challenged wandering third instar larvae from transgenic lines carrying constructs with the *lacZ* reporter gene. Each column represents the activity measured in a pool of five larvae sacrificed 6 h after bacterial challenge, expressed in nmol product formed/min/mg protein. The numbers below the abscissa correspond to the various fly lines carrying a given transgene, which is indicated below as in Figure 1b. (**a**) Transgenic fly lines analysed to study the κB-related sites. (**b**) Transgenic fly lines with various lengths of diptericin promoter sequences. To aid comparisons, we have represented the data on the Dipt2.2−*lacZ* lines in both (a) and (b). Stars indicate X-linked insertions and black dots indicate homozygous lethal insertions which have been analysed as heterozygous balanced lines.

This variability is attributed to position effects, which have also been reported in other studies (see, for instance, Bourouis and Richards, 1985; Laval *et al.*, 1993, for *Drosophila*; Lernbecher *et al.*, 1993, for mice). The level of β-galactosidase activity in control animals was generally hardly detectable: it never exceeded 10 nmol product formed/min/mg protein for the lines carrying the most efficient constructs (I, VI and VII) and was attributable to both a constitutive gut galactosidase and a weak spontaneous activity of the transgene (see Reichhart *et al.*, 1992).

Mutation of the 17 bp motifs

In Figure 2a we show the β-galactosidase activity in various transgenic third instar wandering larvae 6 h after bacterial challenge. A marked inducibility is observed in the four lines carrying 2.2 kb of wild-type upstream sequences (Figure 2a, Dipt2.2−*lacZ*), in agreement with Reichhart *et al.* (1992), who used histochemical detection on whole insect mounts. This inducibility was totally lost when the two 17 bp motifs were replaced by random sequences (Figure 2a, mut−*lacZ*). Again, this result corroborates observations by Kappler *et al.* (1993) and we have therefore only represented two fly lines out of the 20 which were generated. Note that the low levels of β-galactosidase activity indicated for these two lines correspond to background levels in this assay (due to the endogenous galactosidase activity in the gut).

When the most proximal of the two 17 bp motifs was replaced by a random sequence (Figure 2a, mut1−*lacZ*), a low but significant inducibility was evident in five out of six transgenic lines, which corresponded to ~10% of the response induced with a transgene carrying 2.2 kb of wild-type upstream sequences (Figure 2a, Dipt2.2−*lacZ*, and Table I). A similar result was obtained when the more upstream 17 bp motif was replaced (Figure 2a, mut2−*lacZ*, and Table I) and the transgene remained inducible in the eight lines which we generated. We can therefore conclude that a 2.2 kb upstream sequence of the diptericin gene can only confer full inducibility to a reporter gene when it carries both 17 bp motifs in the proximal promoter region. The presence of a single motif can confer a low degree of inducibility, corresponding to ~10% of what we consider as a full response in the present context.

Transgene with a minimal promoter carrying multiple 17 bp repeats

Four independent fly lines were established which carried a transgene in which eight copies of the 17 bp motif were fused to a minimal *hsp70* promoter upstream of the *lacZ* reporter, as explained above. In the absence of bacterial challenge these larvae did not produce β-galactosidase activity (data not shown). In three out of four lines a low but significant activity was induced by pricking (Figure 2a, 8x17bp−*lacZ*); the fourth line could not be induced. In the three inducible lines, the level of induction was lower than 10% of that induced with a wild-type 2.2 kb diptericin upstream sequence and was in the range of, and possibly somewhat lower than, the induction sustained by a 2.2 kb promoter carrying only one copy of the 17 bp motif in the context of an otherwise wild-type sequence (Table I). These results confirm that the 17 bp motifs actually confer *in vivo* bacterial inducibility to a reporter transgene, but that the level of induction is low.

Table I. Comparison of β-galactosidase activities in bacteria-challenged wandering third instar larvae carrying various *lacZ* constructs

Fusion gene	Fly line	β-galactosidase activity m (*n*, s)	M (*n*, s)
Dipt2.2−*lacZ*[a]	1	436.4 (7, 50.5)	
	2	171.7 (5, 41.9)	273.4 (4, 128.7)
	3	316.5 (5, 37.3)	
	4	168.8 (4, 29.7)	
Dipt2.2mut1−*lacZ*[b]	1	1.0 (4, 1.4)	
	2	24.0 (4, 23.4)	
	3	20.0 (4, 16.3)	24.5 (6, 28.6)
	4	15.0 (4, 8.5)	
	5	6.7 (4, 0.9)	
	6	80.4 (10, 57.8)	
Dipt2.2mut2−*lacZ*[c]	1	42.3 (4, 24.5)	
	2	86.3 (4, 20.1)	
	3	30.8 (4, 15.1)	
	4	4.3 (4, 3.3)	35.8 (8, 35.8)
	5	9.0 (4, 3.0)	
	6	93.0 (4, 41.5)	
	7	4.3 (4, 3.3)	
	8	16.0 (4, 11.7)	
8x17bp−*lacZ*[d]	1	38.3 (4, 11.9)	
	2	13.8 (4, 17.0)	17.9 (4, 15.4)
	3	1.2 (4, 2.5)	
	4	18.2 (4, 2.1)	
Dipt0.6−*lacZ*[e]	1	84.0 (3, 31.1)	
	2	10.1 (2, 8.5)	
	3	91.2 (3, 15.0)	50.4 (6, 39.0)
	4	81.4 (5, 13.2)	
	5	25.0 (5, 16.3)	
	6	10.5 (4, 3.0)	
Dipt3.7−*lacZ*[f]	1	331.5 (5, 51.3)	
	2	403.6 (5, 111.8)	
	3	256.8 (4, 97.3)	
	4	285.3 (3, 72.0)	276.6 (8, 89.0)
	5	134.2 (5, 50.8)	
	6	232.6 (4, 28.5)	
	7	366.5 (4, 50.3)	
	8	202.2 (4, 23.5)	
Dipt11−*lacZ*	1	363.8	363.8

Two to 10 pools of five transgenic larvae were analysed 6 h after bacterial challenge for β-galactosidase activity.
Means (m, M) are given in nmol of product formed per min and per mg of proteins, with *n*, the number of pools measured and s, the standard deviation between these pools in parentheses.
The values obtained with the lines [b], [c], [d] or [e] are significantly different from those of lines [a] by a Mann−Whitney test with $P < 0.01$. The values of lines [a] and [f] are not significantly different.

The histochemical analysis of the induced β-galactosidase activity in these three fly lines clearly shows a cell-autonomous expression in the fat body, with some cells reacting strongly and others only poorly or remaining unreactive (Figure 3a). In similar conditions, a wild-type 2.2 kb diptericin promoter region leads to an intense staining reaction in all cells (data not shown, but see Figure 3 in Reichhart *et al.*, 1992). To our surprise, in all three lines the integument reacted in the vicinity of the wound (Figure 3b). This integumental reaction was clearly triggered by the immune challenge and remained localized. The other transgenic lines did not exhibit such a localized reaction. Finally, a reduced number of blood cells (1–5%) showed β-galactosidase activity upon bacterial challenge

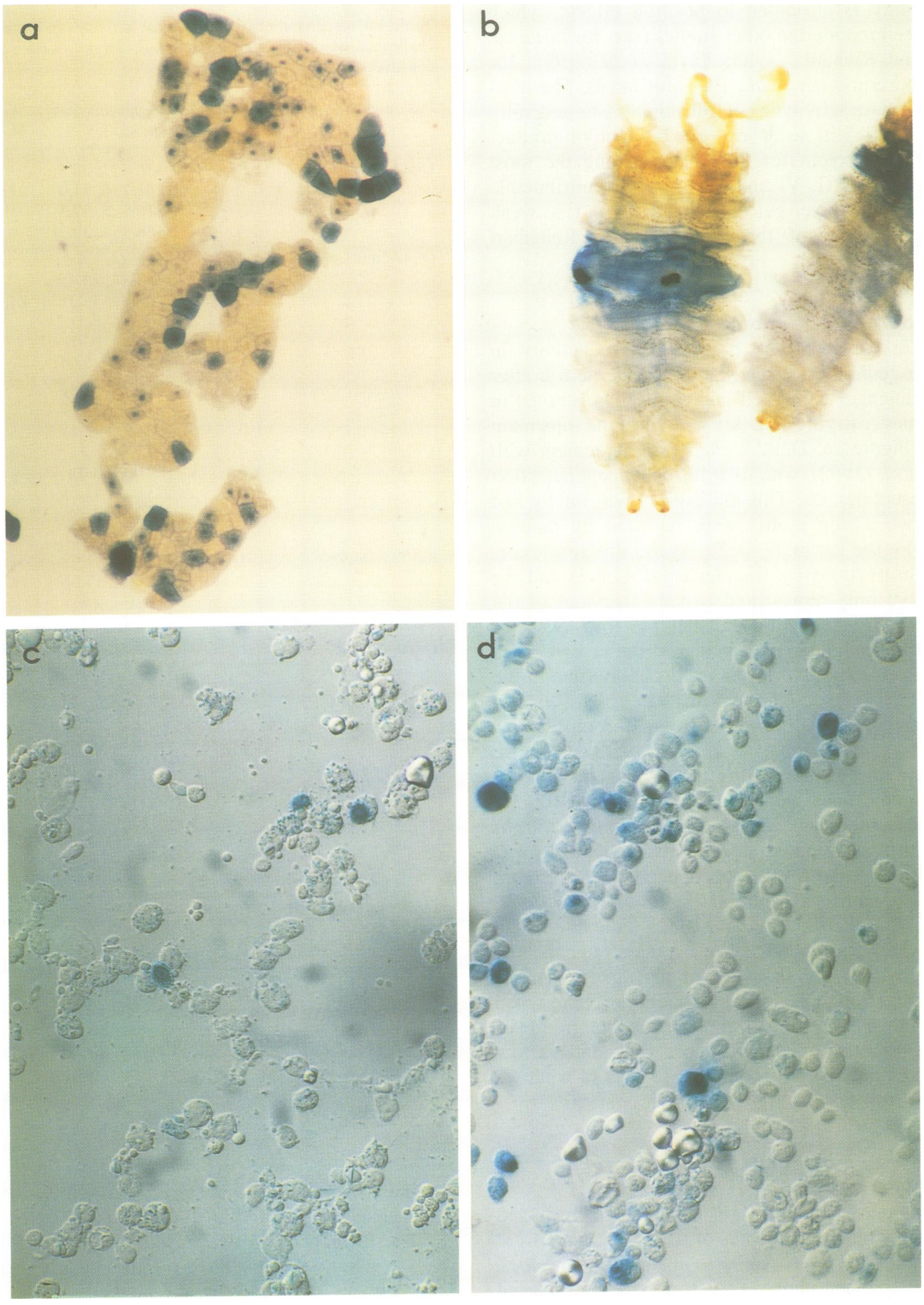

Fig. 3. Histochemical staining of β-galactosidase activity in 8x17bp−*lacZ* and Dipt2.2−*lacZ* transformants. Wandering stage third instar larvae were bacteria-challenged and dissected 6 h later for staining as indicated in Materials and methods. (**a**) Fat body dissected from a challenged 8x17bp−*lacZ*:1 larva. (**b**) Integument from a challenged 8x17bp−*lacZ*:4 larva. The two black spots are the melanization zones at the puncture wounds. (**c** and **d**) Haemocytes from challenged 8x17bp−*lacZ*:2 (c) and Dipt2.2−*lacZ*:1 (d) larvae. A similar percentage of stained cells was routinely observed in the 8x17bp−*lacZ* and Dipt2.2−*lacZ* lines respectively.

in the lines carrying eight copies of the 17 bp motif (Figure 3c). The 2.2 kb diptericin promoter sequence, in comparison, induces under similar conditions a response in some 10% of the blood cells (Figure 3d).

Transgenes with various lengths of upstream and downstream sequences

The experiments described so far prompted the construction of reporter genes with various lengths of promoters

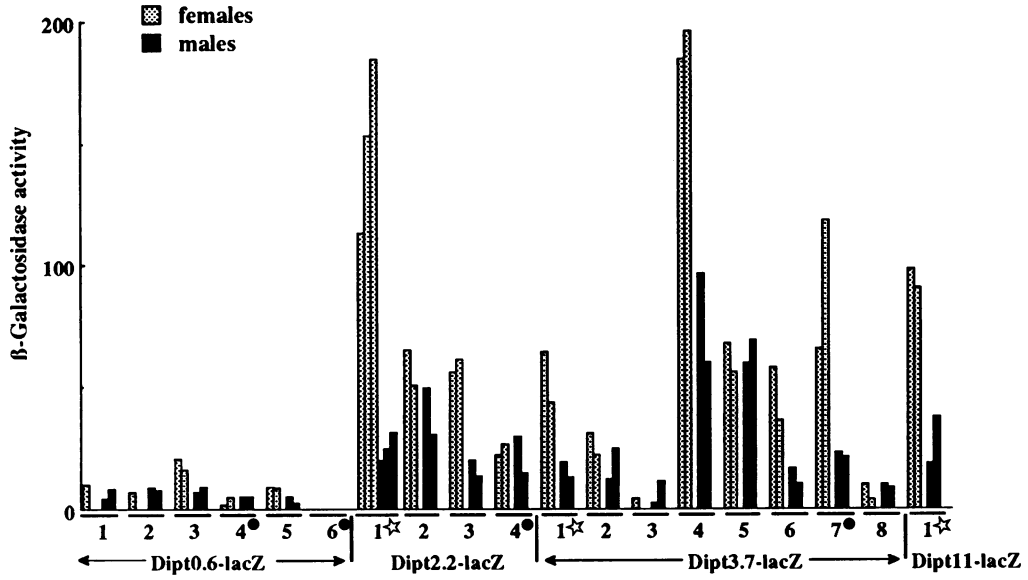

Fig. 4. β-Galactosidase activity in bacteria-challenged adults from transgenic lines carrying constructs with various diptericin promoter sequences upstream of the *lacZ* gene. Each column represents the activity measured in a pool of five females (dotted columns) or males (dark columns) sacrificed 6 h after a bacterial challenge. Other indications are as in Figure 2.

to define regions able to up-regulate the induction conferred to the transgene by the 17 bp motifs. Six fly lines were established which carried a reporter gene under the control of 0.6 kb of diptericin upstream sequences (Figure 2b, Dipt0.6−*lacZ*). In three of these lines, a marked induction was observed, while the three others responded more weakly. When the mean values of β-galactosidase activity obtained with this transgene are compared with those of the transgene carrying eight copies of the 17 bp motif (Figure 2a, 8x17bp−*lacZ*, and Table I), an increase of 2- to 3-fold is apparent. Nevertheless, the 0.6 kb of upstream wild-type sequences are able to sustain only ~20% of the response induced by a transgene with 2.2 kb (Figure 2b, Dipt2.2−*lacZ*, and Table I).

The data obtained with 3.7 and 11 kb respectively of upstream region in the transgenes are also plotted in Figure 2b (Dipt3.7−*lacZ* and Dipt11−*lacZ*, for which we obtained only one transformed fly line). Although there is a variability between the various lines obtained with the same transgene, it is apparent that the transgenic lines carrying 2.2 and 3.7 kb respectively respond similarly to bacterial challenge. The value obtained with the single Dipt11−*lacZ* line does not differ from the previous. We conclude from these experiments that sequences upstream of 2.2 kb do not significantly influence the level of inducibility of the diptericin gene.

Finally, we have established several fly lines with constructs IX and X (see Figure 1b) in which 2.2 kb of upstream sequences were followed by the diptericin coding region carrying a tag sequence. One of the constructs (IX) had the conventional *hsp70* termination element, while the other (X) had 1.1 kb of wild-type downstream sequence of the diptericin gene. We observed no difference in the level of induction compared with those obtained with the Dipt2.2−*lacZ* transgene (see Reichhart *et al.*, 1992), as judged by the signals resulting from Northern blotting experiments (data not shown). This indicates that in our conditions we do not detect regulatory elements within or downstream of the coding sequence that participate in the up-regulation of the bacterial induction of the diptericin gene.

Induction of reporter transgenes in adult males and females

We have also examined whether the reporter *lacZ* was induced in adult male and female flies carrying transgenes with 0.6, 2.2, 3.7 and 11 kb of promoter sequences (constructs VIII, I, VII and VI) as in third instar wandering larvae. Initial experiments showed that in adults both sexes did not respond equally to bacterial challenge; we therefore analysed separately the inducibility of the transgenes in male and female flies. We have previously shown that both larval and adult fat body cells express the transgene (Reichhart *et al.*, 1992). The results of induced β-galactosidase activities are presented in Figure 4. As is the case in larvae, variability is observed among various lines carrying the same transgene. Unexpectedly, some lines (e.g. lines 4 and 6 carrying the transgene Dipt0.6−*lacZ* and lines 3 and 8 for Dipt3.7−*lacZ*) which are inducible in larvae are almost non-induced in adults. Overall, however, the results obtained with adults are consistent with those for larvae in that they show that: (i) transgenes with 2.2–11 kb of upstream sequences yield a roughly similar response (taking into account the variations among lines); (ii) a transgene with 0.6 kb of upstream sequence is induced to a level five to ten times lower than that of the longer constructs. The overall levels of induction are lower in adults as compared with larvae and this reflects the lower inducibility of the transgenes compared with the endogenous diptericin gene in adults. The level of induction is also higher in females than in males in a number of fly lines. Given that the fat body is by far the predominant responsive tissue, this difference might reflect a difference in the relative ratio of fat body tissue to other tissues in females compared with males, the values of β-galactosidase activity being expressed per unit of global protein content. Alternatively, females may be more

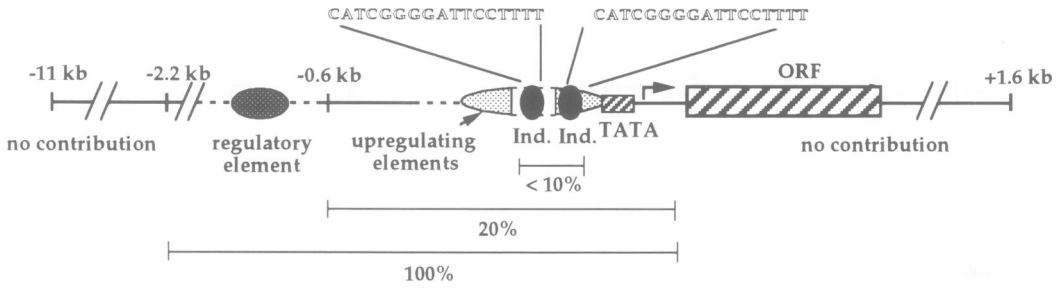

Fig. 5. Regulatory elements in the promoter of the diptericin gene. The sequences of the 17 bp elements are given. The percentages indicated under the bars represent the levels of expression of the reporter gene driven by various promoter elements. Ind, elements conferring inducibility to the gene.

responsive than males, but this proposal should be substantiated by additional experimental approaches.

Discussion

The data presented in this paper provide the first detailed *in vivo* analysis of the relative contribution of various promoter regions to the bacteria-induced expression of an insect gene encoding an antibacterial peptide. They also reveal the existence of a distal regulatory element which had remained undetected in previous studies.

Our results confirm that the two 17 bp motifs present within the 150 nt upstream of the transcription start site are necessary for the immune responsiveness of the diptericin gene. When these motifs are mutated in an otherwise wild-type context, inducibility is lost, indicating that no other region within the promoter can sustain this crucial function. A single copy can mediate a severely reduced induction and cooperativity between the two motifs is clearly essential for the normal induction of the gene. In this respect it is of interest to note that in the promoters of diptericin genes of several *Drosophila* species (*D.simulans*, *D.erecta* and *D.melanogaster*) both 17 bp repeats are conserved in similar positions within the proximal promoter region (Meister *et al.*, 1994).

The *in vivo* relevance of the 17 bp motifs for the inducibility of the transgene is further demonstrated by the first transgenic experiments in *Drosophila*, in which a minimal promoter, containing eight copies of the motifs, is fused to the *lacZ* gene. Such a construct is indeed capable of conferring bacterial inducibility to the reporter transgene, albeit at a level corresponding to 5–10% of the response observed with 2.2 kb of wild-type upstream sequence. These results, taken in conjunction with our previous studies (Kappler *et al.*, 1993), clearly identify the 17 bp motifs as conferring immune responsiveness to the diptericin gene.

The *in vivo* results obtained with reporter constructs carrying 0.6 kb of wild-type upstream sequences show a level of expression ~2- to 3-fold higher than that observed when eight copies of the 17 bp motifs are fused to the reporter. This points to the existence of up-regulatory elements that are distinct from the sequences present in the 17 bp motifs. The proximal upstream region contains, as indicated above, several motifs with sequence homology to regulatory elements of mammalian acute phase response genes, namely IL6 REs and ICREs (see Introduction). Several copies of these sites are protected, together with the 17 bp motifs, in DNase I footprinting assays with nuclear extracts from induced larval fat bodies (Georgel *et al.*, 1993; see Figure 1a). We propose that the 2- to 3-fold up-regulation observed with 0.6 kb of upstream sequence reflects the cooperativity between the decameric κB-related sites in the 17 bp repeats and the various IL6 RE and ICRE elements within the most proximal region.

When the *in vivo* expression level of induced β-galactosidase activity is compared between constructs carrying 0.6 and 2.2 kb of wild-type promoter region, a 5-fold increase is apparent, which indicates the existence of a distal regulatory element between −2.2 and −0.6 kb. We have not precisely mapped this element. It may be relevant to note that at −0.8 kb a third decameric κB-related motif is present in the diptericin promoter. Its sequence differs slightly from the proximal κB-related motifs (Reichhart *et al.*, 1992; Kappler *et al.*, 1993). Obviously, the wild-type distal decameric κB-related motif at −0.8 kb alone cannot confer inducibility to a reporter gene, as illustrated with the transgenic fly lines carrying a 2.2 kb promoter in which the two 17 bp motifs have been mutated. There remains the possibility that this κB-related motif is part of the distal regulatory element.

Finally, we have not been able to increase the level of induced expression of the reporter gene above that obtained with 2.2 kb of wild-type upstream sequence, neither in larvae nor in adults, in any of the experiments extending the constructs either upstream or downstream of the coding region. Similarly, our experiments do not reveal regulatory elements within the coding region of the diptericin gene. We conclude from these data that the *in vivo* expression of the transgene during the immune response is controlled by regulatory elements all of which reside within the 2.2 kb upstream region (Figure 5). This analysis is essentially based on the results obtained with the highly reactive third instar larvae. The data obtained with adult *Drosophila*, whether males or females, are in agreement with this analysis, despite the fact that the levels of induction are lower.

The expression of the resident diptericin gene is observed primarily in the cells of the fat body (Wicker *et al.*, 1990; Reichhart *et al.*, 1992) and in a certain percentage of circulating blood cells (10–20%, as was described for cecropins A and B by Samakovlis *et al.*, 1990). We have systematically dissected transgenic larvae in the present study to analyse whether the various promoter regions under scrutiny affected the pattern of tissue-specific expression. Constructs containing any length of diptericin upstream sequences from 0.6 to 11 kb showed a tissue-specific expression identical to that of the

resident gene. However, transgenic lines carrying only a minimal promoter with eight copies of the 17 bp motifs showed, in addition to a low but significant expression in the fat body and blood cells, an aberrant expression in the integument, which probably reflects transcription in the epidermal cells. This type of expression was not seen with any of the other constructs. Only the regions around the wound express the transgene, the majority of the integument remaining unreactive. We cannot provide an explanation for this result. This may reflect the fact that multimerization of the 17 bp motif constitutes a synthetic promoter element which, although reacting to Rel-related factors, does not faithfully reconstitute the regulation observed in the diptericin promoter. This would be analogous to the observations made in transgenic mice with a synthetic κB-based promoter construct which was activated in several tissues containing different NF−κB/Rel heterodimers (Lernbecher *et al.*, 1993). The integumental reactivity in transgenic *Drosophila* suggests the existence of an induction system localized to the wound site, which logically activates other immunoresponsive genes.

Our information on the control of immune genes in *Drosophila* is restricted so far to the diptericin and cecropin genes. The role of κB-related sequences has also been ascertained by transgenic and transfection experiments for the gene encoding cecropin A1, a 39 residue peptide of *Drosophila* (Engström *et al.*, 1993), and previously in *Hyalophora cecropia* by Sun and Faye (1992a,b). However, the potential role of other regulatory sequences has not yet been reported in that system.

The future challenge for understanding the immune-induced expression of the diptericin gene will be the characterization of the *trans*-regulating proteins which interact with the various regulatory elements identified in this promoter. Experiments are under way to purify these proteins. As regards the decameric κB-related sites, two proteins have recently been shown to bind to these motifs in the diptericin and cecropin promoters: dorsal (Reichhart *et al.*, 1993) and Dif (dorsal-related immunity factor; Ip *et al.*, 1993), which are both 70–80 kDa proteins belonging to the Rel family of inducible transactivating proteins. Both proteins are present predominantly in the cytoplasm of uninduced fat body and translocate rapidly into the nucleus upon bacterial challenge. They have been shown independently to bind to κB-related motifs in the diptericin and the cecropin promoters respectively and in tumorous *mbn-2* blood cells; dorsal can specifically transactivate a reporter gene carrying κB-related motifs. The exact mechanisms by which these proteins exert their functions in the immune response is under investigation.

Finally, we wish to stress that our results pointing to a cooperativity between κB-related, IL6 RE and ICRE sites (or half-sites as in the case of ICRE here) lend further credence to the idea that a basic homology exists between insect host defence and the acute phase reaction in mammals. Cooperativity of similar regulatory sites has indeed been shown in a variety of promoters of genes involved in the mammalian acute phase response (e.g. in LPS-stimulated transcription from the murine IP-10 promoter, Ohmori and Hamilton, 1993; regulation of the interleukins, Matsusaka *et al.*, 1993; Stein and Baldwin, 1993). *Drosophila* may prove a remarkable model system in the future to decipher the fine tuning of gene expression in the innate host defence.

Materials and methods

Construction of plasmids

The construction of C20Dipt2.2−*lacZ* has been described (Reichhart *et al.*, 1992). C20Dipt2.2mut1- or mut2−*lacZ* were constructed as C20Dipt2.2mut−*lacZ* (Kappler *et al.*, 1993) except that only one of the 17 bp motifs was replaced by the random sequence (mut1: the most proximal site; mut2: the most distal site). C20Dipt0.6−*lacZ*, 3.7−*lacZ* and 11−*lacZ* were constructed essentially as C20Dipt2.2–*lacZ* using respectively 0.6, 3.7 and 10.5 kb fragments of the diptericin upstream region. For C20-8x17bp–*lacZ*, an *Eco*RI−*Hin*dIII fragment containing the *hsp70* minimal promoter of glAdE-$H_1H_2H_3$ (Martin *et al.* 1989) was cloned into an M13 TG131 vector downstream of eight repeats of the 17 bp sequence (CATCGGGGATTCCTTTT; see construct D9 in Kappler *et al.*, 1993). This 8x17bp−*hsp70* fragment was then fused upstream of a *Hin*dIII−*Sal*I fragment containing the complete β-galactosidase coding region and the *hsp70* transcription termination signal. The fusion gene was inserted in the appropriate orientation into the *Sal*I site of the Carnegie 20 transformation vector. For C20Dipt2.2-ORF and Dipt2.2-ORF-1.1, a *Xho*I−*Hin*dIII fragment containing 2.2 kb of diptericin upstream sequences followed by the coding sequence was inserted into an M13mp18 vector that had been modified by the insertion of a *Xho*I linker. An additional sequence of 45 nucleotides (tag) was inserted by polymerase chain reaction 3′ to the coding sequence, thus displacing the stop codon and the overlapping *Hin*dIII site (TAAGCTT). Using this restriction site, we fused a 1060 bp *Hin*dIII−*Sal*I fragment of diptericin downstream sequences (Dipt2.2-ORF-1.1) or the *hsp70 Hin*dIII−*Xho*I termination signal (Dipt2.2-ORF) downstream of the tag sequence. The resulting *Xho*I−*Sal*I or *Xho*I−*Xho*I fragments were then inserted into Carnegie 20.

Isolation of transformed strains and immune challenge

The various fusion gene plasmids (300 μg/ml) were 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 or heterozygous lines were established by crosses to appropriate balancers.

The transformed lines were maintained at 25°C on a standard cornmeal medium. Third instar larvae and adults were pricked with a tungsten needle previously dipped in a concentrated bacterial culture (*Escherichia coli* and *Micrococcus luteus*).

β-*Galactosidase assays*

β-*Galactosidase localization.* Dissected larvae were fixed in 0.5% glutaraldehyde, 1 mM $MgCl_2$ in phosphate-buffered saline (PBS), pH 7.5, for 10 min, washed in PBS, then submerged in 0.2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 3.5 mM $K_4Fe(CN)_6$, 3.5 mM $K_3Fe(CN)_6$, 1 mM $MgCl_2$, 150 mM NaCl, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4 and incubated overnight at 37°C (Hiromi *et al.*, 1985). Haemocytes were prepared as follows. Six hours after bacterial challenge, the larvae were punctured and gently squeezed, after which a few microlitres of haemolymph were dropped on a glass slide. After drying, the preparations were fixed for 1 min in glutaraldehyde/PBS solution and treated as above. Staining was allowed to proceed for 24 h at 37°C. Stained tissues and haemocytes were mounted in glycerol.

Quantitative measurements of β-*galactosidase activity.* Five larvae or adults were homogenized in Z buffer (Miller, 1972) and centrifuged for 10 min at 10 000 r.p.m. (4°C) to remove debris. β-Galactosidase activity was measured as described in Miller (1972). The protein concentration was determined by the Bradford assay (Bio-Rad), using bovine serum albumin as a standard. Results are given in nmol product formed/min/mg protein. Internal standard samples were run with each experimental series to allow us to compare the absolute values of β-galactosidase activities.

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